A NEW AND EMERGING MYXOZOAN PARASITE OF FATHEAD MINNOWS: Species description, life- cycle, and effects to the host

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A NEW AND EMERGING MYXOZOAN PARASITE OF FATHEAD MINNOWS:
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To my son Cash, my greatest motivation.

ABSTRACT

This thesis aims to characterize a new species of myxozoan, nominated *Myxobolus rasmusseni* n. sp., parasitizing Fathead Minnows in southern Alberta and to understand its effects on individual hosts. The results of my thesis are the first to document and image the host-parasite interface of *M. rasmusseni* n. sp.- infected minnows and to confirm the identity of both hosts: Fathead Minnow and *Tubifex tubifex* in its complete life cycle. Based upon the observations of distinctive lesions that are associated with the head of infected minnows, I used a combination of approaches to demonstrate that lesion-bearing minnows have reduced physiological performance and poorer survival than non-lesion bearing minnows. My experiments also demonstrate that *M. rasmusseni* n. sp. influences some host behaviours which raise questions regarding parasite-mediated transmission. Ultimately, my results suggest that the lesions caused by the development of *Myxobolus rasmusseni* n. sp. in Fathead Minnow are pathological and an emerging infectious disease that may negatively influence Albertan minnow populations.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 General Background

Myxozoans are an enigmatic subphylum of approximately 2400 species of endoparasitic cnidarians (Alama-Bermejo & Holzer, 2021; Kyslík et al., 2021) that typically infect fishes during one stage of their life cycle (Eiras et al., 2014). Within these hosts, myxozoans have now been reported, depending on the species, from essentially all host tissues and organs (Feist et al., 2015; Feist & Longshaw, 2008; Lom & Dykova, 2006). Recently, the results of phylogenetic and molecular studies have shown that myxozoans provide an outstanding example of the manner in which parasitism evolves from their free-living ancestors. Incredibly, myxozoans have one of the smallest and most reduced genomes in the Animal Kingdom with depletion of genetic material relating to cell differentiation, development, morphogenesis, receptor genes, and signal transducer activity (Chang et al., 2015). In short, myxozoans are highly reduced in comparison to their non-parasitic Cnidarian ancestors especially in features related to body plan, cell-to-cell differentiation, and cell-to-cell communication (Chang et al., 2015). In one extreme example, the myxozoan, *Henneguya salmonica,* is the first multicellular eukaryote that lacks a mitochondrial genome and aerobic respiration, two of the hallmarks of eurkaryotic life (Yahalomi et al., 2020). Beyond the role of myxozoans in advancing our understanding of the evolution of parasitism, they have also garnered attention in recent years in fisheries and aquacultural operations due to the severity of pathological disease caused by certain species to economically important fishes (Gruhl, 2015; Kent et al., 2001; Stilwell, 2021). Furthermore, myxozoans such as *Myxobolus cerebralis* and *Tetracapsuloides bryosalmonae* have been recognized and gained attention from

ecologists and especially wildlife disease ecologists interested in emerging infectious diseases (Hatcher et al., 2012; James et al., 2021).

Although the first detection of myxozoans occurred in the early 1800's (Okamura et al., 2015), the taxonomic designation of these parasites have undergone major reorganizations within the last 40 years (Siddall et al., 1995). Initially, myxozoans were classified as protists (Lom, 1990) and were grouped alongside microsporidians and members of the Apicomplexa, Levine, 1970 (Siddall et al., 1995). Later, ultrastructural studies observed the multicellular nature of myxozoan development and body plan (Desser et al., 1983), and eventually early molecular studies and phylogenies suggested close relatedness with high parsimony to the bilateral animals (Schlegel et al., 1996; Smothers et al., 1994). The discovery of polar capsules in the body wall of several myxozoans provided further morphological evidence that myxozoans were members of the Bilateria (Jimenez-Guri et al., 2007; Nesnidal et al., 2013a; Okamura et al., 2002). The results of combined molecular and ultrastructural investigations further established metazoan features in the myxozoa, such as collagen production, while also organizing the group into a clade within Cnidaria (Siddall et al., 1995). The results of molecular studies then established links between existing morphological associations like minicollagen genes synonymous in both the nematocyst of Cnidarians and the polar capsule of Myxozoans (Holland et al., 2011). Following these advances, the Myxozoa were considered a discrete clade nested within the Phylum Cnidaria. This radically revised phylogenetic tree demonstrated the evolutionary trajectory of parasitism within the Cnidaria, with a divergence from the typical cnidarian body plan to an extremely reduced body plan of the parasitic Myxozoa (Chang et al., 2015).

All myxozoans that have been formally described have complex life-cycles that alternate between two free-living infectious life stages with two obligate parasitic stages, usually in an annelid and a vertebrate host, respectively (Fig 1.1; Feist et al., 2015). The general myxozoan life cycle includes sexual development in the oligochaete host. This stage is followed by a free-living living, motile spore stage, called an actinospore that is released into the water column. The short-lived actinospore penetrates the epidermis of the fish host upon physical contact, which stimulates extrusion of the polar filament; the coiled needle-like structure that is responsible for injecting the infective sporoplasm into host tissues. Following penetration, the actinospore injects an infective sporoplasm into the host tissue (El-Matbouli et al., 1995; Kallert et al., 2010). Following invasion of the sporoplasm into the target tissue of the host, an asexual phase of development occurs that leads to a second free-living infective stage known as a myxospore. Upon liberation from host tissues, which is typically associated with death of the host either by parasite driven pathology or predation (Eszterbauer et al., 2015), myxospores are ingested from the sediment of the pond by the oligochaete host (Fig 1.1). The details of myxozoan life cycles were unresolved until studies confirmed that the two main development stages require two different obligate hosts (Markiw & Wolf, 1983). Markiw and Wolf (1983) demonstrated that transmission of the whirling disease myxozoan, *Myxobolus cerebralis,* to rainbow trout *Onchorynchus mykiss* could only be completed in sediment that contained aquatic tubificid oligochaetes, specifically *Tubifex tubifex.* Following this discovery, the prior interpretation that actinospores were a different clade of myxozoan-like organisms (the Actinosporea) was abandoned.

Myxozoans are highly uniform in morphology, especially within species-rich and well-recognized genera such as *Myxobolus.* This uniformity necessitates the use of combined approaches for species diagnoses that early on, focused on precision measurements of myxospore dimensions and ultrastructure, together with information on host and tissue specificity (Cech et al., 2012; Eiras et al., 2014). This traditional approach has led to a fragmented and confusing taxonomy, including for genera such as *Myxobolus* Butschli 1882 and *Henneguya* Thelohan 1892 that 18S rDNA analyses have determined are distinguishable (Carriero et al., 2013). As a result, myxozoan genera that were once grouped together based on the traditional approach have ended up clustering into different clades when based upon molecular information (Lom & Dykova, 2006). Previous studies that have suggested the relationship of Myxozoa within Cnidaria still struggle to elucidate the relationship with other classes such as hydrozoa, cubozoa, and scyphozoa; which together are considered the Medosozoa, making the progress of Myxozoan phylogenetics a complicated and slow-moving process (Nesnidal et al., 2013b). The discrepancy between studies regarding the placement of Myxozoans highlights the importance of using multiple sources of evidence to support taxonomic designations at the species level and at higher levels of taxonomic resolution.

Their small size and the cryptic nature of free-living and parasitic stages can explain this complex history of discoverey of Myxozoans. Yet their small size and cryptic nature has also led to gaps in our understanding of fundamental epidemiological parameters associated with transmission, and gaps in our understanding of the effects of myxozoans on their hosts (Fontes et al., 2015). For example, whereas numerous biotic and abiotic factors are known to affect rates of transmission of actinospores, the role of

factors within particular myxozoan/fish interactions is poorly known. Actinospore production and release by infected oligochaetes has been shown to be strongly influenced by water temperature. Some myxozoans, such as *Ceratomyxa shasta* and *Parvicapsula minibicornis* increase actinospore production when water temperature exceeds 7◦C and the spores remain viable for at least 7 days (Foott et al., 2007). Similar requirements for optimal actinospore release and viability have been reported for other species of myxozoan (El-Matbouli et al., 1999; Kallert & El-Matbouli, 2008), a result that emphasizes the influences that the environment plays in actinospore transmission. Moreover, the myxospore life stage of the myxozoans are also subject to environmental influences. The myxospores of *Myxobolus cerebralis* require a period of aging in the sediment to become infective to the final worm host (El-Matbouli & Hoffmann, 1991), with viability remaining, yet decreasing, up to one year after liberation from the fish host (Nehring et al., 2015). Additionally, myxozoans share the life history challenges of many other parasites of freshwater fishes that have multiple host life cycles; the problem of transmission from one host to the next. Some parasites solve this problem through trophic level exchanges that involve multiple ingestion events of parasite life stages by multiple hosts, such as with larvae of the cestode *Schistocephalus solidus* (Barber & Scharsack, 2010). Myxozoans may benefit from trophic exchanges by predation of infected hosts which then accelerates the liberation of the myxospores, such as has been implicated in the dissemination of *Myxobolus cerebralis*- infected salmonids by birds (Arsan & Bartholomew, 2008; Bartholomew & Reno, 2002). Yet, some infected hosts may passively release infective myxospores from living hosts, such as through the expulsion of wastes from the alimentary and renal canal (Fontes et al., 2015). Other abiotic and

biotic factors that influence transmission success of myxozoans, include water turbidity and eutrophication (Marcogliese & Cone, 2021), sediment character for the establishment of oligochaete populations (McGinnis & Kerans, 2013), as well as the presence of other aquatic hosts and non-host, are known to affect rates of myxozoan transmission.

Once transmission of actinospores into fish is successful, some myxozoan species cause severe pathology within host tissues. Examples of such pathology include the skeletal deformities to juvenile Rainbow Trout *Onchorynchus mykiss* by developing *Myxobolus cerebralis* feeding on the cartilage of their salmonid hosts (Bechara et al., 2003; El-Matbouli et al., 1995), the mottled and swollen kidneys and immunosuppression of salmonids caused by sporogonic stages of *Tetracapsuloides bryosalmonae* (Gorgoglione et al., 2020; Kent et al., 2000; Kotob et al., 2017), and the severe branchial inflammation, epithelial hyperplasia and chondrolytic lesions of the gills of channel catfish caused by *Henneguya ictaluri* (Wise et al., 2008). These well-studied myxozoans have been recognized as stressors that can affect the health of individual fish, and for some, premature host morbidity and mortality (Gorgoglione et al., 2020; James et al., 2021; Nehring & Walker, 1996; Stilwell, 2021; Wise et al., 2008). The results of population-level studies of Rainbow Trout completed along a stretch of the Colorado River showed a significant decline in the survival of juvenile fish between 1981-1995 (Nehring & Walker, 1996). In southern Alberta, up to 85% of wild Rainbow trout fingerlings recovered from multiple sites of the Crowsnest River showed pathological symptoms of whirling disease (James et al., 2021). In the latter study, older yearling cohorts were absent at most sites, suggesting that the population of the yearling cohort was significantly reduced. Results from the study on the Crowsnest River are especially

concerning because Rainbow Trout in the river are sympatric with endangered populations of westslope cutthroat trout that are highly susceptible to *M. cerebralis* (Koel et al., 2006). These combined results indicate that some species of myxozoans are critically important both from an economic and conservation point of view.

The results of host surveys completed in 2017 of waterbodies in southern Alberta detected Fathead Minnows with large, conspicuous lesions on the head region. The lesions had not been observed in intensive host surveys completed on the same waterbodies between 1997 and 2017 (Ahn, 2019; Sandland et al., 2001). Nor had the lesions been reported from host surveys, several of which specifically targeted myxozoans in other sites (Ahn, 2019). The lesions observed on Fathead Minnows in 2017 were whitish in colour and followed general pathology that was associated with conspicuous bulging of the eyes, obstruction of the nose, mouth and opercula, and growth along the pectoral girdle of fish (Fig. 1.2). Investigation of lesions under light microscopy yielded thousands of spores that were later determined to be myxospores, consistent with those in the genus *Myxobolus.* These observations of a putative emerging myxozoan in Fathead Minnow led to the current study.

1.2 Thesis objectives

The aim of Chapter 2 is to characterize the putatively new *Myxobolus* sp. that was found infecting Fathead Minnows in southern Alberta (Fig 1.1). I thus combined contemporary standard protocols for naming and classification for myxozoan parasites, including combining imaging, standard histology of infected host tissue, 18S rDNA gene sequencing and phylogenomic analyses to determine its taxonomic status. I compared 18S rDNA gene sequences of myxospores isolated from the lesions of *Myxobolus* sp.-

infected Fathead Minnows, to the sequences of free-living actinospore (triactinomyxons or TAMs) released from two species of oligochaete worms. The purpose of this comparison was to test the hypothesis that TAMs released from oligochaetes collected from the substrate of ponds that contained Fathead Minnows with lesions were identical. Support for this hypothesis would demonstrate the complete life cycle of this putative new species of myxozoan. An additional aim of this chapter is to describe the overall myxozoan assemblage found in Fathead Minnows collected from six sites in southern Alberta.

The aim of Chapter 3 is to evaluate the consequences of *Myxobolus* sp. on the overall performance of individual Fathead Minnows. Intuitively, the large and potentially immobilizing lesions that affect the head region of Fathead Minnows may cause impairment to important sensory systems such as vision and olfaction. In this chapter, I thus compared the physiological and behavioural performance of lesion-bearing Fathead Minnows to non-lesioned and uninfected control minnows. I evaluated the optomotor response, an innate response of fish defined by impulsive reorienting behaviour to visual stimuli (Rock et al., 1965). Although the optomotor response has been used as a tool to measure the impact of parasite development on visual acuity in Fathead Minnows infected with the trematode parasite *Ornithodiplostomum ptychochelius* (Shirakashi & Goater, 2005; Shirakashi & Goater, 2001), this is the first study to evaluate consequences to host vision by myxozoan induced lesions. I next evaluated, the minnow electro-olfactory response to assess olfactory acuity. Here, electrodes are placed on the surface of the olfactory epithelium to record the negative electrical potential change (mV) of the olfactory receptor neurons (ORNs) in response to an odourant (Scott & Scott-Johnson,

2002). Whereas the effects of other types of parasites on host olfaction have been assessed in fish (Lari & Pyle, 2017; Lari et al., 2017; Mizuno et al., 2018), there are no studies on the effects of myxozoans. Lastly, I also assessed the impact of *Myxobolus* sp.-induced lesions on the swimming performance of Fathead Minnows. The large lesions located along the pectoral girdle, on the opercular region and on the head region may impede lesion-bearing minnows' mobility in flowing water and ultimately their overall swimming endurance in a fixed velocity style challenge (Hammer, 1995). Finally, I ran an outdoor mesocosm study that evaluated the survival of lesioned and non-lesioned minnows collected from the field over a 2-week interval.

Altogether, I used the results of this set of experiments to test the idea that *Myxobolus* sp. lesions impose consequences to individual Fathead Minnow physiology and behaviour and impede Fathead Minnows' capacity to respond to external stimuli.

1.3 Literature Cited

Ahn, S. 2019. *Effects of host community structure on parasite transmission and disease risk.* [Master of Science]. University of Lethbridge.

Alama-Bermejo, G., & Holzer, A. S. 2021. Advances and discoveries in myxozoan genomics. Trends in Parasitology 37: 552–568.

Arsan, E. L., & Bartholomew, J. L. 2008. Potential for dissemination of the nonnative salmonid parasite *Myxobolus cerebralis* in Alaska. Journal of Aquatic Animal Health 20: 136–149.

Barber, I., & Scharsack, J. P. 2010. The three-spined stickleback-*Schistocephalus solidus* system: an experimental model for investigating host-parasite interactions in fish. Parasitology 137: 411–424.

Bartholomew, J. L., & Reno, P. W. 2002. The history and dissemination of whirling disease. American Fishereies Society Symposium 26:1-26.

Bechara, I. J., Youssef, N. N., & Roberts, D. W. 2003. Histologic study of head cartilage degeneration in rainbow trout (*Oncorhynchus mykiss*) infected with the parasite *Myxobolus cerebralis*. Journal of Submicroscopic Cytology and Pathology 35: 111-116.

Carriero, M. M., Adriano, E. A., Silva, M. R. M., Ceccarelli, P. S., & Maia, A. A. M. 2013. Molecular phylogeny of the *Myxobolus* and *Henneguya* genera with several new South American species. PLoS ONE 8:1-12.

Chang, E. S., Neuhof, M., Rubinstein, N. D., Diamant, A., Philippe, H., Huchon, D., & Cartwright, P. 2015. Genomic insights into the evolutionary origin of Myxozoa within Cnidaria. Proceedings of the National Academy of Sciences 112: 14912–14917.

Desser, S. S., Molnar, K., & Horvath, I. 1983. An ultrastructural study of the Myxosporeans, *Sphaerospoa angulata* and *Sphaerospora carassii*, in the common carp, *Cyprinus carpio* L. The Journal of Protozoology 30: 415–422.

Eiras, J. C., Zhang, J., & Molnár, K. 2014. Synopsis of the species of *Myxobolus* Bütschli, 1882 (Myxozoa: Myxosporea, Myxobolidae) described between 2005 and 2013. Systematic Parasitology 88: 11–36.

El-Matbouli, M., & Hoffmann, R. W. 1991. Effects of freezing, aging, and passage through the alimentary canal of predatory animals on the viability of *Myxobolus cerebralis* spores. Journal of Aquatic Animal Health 3: 260–262.

El-Matbouli, M., Hoffmann, R. W., & Mandok, C. 1995. Light and electron microscopic observations on the route of the triactinomyxon-sporoplasm of *Myxobolus cerebralis* from epidermis into rainbow trout cartilage. Journal of Fish Biology, 46: 919–935.

El-Matbouli, M., McDowell, T. S., Antonio, D. B., Andree, K. B., & Hedrick, R. P. 1999. Effect of water temperature on the development, release, and survival of the triactinomyxon stage of *Myxobolus cerebralis* in its oligochaete host. International Journal for Parasitology 29: 627-641.

Eszterbauer, E., Atkinson, S. D., Diamant, A., Morris, D. J., El-Matbouli, M., & Hartikainen, H. 2015. Myxozoan life cycles: practical approaches and insights. In B. Okamura, A. Gruhl, & J. L. Bartholomew (Eds.), *Myxozoan Evolution, Ecology and Development*. (pp. 175-200). Springer International Publishing.

Fontes, I., Hallett, S. L., & Mo, T. A. 2015. Comparative epidemiology of myxozoan diseases. In B. Okamura, A. Gruhl, & J.L. Bartholomew (Eds.), *Myxozoan Evolution, Ecology and Development* (pp. 317–342). Springer International Publishing.

Foott, J. S., Stone, R., Wiseman, E., True, K., & Nichols, K. 2007. Longevity of *Ceratomyxa shasta* and *Parvicapsula minibicornis* actinospore infectivity in the Klamath River. Journal of Aquatic Animal Health 19: 77–83.

Gorgoglione, B., Bailey, C., & Ferguson, J. A. 2020. Proliferative kidney disease in Alaskan salmonids with evidence that pathogenic myxozoans may be emerging north. International Journal for Parasitology 50: 797–807.

Gruhl, A. 2015. Myxozoa. In A. Wanninger (Ed.), *Evolutionary Developmental Biology of Invertebrates 1* (pp. 165–177). Springer Vienna.

Hammer, C. 1995. Fatigure and exercise tests with fish. Comparative Biochemistry and Physiology 112: 1–20.

Hatcher, M. J., Dick, J. T. A., & Dunn, A. M. 2012. Disease emergence and invasions. Functional Ecology 26: 1275–1287.

Holland, J. W., Okamura, B., Hartikainen, H., & Secombes, C. J. 2011. A novel minicollagen gene links cnidarians and myxozoans. Proceedings of the Royal Society B: Biological Sciences 278: 546–553.

James, C. T., Veillard, M. F., Martens, A. M., Pila, E. A., Turnbull, A., Hanington, P., Luek, A., Alexander, J., & Nehring, R. B. 2021. Whirling disease in the Crowsnest River: an emerging threat to wild salmonids in Alberta. Canadian Journal of Fisheries and Aquatic Sciences 78: 1–14.

Jimenez-Guri, E., Philippe, H., Okamura, B., & Holland, P. W. H. 2007. *Buddenbrockia* is a Cnidarian worm. Science 317: 116–118.

Kallert, D. M., & El-Matbouli, M. 2008. Differences in viability and reactivity of actinospores of three myxozoan species upon ageing. Folia Parasitologica 55: 105-110.

Kallert, D. M., Ponader, S., Adelt, S., Kaese, P., Geyer, R., Haas, W., & EL-Matbouli, M. 2010. Analysis of rainbow trout *Oncorhynchus mykiss* epidermal mucus and evaluation of semiochemical activity for polar filament discharge in *Myxobolus cerebralis* actinospores. Journal of Fish Biology 77: 1579–1598.

Kent, M. L., Andree, K. B., Bartholomew, J. L., EL-Matbouli, M., Desser, S. S., Devlin, R. H., Feist, S. W., Hedrick, R. P., Hoffmann, R. W., Palenzuela, O., Siddall, M. E., & Xiao, C. 2001. Recent advances in our knowledge of the Myxozoa. The Journal of Eukaryotic Microbiology 48: 395–413.

Kent, M. L., Khattra, J., Hedrick, R. P., & Devlin, R. H. 2000. *Tetracapsula renicola* n. sp. (Myxozoa: Saccosporidae) the PKX myxozoan: the cause of proliferative kidney disease in salmonid fishes. Journal of Parasitology 86: 103–111.

Koel, T. M., Mahony, D. L., Kinnan, K. L., Rasmussen, C., Hudson, C. J., Murcia, S., & Kerans, B. L. 2006. *Myxobolus cerebralis* in native cutthroat trout of the Yellowstone Lake ecosystem. Journal of Aquatic Animal Health 18: 157–175.

Kotob, M. H., Gorgoglione, B., Kumar, G., Abdelzaher, M., Saleh, M., & El-Matbouli, M. 2017. The impact of *Tetracapsuloides bryosalmonae* and *Myxobolus cerebralis* co- infections on pathology in rainbow trout. Parasites & Vectors 10: 442-456.

Kyslík, J., Kosakyan, A., Nenarokov, S., Holzer, A. S., & Fiala, I. 2021. The myxozoan minicollagen gene repertoire was not simplified by the parasitic lifestyle: computational identification of a novel myxozoan minicollagen gene. BMC Genomics 22: 198-212.

Lari, E., Goater, C. P., Cone, D. K., & Pyle, G. G. 2017. Parasites and a host's sense of smell: reduced chemosensory performance of fathead minnows (*Pimephales promelas*) infected with a monogenean parasite. Journal of Animal Ecology 86: 434–441.

Lari, E., & Pyle, G. G. 2017. *Gyrodactylus salmonis* infection impairs the olfactory system of rainbow trout. Journal of Fish Diseases 40: 1279–1284.

Lom, J. 1990. Phylum Myxozoa. In *Handbook of Protoctista* (pp. 36–52). Jones and Bartlett.

Lom, J., & Dykova, I. 2006. Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. Folia Parasitologica 53: 1–36.

Marcogliese, D. J., & Cone, D. K. 2021. Myxozoan communities in two cyprinid fishes from mesotrophic and eutrophic rivers. Journal of Parasitology 107: 39–47.

Markiw, M. E., & Wolf, K. 1983. *Myxosoma cerebralis* (Myxozoa: Myxosporea) etiologic agent of salmonid whirling disease requires tubificid worm (Annelida: Oligochaeta) in its life cycle. Journal of Protozoology 30: 561–564.

McGinnis, S., & Kerans, B. L. 2013. Land use and host community characteristics as predictors of disease risk. Landscape Ecology 28: 29–44.

Mizuno, S., Urawa, S., Miyamoto, M., Hatakeyama, M., Sasaki, Y., Koide, N., Tada, S., & Ueda, H. 2018. Effects of dietary supplementation with oregano essential oil on prevention of the ectoparasitic protozoans *Ichthyobodo salmonis* and *Trichodina truttae* in juvenile chum salmon *Oncorhynchus keta*. Journal of Fish Biology 93: 528–539.

Nehring, R. B., Schisler, G., Chiaramonte, L., Horton, A., & Poole, B. 2015. Assessment of the long-term viability of the myxospores of *Myxobolus cerebralis* as determined by production of the actinospores by *Tubifex tubifex*. Journal of Aquatic Animal Health 27: 50–56.

Nehring, R. B., & Walker, P. G. 1996. Whirling disease in the wild: the new reality in the intermountain west. Fisheries 21: 28–30.

Nesnidal, M. P., Helmkampf, M., Bruchhaus, I., El-Matbouli, M., & Hausdorf, B. 2013. Agent of whirling disease meets orphan worm: phylogenomic analyses firmly place Myxozoa in Cnidaria. PLoS ONE 8: 1-6.

Okamura, B., Curry, A., Wood, T. S., & Canning, E. U. 2002. Ultrastructure of *Buddenbrockia* identifies it as a myxozoan and verifies the bilaterian origin of the Myxozoa. Parasitology 124: 215–223.

Okamura, B., Gruhl, A., & Bartholomew, J. L. 2015. An introduction to myxozoan evolution, ecology and devlopment. In B. Okamura, A. Gruhl, & J. L. Bartholomew (Eds.), In *Myxozoan Evolution, Ecology and Developement* (pp. 1–22). Springer International Publishing.

Rock, I., Tauber, E. S., & Heller, D. P. 1965. Perception of stroboscopic movement: evidence for its innate basis. Science 147: 1050–1052.

Sandland, G. J., Goater, C. P., & Danylchuk, A. J. 2001. Population dynmaics of *Ornithodiplostomum ptychocheilus* metacercariae in fathead minnows

(*Pimephales promelas*) from four northern-Alberta lakes. Journal of Parasitology 87: 744–748.

Schlegel, M., Lom, J., Stechmann, A., Bernhard, D., Leipe, D., Dyková, I., & Sogin, M. L. 1996. Phylogenetic analysis of complete small subunit ribosomal RNA coding region of *Myxidium lieberkuehni*: Evidence that Myxozoa are metazoa and related to the Bilateria. Archiv Für Protistenkunde 147: 1–9.

Scott, J. W., & Scott-Johnson, P. E. 2002. The electroolfactogram: a review of its history and uses. Microscopy Research and Technique 58: 152–160.

Shirakashi, S., & Goater, C. P. 2001. Brain-encysting parasites affect visually-mediated behaviours of fathead minnows. Écoscience 8: 289–293.

Shirakashi, S., & Goater, C. P. 2005. Chronology of parasite-induced alteration of fish behaviour: effects of parasite maturation and host experience. Parasitology 130: 177–183.

Siddall, M. E., Martin, D. S., Bridge, D., Desser, S. S., & Cone, D. K. 1995. The demise of a phylum of protists: phylogeny of Myxozoa and other parasitic Cnidaria. The Journal of Parasitology 81: 961-967.

Smothers, J. F., von Dohlen, C. D., Jr., L. H. S., & Spall, R. D. 1994. Molecular evidence that the myxozoan protists are metazoans. Science 265: 1719–1721.

Stilwell, J. M. 2021. *Pathology, pathogenesis, and metagenomic characterization of myxozoan infections in catfish aquaculture* [Ph.D., University of Georgia].

Wise, D. J., Griffin, M. J., Terhune, J. S., Pote, L. M., & Khoo, L. H. 2008. Induction and evaluation of proliferative gill disease in channel catfish fingerlings. Journal of Aquatic Animal Health, 20: 236–244.

Yahalomi, D., Atkinson, S. D., Neuhof, M., Chang, E. S., Philippe, H., Cartwright, P., Bartholomew, J. L., & Huchon, D. 2020. A cnidarian parasite of salmon (Myxozoa: Henneguya) lacks a mitochondrial genome. Proceedings of the National Academy of Sciences 117: 5358–5363.

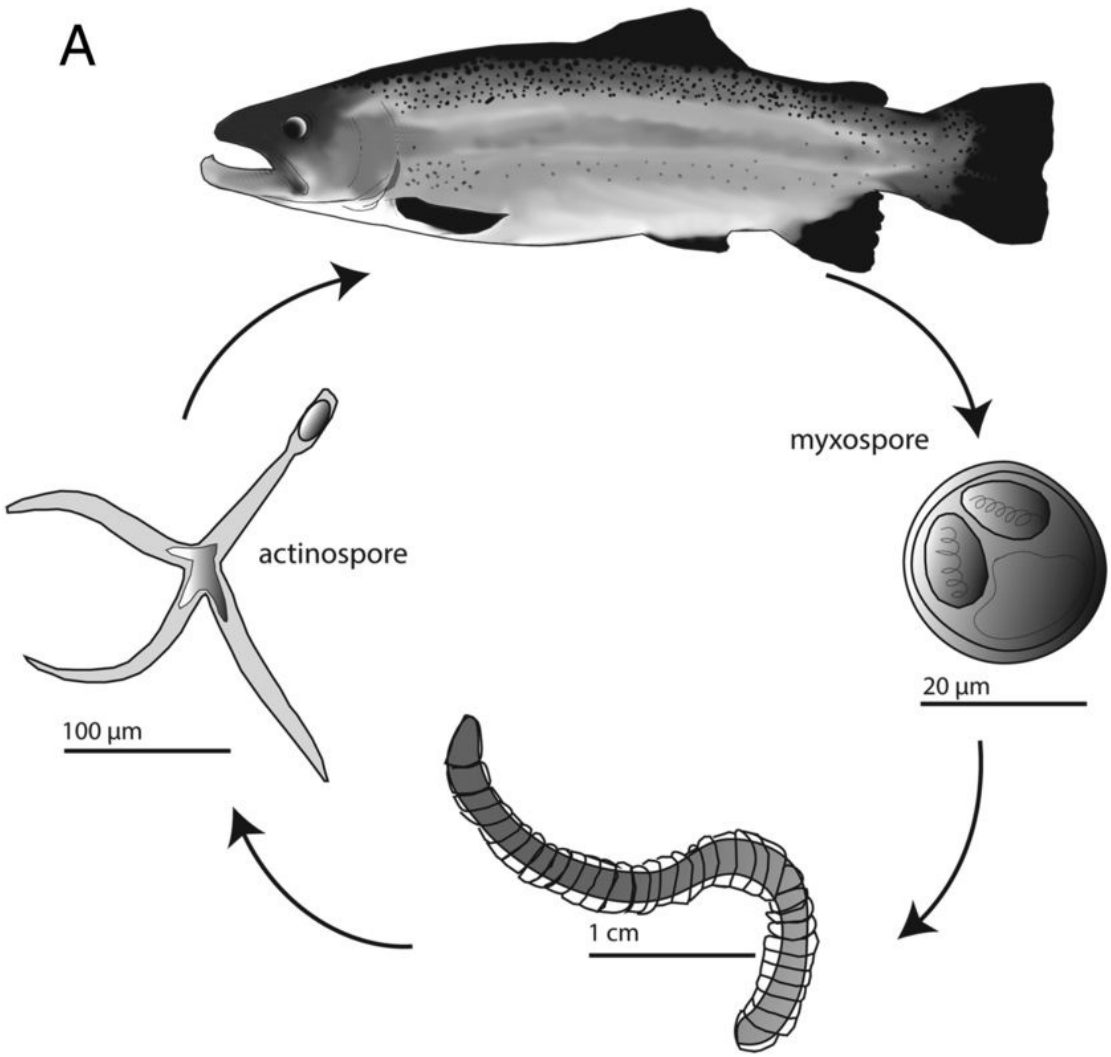


Figure 1.1: A generalized myxozoan life-cycle with both fish (e.g. Fathead Minnow) and oligochaete (e.g. *Tubifex tubifex*) hosts (from Chang et al. 2015).

Figure 1.2: Fathead Minnow shoals from Stirling Children's Pond, southern Alberta. Each minnow contains large whitish lesions located on the head, especially around the eyes, associated with development of the myxozoan *Myxobolus rasmusseni* n. sp.

CHAPTER 2: DESCRIPTION, LIFE-CYCLE, AND DEVELOPMENT OF THE MYXOZOAN, MYXOBOLUS RASMUSSENI N. SP. IN FATHEAD MINNOWS, PIMEPHALES PROMELAS, EMERGING IN SOUTHERN ALBERTA

2.1 Abstract

A new species of myxozoan *Myxobolus rasmusseni* n. sp. from the genus *Myxobolus* is described from populations of Fathead Minnow *Pimephales promelas* in southern Alberta using morphological criteria and the analysis of 18S rDNA gene sequences. Histological analyses of whole-head tissue showed that parasite development occurred within the ocular, sinus, oral cavities, and within epithelial tissues of the mandibles, pectoral girdle, opercula, and anal fin. Myxospores were absent from the gill apparatus. Mature pseudocysts that contained thousands of myxospores were associated with conspicuous lesions that deformed the head region of infected hosts. The ovoid myxospores located within pseudocysts were 15µm ± 0.4 long, 7.6µm ± 0.4 wide; the polar capsule was 7.6µm ± 0.6 long and 2.6µm ± 0.3 wide. Mature myxospores had 7-8 turns in the polar filament coil. BLASTn search in GenBank of the consensus sequence of a 1974 bp sequence of the 18S rDNA gene isolated from 12 infected minnows indicated that *M. rasmusseni* n. sp. was distinct from other myxozoan sequences and aligned with other cyprinid-infecting members of the genus *Myxobolus.* The 18S rDNA gene sequences from triactinomyxons that were released from the oligochaete *Tubifex tubifex* were 100% identical to sequences from myxospores contained within pseudocysts of infected minnows. Peaks in prevalence of lesion-bearing Fathead Minnows occurred in late summer/early fall (85.5%, 29.4%) at two infected sites. Peaks in prevalence in 18–20-month-old fish likely stemmed from exposure to TAMs as young-of-the-year

juveniles, followed by a 12-18 month period of development to the lesion stage. *Myxobolus rasmusseni* n. sp. appears to be emerging infectious disease within Fathead Minnow populations in southern Alberta, Canada.

2.2 Introduction

Species-level identification of myxozoans of freshwater fishes remains a challenge due to their small size and uniform morphology. Thus, new species diagnoses require information from a combination of traditional and molecular approaches. Evaluation of life-stage morphological dimensions, ultrastructural analyses, host tissue selection, and molecular sequencing are often required to differentiate among the many cryptic species (Cech et al., 2012; Eiras et al., 2014). Presently, approximately 850 species within the speciose genus *Myxobolus* have been described and new species descriptions are frequently being published (Eiras et al., 2014; Lom & Dykova, 2006; Naldoni et al., 2019). Furthermore, the life cycles of many of these species are not fully resolved, making identification and interpretation of complex developmental life-stages difficult (Eiras et al., 2014; Feist et al., 2015). Of the approximately 2400 myxozoan species known (Alama-Bermejo & Holzer, 2021), only about 50 have even a partially described life-cycle and fewer of these include a successful laboratory transmission study. Indeed, only six species of myxozoans are known in which both hosts in the life-cycle have been molecularly confirmed and in which the life cycle has been experimentally completed in the laboratory (Eszterbauer et al., 2015; Fontes et al., 2015). Thus, almost 40 years following the initial life cycle confirmation of *Myxobolus cerebralis* (Markiw & Wolf, 1983), the causative agent of whirling disease in salmonid fishes, these important parasites of fishes remain enigmatic.

Fathead Minnows, *Pimephales promelas,* are infected with numerous species of nematodes, cestodes, digenean and monogenean trematodes, microsporidians, and myxozoans (Sandland, 1999; Purdy, 2011; Wisenden et al., 2012; Lari et al., 2017; Ahn, 2019). The myxozoan fauna in Fathead Minnows sampled from across their extensive geographical range is reasonably-well characterized and includes: *Myxobolus hyborhynchi* (Cone & Frasca, 2002)*, Myxobolus hendricksoni* (Mitchell et al., 1985), *Unicauda magna* (Minchew, 1981)*, Myxobolus angustus* (Kudo, 1934; Purdy, 2011)*, Myxobolus aureatus,* and *Myxobolus hoffmanus* (Eiras et al., 2005; Lom & Dykova, 2006; Purdy, 2011; Eiras et al., 2014). The results of previous research completed in the Goater laboratory over the past 20 years has described various components of the parasite assemblages in Fathead Minnows at the northern edge of their range in Alberta, Canada. Annual host surveys have been completed at targeted sites in the region since 2005 (Ahn, 2019). During routine parasitological surveys of Fathead Minnows from several sites in southern Alberta in 2017 (Ahn, 2019), fish with distinctive, white lesions in the head region, most notably surrounding the eyes and mouth, were first observed. Microscopic investigation of these characteristic lesions from the head of infected minnows revealed thousands of mature myxospores. My initial determinations suggested that these were distinct from other reported cyprinid-infecting myxozoans based on tissue site selection and spore morphology.

In this study, I describe *Myxobolus rasmusseni* n. sp. isolated from lesions located in the head region of Fathead Minnows collected from sites in southern Alberta where this forage fish tends to dominate the piscine community. As part of ongoing parasitological surveys of fish in this region (Sandland et al. 2001; Ahn, 2019), I first

describe the myxozoan assemblage in Fathead Minnows. Next, I combine standard morphological, molecular, and phylogenetic procedures to describe *M. rasmusseni* n. sp. in Fathead Minnows and use molecular tools to characterize its life-cycle. Lastly, I monitor the time-course of development between exposure and lesion development in Fathead Minnows by following patterns of infection within the 2020 cohort within 2 infected ponds over a period of 18 months.

2.3 Materials and Methods

2.3.1 Host collection

Fathead Minnows infected with mature *Myxobolus rasmusseni* n. sp. pseudocysts had ocular, sinus, and oral cavities that were often grossly disfigured (Fig.2.1). More rarely, the epithelial layers of the opercula, the mandibles, the pectoral girdle, and the anal fin were also disfigured. To obtain appropriate parasite and host material for morphological and molecular diagnoses, I collected minnows from sites where disfigured minnows were visually obvious in fish observed from the shoreline (Table 2.1). University Pond (49.68107, -112.87152) was the main collection site. This pond is adjacent to the Alberta Water and Environmental Science Building on the University of Lethbridge campus and minnows showing clear signs of infection could easily be collected. Coulee Creek (49.65635 N, -112.78519 W), located 6 km south-east of University Pond, was also a common and convenient sampling site. Minnows from other known infected sites were collected to supplement my samples of host and parasite tissues (Table 2.1). At each of the two sites, juvenile and adult minnows were collected from overnight sets of Gee minnow traps following standard methods (Sandland et al., 2001).

Tubificid worms were collected from University Pond in the last week of August 2019. Collections involved 15-20 kick-net samples taken approximately 1 m apart along the eastern edge of the pond. All contents of each net sweep were washed into a bucket, filtered through a 2mm mesh, and then resuspended in dechlorinated water. Tubificid worms were isolated from the bulk contents, divided into tissue culture test plates (SPL life sciences) with subsets of ten worms, and left overnight in 3 ml of dechlorinated water. The subsets were then screened for the presence of TAM's using crystal violet stain (Nehring et al., 2003). Subsets of worms that were confirmed to contain TAM's were then divided individually into vials with dechlorinated water and the previously described methods were repeated so that individual TAMs could be matched with individual infected worms.

The course of development of *Myxobolus*-induced lesions was evaluated qualitatively by monitoring infection in the 2020 cohort of minnows at two sites. The sampling procedure was designed to follow the onset of lesion development over the two-year life span of minnows in this region. Collections originated from two sites where high densities of lesion-infected adult fish were observed in fall, 2019 – Coalhurst Stormwater Pond (49.744667 N, -112.93666 W) and McQuillan Reservoir (49.6471135 N, -112.4593580 W) (Table 2.1). Samples of fish from the 2020 cohort were collected in fall, 2020, spring 2021, and fall, 2021 from the two sites. Adults were collected from overnight sets of six Gee minnow traps set along the shoreline approximately 2 m apart and at approximately 1.5 m depth (Sandland et al., 2001). All adults collected in each trap were pooled into a single aerated container. Individuals were then removed haphazardly (n=100), measured for standard length, scored for the presence of lesions on the body

surface, and then released. An additional sample of young-of-the-year was collected using a dip net from the same region of the pond that the Gee traps were set. Samples of Young of the year (YOY) were pooled into a single aerated container located at the edge of the pond. Individuals were removed haphazardly (n=50), photographed, and then released. Standard length of each YOY was measured from the digital images with ImageJ.

2.3.2 Histological procedures

Following capture, live minnows were transported to the University of Lethbridge Alberta Water and Environmental Science Building where they were euthanized and immediately fixed in 10% neutral buffered formalin. Minnows were removed from the fixative after three days. To ensure optimal penetration of the chelating agent, Ethylenediaminetetraacetic acid (Matisz & Goater, 2010), the tip of the snout and a section located approximately 3 mm behind the anal fins was removed with a scalpel. The fish were then placed into 10% ethylenediaminetetraacetic acid to decalcify the bones following standard protocols (Matisz & Goater, 2010).

Individual minnows were coronally sectioned into 4 mm pieces and dehydrated, infiltrated with paraffin wax, and embedded into paraffin blocks following standard histological tissue processing methods (Matisz & Goater, 2010). The fish were then sectioned into 5 μm pieces using a microtome (American Optical Corporation “820” Spencer). Sections were mounted onto microscope slides precoated in subbing solution and stained following Gill’s 3 III Hematoxylin and Eosin Protocol (Cone & Frasca, 2002). The stained slides were then sequentially viewed using light microscopy and

imaged using Zeiss Axioskop Imager MI and measured using MBF stereoinvestigator software, and then formatted in ImageJ.

2.3.3 Light and transmission electron microscopy

Fathead Minnows with obvious pseudocyst swellings in the head region (Fig. 2.1) were euthanized and infected tissues were observed using light microscopy. Smears were prepared from fresh dissections of pseudocyst tissue, viewed under light microscopy (Zeiss Axioskop 40, Carl Zeiss Microimaging, Germany), and imaged using Canon Powershot A640. Individual mature myxospores (n = 50) from 10 Fathead Minnows were measured for spore dimensions using an ocular and stage micrometer. Dimensions measured were total spore length, total spore width, polar capsule length, polar capsule width, and the number of turns in the polar filament coil. Each metric is reported as mean ± standard deviation. Methylene Blue stain was applied to slides to facilitate observations of the polar capsule in mature myxospores and then imaged with Canon Powershot A640. Similarly, Lugol's iodine was applied to selected slides and observed under light microscopy to determine the shape and character of the mucous coat surrounding myxospores and iodinophilous vacuole (Liu et al., 2016).

To prepare host and parasite tissue for TEM, four infected Fathead Minnows were euthanized, and the heads were dissected diagonally to isolate the eyes and the ocular cavity. The isolated tissue was fixed in Karnovsky's fixative for 24 hours and then transferred into 0.1% sodium cacodylate overnight (Matisz et al., 2010). These tissues were then post-fixed in cacodylate buffered (0.1M, pH 7.2) 1% osmium tetraoxide for one hour. The tissues were then rinsed for 15 minutes in reverse osmosis water before dehydration through a graded ethanol series. The tissues were then transitioned through a

graded series of ethanol and propylene oxide, and eventually transitioned into pure Epon resin (Matisz et al., 2010; Doug Bray personal communication). Once embedded in Epon resin, the tissues were sliced at 60 nm on Reichart OM-U2 ultramicrotome and stained with uranyl acetate and Reynold's lead citrate. The slices were then imaged using the Hitachi transmission electron microscope in the Central Analytical Facility of the Science Commons Building at the University of Lethbridge with an acceleration of 100 kV.

2.3.4 DNA extraction, PCR, and sequencing

I extracted DNA from three tissue sources. First, I extracted myxozoan DNA from the lesion tissues of minnows, then DNA from the infected worms to identify the worm species, and third the myxozoan DNA from within the infected worms. Primers that target the 18S rDNA gene of myxozoans, ERIB1-ACT1r and Myxgen4f-ERIB10, were used to transcribe myxozoan sequences, and the LCO and HCO primers were used to transcribe worm DNA. First, putative myxozoan DNA was extracted from pseudocyst tissue from Fathead Minnows (n = 6, University Pond) using the Qiagen DNeasy Blood and Tissue Kit. Two minor modifications included an elution of 50 µl and a minimum digestion of 2 hours at 56◦C. Further, to confirm the final host of the life cycle of *M. rasmusseni* n. sp., tubificids that were known to be releasing TAM's were selected haphazardly for molecular analyses. To assist in host identification, DNA was extracted from the tubificid worms (n=10) from University Pond and then also extracted the DNA from the myxozoan parasites that infected these individual worms (n =10) according to the manufacturer's guidelines using the DNeasy Blood and Tissues extraction kit from Qiagen. One modification included an elution of 50 µl. Prior to tissue digestion, ethanol was removed by opening the microcentrifuge tube and allowing for evaporation for

approximately 16 hours. The University Pond site was selected for its high density of pseudocyst-induced deformities in yearling minnows and for its high densities of tubificid worms in the muddy substrate.

Myxozoan DNA isolated from the pseudocyst tissue from Fathead Minnow lesions and from within the infected oligochaetes was amplified using the ERIB1-ACT1r and Myxgen4f-ERIB10 primer sets that target the 18S rDNA gene. For the samples extracted from Fathead Minnow, the sets of primers ERIB1-ACT1r and Myxgen4f-ERIB10 (Barta et al., 1997; Diamant et al., 2004; Hallett & Diamant, 2001) produced an overlapping region of the 18S rDNA gene and combined to yield 25 µl of total reagent. The initial PCR denaturation was 95◦C for 3 minutes, followed by 35 cycles of 95◦C for 30 seconds, 55◦C for 45 seconds, 72◦C for 1 minute, 15 seconds, and the final elongation at 72◦C for 7 minutes. The product was run on 1% agarose gel, then the appropriate band size was cut out and extracted with GeneJet Gel Extraction Kit. For the samples extracted from infected tubificids, PCR reaction volumes were 12.5 µl, with 6.25 µl of 2X enzyme mix, 1µl of nuclease free water, 5 µl DNA, and 125 nM primer concentrations. The thermocycle profile was: 95◦C for three minutes, followed by 45 cycles of 94◦C for 30 seconds, 55◦C for 45 seconds, 72◦C for one minute and 15 seconds, and a final elongation at 72◦C for seven minutes. The amplicons were run on a 1% agarose gel, extracted using the GeneJet Gel Extraction Kit.

PCR-amplified partial sequences of the Folmer region of the CO1 gene of the tubificid worms using 10 µl IDT PrimeTime master mix reaction volumes with 4 µl of extracted oligochaete DNA and LCO and HCO primers (Folmer, 1994) of 250 nM concentrations. Denaturing began with 95◦C for 5 minutes, followed by a series of cycles:

35 cycles of 95◦C for 40 seconds, 44◦C for 45 seconds, 72◦C for one minute, with a final elongation of 72◦C for 8 minutes. Amplicon products obtained from amplification with the LCO and HCO primers were run on 1% agarose gel and subsequently extracted with the GeneJet Gel Extraction Kit.

The purified amplicon sequences of the extracted oligochaete and parasite DNA were Sanger sequenced by Macrogen (Korea), where the forward and reverse sequences were produced using the same LCO and HCO primers, and ERIB1-ACT1r and Myxgen4f-ERIB10 primers, respectively. All PCR products were sequenced in both directions with their respective PCR primers.

Sequence quality of the oligochaete DNA was evaluated and trimmed using the 4peaks (Nucleobytes) software and snapgene viewer, and the primer regions were trimmed prior to alignment of the forward and reverse segments in Geneious Prime2019 (https://www.geneious.com, 2019). The consensus sequence from each oligochaete sample was then compared against other catalogued species in the NCBI GenBank BLASTn database. Finally, each consensus sequence was aligned with every representative species in GenBank that shared greater than 80% sequence homology to produce a percent identity matrix.

2.3.5 Phylogenetic analyses

18 S rDNA sequences that originated from pseudocyst tissue were aligned in MEGA 7.0, and manually edited to obtain a consensus sequence of 1974 bp from the overlapping of the two 18S rDNA gene primer sets. GenBank BLAST confirmed the sequences to be in the *Myxobolus* genus. The consensus sequences were aligned in MEGA 7.0 (Kumar et al., 2016) using MUSCLE with the 18S rDNA gene sequences of

41 other cyprinid-infecting myxobolid species available in GenBank. This overall set of species was chosen based on sequence similarity. The sequences were manually edited in MEGA 7.0 which resulted in a 962 bp alignment of the 42 species. Phylogenetic analyses were carried out using the general time reversible model (GTR+G) as determined by Akaike information criterion values in MEGA 7.0, and a maximum likelihood analysis tree was generated with confidence bootstrap estimates (Felsenstein, 1985; Nei & Kumar, 2000). Furthermore, Bayesian analyses were performed in MrBayes using the general time reversible model (GTR+G+I) as determined by Akaike Information Criterion. The Bayesian analyses performed two independent runs for one million generations with sampling at every 100 generations. As per software defaults, the cold chain abandoned the first 25% as a relative burnin (Ronquist & Huelsenbeck, 2003). *Ceratomyxa shasta* was chosen as the outgroup for phylogenetic analyses of the 18S rDNA gene dataset (Bartholomew et al., 1997).

2.4 Results

2.4.1 Description of Myxobolus rasmusseni n. sp.

Family *Myxobolidae Thélohan, 1892*

Genus *Myxobolus Bütschli, 1882*

Myxobolus rasmusseni n. sp.

Type host: Fathead Minnow *Pimephales promelas* (Cypriniformes: Cyprinidae)

Type locality: Several sites in Southern Alberta: Reesor Lake and Spruce Coulee Reservoir, Cypress Hills Interprovincial Park, University Pond, Coulee Creek Stormwater

Pond, McQuillan Reservoir, Stirling Children's Pond. Site coordinates and site characteristics are described in Ahn (2019).

Host Tissue: ocular, sinus and oral cavities, epithelium of the inner and outer surface of the operculum, epithelial tissues associated with the mandibular region, along the pectoral girdle, base of anal fin.

Etymology: The new species is named after Dr. Joseph Rasmussen in recognition of his important contributions to our understanding of aquatic ecosystem ecology.

2.4.2 Morphological description

Myxospores have a general ovoid shape, 14.7 – 15.5 μm long (15.1 ± 0.4 μm; n = 50) and 7.2 – 8.0 μm (7.6 ± 0.4 μm; n = 50) wide (Table 2.4). They are composed of two shell valves with a suture that runs lengthwise along the midline. The suture has two additional projections that run lengthwise directly above, and beneath, which are most visible at the posterior end of the myxospore (Fig. 2.3). The outer surface of the spore appears to have additional projections that run lengthwise proximal to the ventral surface of the myxospore in frontal view. The mature myxospore has two tear-drop shaped polar capsules 7.0– 8.2 μm long (7.6 ± 0.6; n = 50), 2.3 – 2.9 μm wide (2.6 ± 0.3; n = 50), that contain a polar filament with 7-8 turns in the coil. The sporoplasm is binucleate and located basally in the myxospore. The sporoplasm occupies most of the posterior region of the myxospore and contains a large iodinophilous vacuole (Fig. 2.3). The surface of the relatively thin-walled capsule shell contains a small mucous coat that is thicker near the posterior end.

2.4.3 Myxozoan assemblages in Fathead Minnows in southern Alberta

Adult Fathead Minnows collected from four ponds/lakes in southern Alberta were infected with at least 5 species of myxozoan (Table 2.5). Three species were identifiable to species based on spore morphology and host tissue site specificity: *Myxobolus hendricksoni* (Mitchell et al., 1985)*, Myxobolus hyborhynchi* (Cone & Frasca, 2002)*,* and *Unicauda magna* (Minchew, 1981)*.* Examination of histological sections revealed multiple cases of coinfection of individual fish. One Fathead Minnow from Bathing Lake was infected with myxospores of *M. hendricksoni,* and *M. hyborhynchi.* Another minnow from University Pond was infected with *M. rasmusseni* n. sp. and *Myxobolus* sp. One minnow from Spruce Coulee Reservoir was co-infected with *M. rasmusseni* n. sp. and *Unicauda magna.* Coulee Creek Stormwater Pond contained minnows infected with *M. rasmusseni* n. sp., *Unicauda magna,* and *Myxobolus* sp.

2.4.4 Pseudocyst description and histopathological observations

The lesions most commonly present as exopthalamia, or bulging of the eyes, and whitish mucous swellings of the sinus, the oral cavity, and the epithelial tissues of the opercula, pectoral girdle, mandibles, and anal fin (Fig. 2.1). In many cases, the development of lesions adjacent to the ocular cavity was associated with obvious protrusion of the eyes (Fig. 2.1). Similarly, lesions associated with the nasal and oral cavities were associated with complete obstruction of the olfactory chamber in which the olfactory lamellae protrude from the chamber itself. In yet other minnows, the oral cavity was completely obstructed by pseudocyst tissue such that the mouth was either permanently open, or permanently closed. Dissection of these tissues led to the release of white/yellow fluid.

2.4.5 Histology of the host-parasite interface

Myxobolus rasmusseni n. sp. myxospores were observed in histological sections of minnows collected from three of the four sites sampled: Coulee Creek Stormwater Pond, University Pond, and Spruce Coulee Reservoir. *Myxobolus rasmusseni* n. sp. myxospores were observed in the epithelial layers of the opercula, in the epidermal layers of the mandibles, along the epidermal tissues of the pectoral girdle, and in the pharangeal mucosal layers of the mouth (Fig. 2.2A). The sequence of images in Fig. 2.2 indicate that the internal structure of pseudocysts involves loose aggregates of discrete package-like structures (Fig. 2.2B) containing myxospores (Fig. 2.2C). Transmission electron imaging of host and parasite tissue from the ocular cavity of infected minnows revealed the presence of host macrophages, most recognizable of these being rodlet cells, aggregated within the pseudocyst tissue interspersed with mature myxospores (Fig. 2.4). Within the ocular cavity, the pseudocyst tissues come into close contact with identifiable host tissues, such as the optic nerve and striated muscles, where there are no obvious signs of parasite invasion or host immune response. However, there were obvious signs within the ocular cavity of hemorrhaging of blood vessels that are in close association with the pseudocyst (Tilley, unpublished data).

2.4.6 LM and TEM observations

Tissues of the ocular cavity of minnows infected with *Myxobolus rasmusseni* n. sp. were infested with myxospores. The myxospores were abundant and easily identified by their overall shape and the characteristic polar capsules (PC) which encapsulate the polar filament (PF) (Fig. 2.3). The myxospores were completely encapsulated by a membrane that appeared to be relatively thin. The sporoplasm occupied the posterior

space of the spores. The mature myxospores had a binucleate sporoplasm and an additional nucleus was visible in the anterior region of the spore. The anterior nucleus was located directly beneath the polar capsules (Fig. 2.3). Several myxospores were found in doublets and appeared to share a mucous membrane (Fig. 2.3).

2.4.7 Molecular characterization and phylogenetic analyses

Maximum likelihood analyses of the *M. rasmusseni* n. sp. consensus 18S rDNA gene sequence with other selected species of *Myxobolids* showed that *M. rasmusseni* n. sp. groups into a larger clade of gill-infecting species of cyprinid fishes (Fig. 2.7). This clade included: *M. bilobus* (Cone et al., 2005)*, M. n. sp. EzoUgui* (Yustinasari et al. unpublished; LC544125, 93.1%), *M. obesus* (Gurley, 1893)*, M. hungaricus* (Baska & Molnár, 1999)*, M. szentendrensis* (Cech et al., 2015)*, M. dujardini* (Mitchell, 1989)*, M. alvarezae* (Cech et al., 2012) *M. eirasianus* (Cech et al., 2012), *M. sitjae* (Cech et al., 2012), and *M. intimus* (Cech et al. 2012). A BLASTn search of the 18S rDNA gene sequence in GenBank showed that *Myxobolus rasmusseni* n. sp. was dissimilar to all other myxozoan sequences catalogued. The *M. rasmusseni* n. sp. sequence was 93.1% similar to an unnamed new Myxobolid species, *Myxobolus* sp. EzoUgui, reported from fishes in Japan (Yustinasari et al. unpublished; LC544125), and 92.2% similar to *M. bilobus* (Cone et al., 2005; DQ008579) (Fig. 2.7).

Phylogenetic analyses placed *M. rasmusseni* n. sp. in a clade of morphologically-similar species possessing an ovoid mature myxospore with a tapered anterior end and two interior polar capsules. The dimensions of the myxospores and polar capsules varied in the species that molecular analyses identified as closely related (Table 2.3). The site

selection of Myxobolids in this clade was generally the respiratory tissues of cyprinid fishes (Table 2.3).

2.4.8 Confirmation of the Myxobolus rasmusseni n. sp. lifecycle

18S rDNA gene sequences amplified from the ten TAM-releasing worms identified two species of myxozoan that corresponded to two species of annelid worm in the family Tubificidae identified by sequencing of the Folmer region of the COI gene through the LCO and HCO primers (Table 2.3). Sequences from eight of the worms were between 96-99% similar to known sequences of *Ilyodrilus templetoni,* and two of the ten worms were 100% similar to *Tubifex tubifex*. Both myxozoan DNA sequences were compared against the consensus sequence generated for *M. rasmusseni* n. sp. and to other sequences available in GenBank. The myxozoan 18S rDNA gene sequences recovered from the two *Tubifex tubifex* worms were a 100% match with the consensus sequence of *Myxobolus rasmusseni* n. sp. that was sequenced from the pseudocyst from tissue in minnows (Table 2.2). The myxozoan 18S rDNA gene sequences recovered from the eight *Ilyodrilus templetoni* worms had closest sequence similarity between 93-94% in GenBank to *Dicauda athernoidi* (Loch et al., 2017), a myxozoan that infects the head region of emerald and mimic shiners in North America.

2.4.9 Time course of lesion development

Lesions were absent in samples of YOY fathead minnows collected at McQuillan Reservoir and Coalhurst Stormwater Pond (Fig 2.6A, B). There was a single exception (1% of a total of 100 YOY minnows collected from both ponds) in a larger sized YOY from Coalhurst Stormwater Pond. In contrast, all 1-yr old minnows collected from Coalhurst Stormwater Pond had characteristic lesions, whereas lesions were absent in 1-

yr old fish from McQuillan Reservoir sampled at the same time (June 2021; Fig 2.6B). Four months later (Sept. 2021), 29.4% and 85.5% of 16 mo. old minnows harboured lesions at McQuillan Reservoir and Coalhurst Stormwater Pond, respectively.

2.5 Discussion

Myxospores of *Myxobolus rasmusseni* n. sp. have distinct dimensions, they target host tissue other than the gills, and the development of pseudocysts is associated with conspicuous inflammatory lesions that are distinct from other species of cyprinid-infecting myxozoan (Minchew, 1981; Mitchell et al., 1985; Cone & Frasca, 2002). Furthermore, compared to other closely- related species found in GenBank such as *M. tanakai* and *M. bilobus* (Cone et al., 2005; Kato et al., 2017). Although *M. rasmusseni* n. sp. myxospores share similarities in tissue site selection to *M. mutabilis,* described from bluntnose minnow *Pimephales notatus,* overall myxospore morphology and pseudocyst development in the ocular, sinus, and oral cavities are not consistent with this species (Kudo, 1934). Similarly, *M. angustus* shares similarities to *M. rasmusseni* n. sp. in myxospore dimensions but this species forms indistinct pseudocysts within the gill filaments of its host, the bullhead minnow, *Pimphales vigilax* (Kudo, 1934). Unfortunately, there is no sequence datum available for *M. angustus,* or for other myxozoans that have been reported from Fathead Minnows such as *M. hyborhynchi, M. hendricksoni,* or *Unicauda magna.* The combined evidence from interspecific morphological comparisons, patterns of within-host parasite development, and from comparative 18S rDNA gene sequence analyses support the new-species designation for *M. rasmusseni* n. sp.

Myxozoan biodiversity within Fathead Minnows in southern Alberta was undocumented prior to this study. Histology of Fathead Minnows from the four sites reveal that there is a species richness of at least 5 species of myxozoans in southern Alberta. This richness is likely conservative considering the small sample size and the limited number of sites. The myxozoans that were identifiable to the species level in Albertan Fathead Minnows: *Myxobolus hyborhynchi* (Cone & Frasca, 2002), *Unicauda magna* (Minchew, 1981), and *Myxobolus hendricksoni* (Mitchell et al., 1985) have only previously been documented from fathead minnows in Minnesota, USA, Pennsylvania, USA, and Iowa, USA, respectively. Furthermore, it appears that concurrent infections of minnows with more than one species of myxozoan is common.

Emerging infectious diseases are defined as pathogens that have either recently experienced expansion in incidence or geographic range or have recently been discovered or evolved (Daszak et al., 2001). The recent detection of *M. rasmusseni* n. sp., in Fathead Minnows from multiple waterbodies since 2016 where it had never been detected before suggests that it is emerging in Fathead Minnow populations in southern Alberta. Previous host surveys involving complete necropsies of individual minnows have occurred at these sites, intermittently, for over 20 years (Ahn, 2019; Sandland et al., 2001, Goater, personal communication). In addition, intensive surveys completed by researchers in our laboratory have been done annually on samples of minnows at McQuillan Reservoir since 2005 (James et al., 2009; Matisz & Goater, 2010; Stumbo et al., 2012). The distinctive lesions that contain *M. rasmusseni* n. sp. pseudocysts were first observed in minnows in 2017 at this site and have been observed each year since. Despite this extensive survey effort spanning numerous sites over numerous years, myxospores of *M. rasmusseni* n. sp.

have never been observed, nor have fish with the characteristic lesions been observed prior to 2017. Given the cryptic nature of myxozoans in general, particularly involving their tiny myxospores within host tissues, under-reporting should be expected. Yet the conspicuous nature of pseudocyst development on the head and body, the persistence of the deformities, and the erratic swimming behaviour of these pseudocyst-infected fish makes it unlikely that infection would have been missed. Although I recognize that direct demonstration of parasitic emergence is difficult, my evidence from host surveys across broad temporal and spatial scales support the idea that *M. rasmusseni* n. sp is an emerging parasite of this important species of forage fish. Unfortunately, the origin of *M. rasmusseni* n. sp. in Fathead Minnows is unknown. Furthermore, there are no datum available regarding infection in other species of sympatric cyprinids that are present at sites where *M. rasmusseni* n. sp. occurs in Fathead Minnows.

The distinctive lesions that are characteristic of infected minnows that contain mature pseudocysts appear to be associated with a host inflammatory response. Indirect evidence supports the idea that the swellings are caused by a component of the minnow inflammatory response. Tissues of the ocular cavity that were visualized with TEM revealed thousands of myxospores interspersed with a variety of host cells, such as eosinophils, macrophages, neutrophils, mast cells, and rodlet cells. Although enigmatic, there is a general consensus that rodlet cells are a key component of the inflammatory immune response to parasitic infections in tissue of teleost fishes (Manera & Dezfuli, 2004; Matisz et al., 2010; Dezfuli et al., 2015). Rodlet cells were abundant within pseudocyst tissues of Fathead Minnows infected with *M. rasmusseni* n. sp. Previous studies have identified rodlet cells in Fathead Minnows as a significant component of the

host's inflammatory response to other parasitic infections (Matisz et al., 2010). Similarly, Matisz et al. (2010) found that rodlet cell density increased in the optic lobes of Fathead Minnows that were exposed to cercariae of the trematode *Ornithodiplostimum ptychochelius.*

The complete life cycles of species in the genus *Myxobolus* have been established for approximately 5% of the described species in the genus (Li et al., 2012; Eszterbauer et al., 2015). My results confirm the life cycle of *M. rasmusseni* n. sp within at least one known site of infection. DNA sequences amplified from the pseudocysts of infected Fathead Minnows were an identical match to the sequences amplified from TAM-releasing *Tubifex tubifex* that were collected from University Pond. Confirmation of the utilization of *T. tubifex* for the sexual stages of *M. rasmusseni* n. sp. is not a surprising result. *Tubifex tubifex/*fish life cycles have been described for other myxozoans in the genus *Myxobolus*, including whirling-diseasing causing *M. cerebralis* (Lom & Dykova, 2006; Eszterbauer et al., 2015). Unfortunately, my efforts to further confirm the life cycle by exposing egg-reared juvenile Fathead Minnows to the matching TAMs were interrupted by Covid-19 restrictions in fall 2020.

Qualitative analyses of serial size-frequency distributions and infection data at two sites show that the time-course of development of lesions is approximately 12 mo. to 16 mo. Minnows that hatch in May-June each year are likely first exposed to TAMs during their peak release from *T. tubifex* in June/July (Tilley, unpublished observations) but do not appear to present lesions until the following June, approximately 12-mo later. The alternative, that the June collected minnows (approximately 1-yr old) from Coalhurst Stormwater Pond that had lesions were exposed a few weeks prior, seems to be unlikely

given what is known about myxospore development in other fish. Thus, lesion development appears to require at least one year and is fully expressed in minnows approaching the second winter. These results parallel those from laboratory studies that have shown that myxospore development times vary in fish and many require between 40-80- days post TAM exposure (El-Matbouli et al., 1995; Szekely et al., 2009). Similarly, surveys of juvenile cyprinid fishes in the UK found mature myxospores in fish that were approximately three to four months of age (Longshaw et al., 2005).

The results of the field sampling suggests that timing of hatching may also be an important component of lesion development. Average fork length of young-of-the-year sampled in September 2020 from Coalhurst Stormwater Pond, was 29.2 mm whereas fork length of the young-of-the-year at McQuillan Reservoir sampled at the same time was only 13.6 mm. Fathead Minnows are known to be variable in spawning time and those that are hatched in early summer tend to be larger than those spawned later in the summer (Divino & Tonn, 2007). Therefore, the June 2021 fish from McQuillan Reservoir may not have developed lesions because they were spawned later in the summer and missed the window of peak TAM transmission. Their smaller size may have also reduced their probability of encountering swimming TAMs. Taken together, these results indicate that both the timing of TAM exposure and the timing of spawning and egg hatch are important components of the expression of lesions in wild fish.

The establishment of *M. rasmusseni* n. sp. in University Pond and other sites in southern Alberta (Ahn, 2019) highlights the ability for myxozoans to rapidly colonize disconnected habitats. University Pond was constructed in 2009 as a habitat feature following the construction of the Alberta Water and Environmental Science Building on

the University of Lethbridge campus. The pond was drained in 2015 to accommodate repairs to the lining of the pond. Fathead Minnows recolonized the pond in 2016, their introduction was facilitated by the movement of water between source bodies as part of the LNID irrigation system. The lesions associated with the development of *M. rasmusseni* n. sp. pseudocysts were first observed in one-year old Fathead Minnows in 2017. This sequence of wetland development requires that the young-of-the-year of the 2016 cohort were exposed in the same summer that the wetland was filled. This, in turn, requires that either infected *T. tubifex* or infective myxospores survived the draining process in 2015, or that the oligochaetes were exposed to infective stages in the same year that the pond was filled. Regardless of which of these scenarios occurred, *M. rasmusseni* n. sp. established in both of its required hosts within a single year and have persisted in the minnow population since. The establishment of the myxozoan *Dicauda athernoidi* in the other species of oligochaete, *Ilyodrilus templetoni* in University Pond, further confirms the remarkable colonization ability of freshwater myxozoans.

2.6 Literature Cited

Ahn, S. 2019. *Effects of host community structure on parasite transmission and disease risk.* [Master of Science]. University of Lethbridge.

Cone, D. K., & Frasca, S. 2002. Revised diagnosis, site of development, and lesions due to *Myxobolus hyborhynchi* (Myxozoa) parasitizing fathead minnow in Minnesota. Journal of Aquatic Animal Health 14: 209–215.

Cone, D., Yang, J., Sun, G., & Easy, R. 2005. Taxonomy and molecular phylogeny of *Myxobolus bilobus* n. sp. (Myxozoa) parasitizing *Notemigonus crysoleucas* (Cyprinidae) in Algonquin Park, Ontario, Canada. Diseases of Aquatic Organisms 66: 227–232.

Divino, J. N., & Tonn, W. M. 2007. Effects of reproductive timing and hatch date on fathead minnow recruitment. Ecology of Freshwater Fish 16*:* 165-176.

Daszak, P., Cunningham, A. A., & Hyatt, A. D. 2001. Anthropogenic environmental change and the emergence of infectious diseases in wildlife. Acta Tropica, 78: 103–116.

Dezfuli, B. S., Manera, M., Giari, L., DePasquale, J. A., & Bosi, G. 2015. Occurrence of immune cells in the intestinal wall of *Squalius cephalus* infected with *Pomphorhynchus laevis*. Fish & Shellfish Immunology 47: 556–564.

Eiras, J. C., Molnár, K., & Lu, Y. S. 2005. Synopsis of the species of *Myxobolus* Bütschli, 1882 (Myxozoa: Myxosporea: Myxobolidae). Systematic Parasitology 61: 1–46.

Eiras, J. C., Zhang, J., & Molnár, K. 2014. Synopsis of the species of *Myxobolus* Bütschli, 1882 (Myxozoa: Myxosporea, Myxobolidae) described between 2005 and 2013. Systematic Parasitology 88: 11–36.

El-Matbouli, M., Hoffmann, R. W., & Mandok, C. 1995. Light and electron microscopic observations on the route of the triactinomyxon-sporoplasm of *Myxobolus cerebralis* from epidermis into rainbow trout cartilage. Journal of Fish Biology 46: 919-935.

Eszterbauer, E., Atkinson, S. D., Diamant, A., Morris, D. J., El-Matbouli, M., & Hartikainen, H. 2015. Myxozoan Life Cycles: Practical Approaches and Insights. In B. Okamura, A. Gruhl, & J. L. Bartholomew (Eds.), *Myxozoan Evolution, Ecology and Development*. (pp.175-200). Springer International Publishing.

Feist, S. W., Morris, D. J., Alama-Bermejo, G., & Holzer, A. S. 2015. Cellular Processes in Myxozoans. In B. Okamura, A. Gruhl, & J. L. Bartholomew (Eds.), *Myxozoan*

Evolution, Ecology and Development (pp. 139–154). Springer International Publishing.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3: 294-299.

Fontes, I., Hallett, S. L., & Mo, T. A. 2015. Comparative epidemiology of myxozoan diseases. In B. Okamura, A. Gruhl, & J. L. Bartholomew (Eds.), *Myxozoan Evolution, Ecology and Development* (pp. 317–342). Springer International Publishing.

James, C. T., Wisenden, B. D., & Goater, C. P. 2009. Epidermal club cells do not protect fathead minnows against trematode cercariae: a test of the anti-parasite hypothesis. Biological Journal of the Linnean Society 98: 884–890.

Kato, E., Kasai, A., Tomochi, H., Li, Y.-C., & Sato, H. 2017. Four *Myxobolus* spp. (Myxosporea: Bivalvulida) from the gill lamellae of common carp (*Cyprinus carpio*) and Japanese silver crucian carp (*Carassius langsdorfii*) in the western part of Japan, with the description of three new species (*M. tanakai* n. sp., *M. paratoyamai* n. sp., and *M. ginbuna* n. sp.). Parasitology Research 116: 2427–2441.

Kudo, R. R. 1934. Studies on some protozoan parasites of fishes in Illinois. In *Illinois Biological Monographs* (Vol. 13, pp. 1–44). University of Illinois.

Lari, E., Goater, C. P., Cone, D. K., & Pyle, G. G. 2017. Parasites and a host's sense of smell: reduced chemosensory performance of fathead minnows (*Pimephales promelas*) infected with a monogenean parasite. Journal of Animal Ecology 86: 434–441.

Li, L., & Desser, S. S. 1985. The protozoan parasites of fish from two lakes in Algonquin Park, Ontario. Canadian Journal of Zoology 63: 1846–1858.

Li, Y.-C., Sato, H., Kamata, Y., Ohnishi, T., & Sugita-Konishi, Y. 2012. Three novel myxobolid species of genera *Henneguya* and *Myxobolus* (Myxosporea: Bivalvulida) from marine fish in Japan. Parasitology Research 111: 819–826.

Liu, X.-H., Batueva, M.-D., Zhao, Y.-L., Zhang, J.-Y., Zhang, Q.-Q., Li, T.-T., & Li, A.-H. 2016. Morphological and molecular characterisation of *Myxobolus pronini* n. sp. (Myxozoa: Myxobolidae) from the abdominal cavity and visceral serous membranes of the gibel carp *Carassius auratus gibelio* (Bloch) in Russia and China. Parasites & Vectors 9: 562-573.

Lom, J., & Dykova, I. 2006. Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. Folia Parasitologica 53: 1–36.

Longshaw, M., Frear, P. A., & Feist, S. W. 2005. Descriptions, development, and pathogenicity of myxozoan (Myxozoa: Myxosporea) parasites of juvenile cyprinids. Journal of Fish Diseases 28: 489-508.

Manera, M., & Dezfuli, B. S. 2004. Rodlet cells in teleosts: a new insight into their nature and functions. Journal of Fish Biology 65: 597–619.

Markevitsch, A. P. 1932. Zur kenntnis der myxosporidien von suswasserfischen der Ukraine. Zoologischer Anzeiger 99: 297–303.

Markiw, M. E., & Wolf, K. 1983. *Myxosoma cerebralis* (Myxozoa: Myxosporea) etiologic agent of salmonid whirling disease requires tubificid worm (Annelida: Oligochaeta) in its life cycle. Journal of Protozoology 30: 561–564.

Matisz, C. E., & Goater, C. P. 2010. Migration, site selection, and development of *Ornithodiplostomum* sp. metacercariae (Digenea: Strigeoidea) in fathead minnows (*Pimephales promelas*). International Journal for Parasitology 40: 1489–1496.

Matisz, C. E., Goater, C. P., & Bray, D. 2010. Density and maturation of rodlet cells in brain tissue of fathead minnows (*Pimephales promelas*) exposed to trematode cercariae. International Journal for Parasitology 40: 307–312.

Minchew, C. D. 1981. *Unicauda magna* sp. n. (Protozoa: Myxozoa): a new myxozoan from the fin tissues of the fathead minnow*, Pimphales promelas* Rafinesque. Journal of Fish Diseases 4: 513–518.

Mitchell, L. G., Seymour, C. L., & Gamble, J. M. 1985. Light and electron microscopy of *Myxobolus hendricksoni* sp.nov. (Myxozoa: Myxobolidae) infecting the brain of the fathead minnow, *Pimephales promelas* Rafinesque. Journal of Fish Diseases 8: 75–89.

Naldoni, J., Zatti, S. A., da Silva, M. R. M., Maia, A. A. M., & Adriano, E. A. (2019). Morphological, ultrastructural, and phylogenetic analysis of two novel Myxobolus species (Cnidaria: Myxosporea) parasitizing bryconid fish from São Francisco River, Brazil. Parasitology International 71: 27–36.

Purdy, A. M. 2011. *Parasite survey of fathead minnows, golden shiners, and white suckers used as bait in Wisconsin*. [Master of Science]. University of Wisconsin-La Crosse.

Sandland, G. J. 1999. *Interactions between a brain-encysting trematode and its intermediate host, the fathead minnow*. [Master of Science]. University of Lethbridge.

Sandland, G. J., Goater, C. P., & Danylchuk, A. J. 2001. Population dynmaics of *Ornithodiplostomum ptychocheilus* metacercariae in fathead minnows (*Pimephales promelas*) from four northern-Alberta lakes. Journal of Parasitology 87: 744–748.

Stumbo, A. D., James, C. T., Goater, C. P., & Wisenden, B. D. 2012. Shoaling as an antiparasite defence in minnows (*Pimephales promelas*) exposed to trematode cercariae. Journal of Animal Ecology 81: 1319–1326.

Szekely, C., Hallett, S. L., Atkinson, S. D., & Molnar, K. 2009. Complete life cycle *Myxobolus rotundus* (Myxosporea: Myxobolidae), a gill myxozoan of common bream *Abramis brama.* Diseases of Aquatic Organisms 85: 147-155.

Wisenden, B. D., Martinez-Marquez, J. Y., Gracia, E. S., & McEwen, D. C. 2012. High intensity and prevalence of two species of trematode metacercariae in the fathead minnow (*Pimephales promelas*) with no compromise of minnow anti-predator competence. Journal of Parasitology 98: 722–727.

Table 2.1: Collection sites and sample sizes for Fathead Minnows and tubificid worms collected for this study.

Site	Year	Host	Sample size (n)	Fate of Hosts
University Pond/ Coulee Creek Stormwater Pond	2018/2019	Fathead Minnow	10	Preparation of hosts and parasite tissues
University Pond	2019	Fathead Minnow	6	18SrDNA sequencing of parasite tissues
Bathing Lake, University Pond, Coulee Creek Stormwater Pond, Spruce Coulee Reservoir	2018	Fathead Minnow	12	Histology
University Pond	2020	Fathead Minnow	4	Transmission Electron Microscopy
University Pond	2019	Triactinomyxon (TAM)-releasing oligochaetes	10	DNA sequencing
Mcquillan reservoir/ Coalhurst Stormwater Pond	2020/2021	Fathead Minnow	900	Cohort sampling

Table 2.2: Primers used to amplify the 18S rDNA gene of *M. rasmusseni* n. sp. obtained from myxospores dissected from pseudocysts from Fathead Minnows.

PRIMER	SEQUENCE (5'-3')	REFERENCE
ERIB1	ACCTGGTTGATCCTGCCAG	Barta et al. 1997
ACT1r	AATTTCACCTCTCGCTGCCA	Barta et al. 1997
Myxgen4f	GTGCCTTGAATAAATCAGAG	Bartosova et al. 2009
ERIB10	CTTCCGCGAGGTTCACCTACGG	Bartosova et al. 2009

Table 2.3: The individual sequence identity returned from GenBank for 10 TAM-releasing oligochaetes collected from University Pond, southern Alberta, and their corresponding Myxozoan sequence identity.

Worm sample number	Worm identity (% homology in GenBank)	Myxozoan Identity
MT1	*Ilyodrilus templetoni* (98%)	*Dicauda athernoidi* (94%)
MT2	*Ilyodrilus templtoni* (96%)	*Dicauda athernoidi*
MT3	*Ilyodrilus templtoni* (98%)	*Dicauda athernoidi* (93%)
MT4	*Ilyodrilus templetoni* (97%)	*Dicauda athernoidi* (93%)
MT5	*Ilyodrilus templetoni* (99%)	*Dicauda athernoidi* (94%)
MT6	*Ilyodrilus templetoni* (98%)	*Dicauda athernoidi* (93%)
MT7	*Tubifex tubifex* (100%)	*Myxobolus tanakai* (91%)
MT8	*Ilyodrilus templetoni* (98%)	*Dicauda athernoidi* (93%)
MT9	*Tubifex tubifex* (100%)	*Myxobolus tanakai* (91%)
MT10	*Ilyodrilus templetoni* (97%)	*Dicauda athernoidi*

Table 2.4: Summary of spore dimensions for species of *Myxobolus* that are morphologically and genetically similar to *Myxobolus rasmusseni* n. sp.

species	host	site	TSL	TSW	PCL	PCW	PFC	reference
***M. rasmusseni* n. sp.**	Fathead Minnow *(Pimphales promelas)*	head region	15.1± 0.4	7.6± 0.4	7.6±0.6	2.6±0.3	~7	This study
M.branchialis	Common Barbel *(Barbus barbus borysthenicus)*	gills	6.8-8.4	5.8-6.4	4.0-4.8	2.5-3.2	-	Markevitsch, 1932
M. bilobus	Golden shiner (*Notemigonus crysoleucas)*	distal gill filaments	21±0.6	8.4± 0.5	large: 10.8± 0.7 small: 10.1±0.7	large: 2.7±0.2 small 2.8±0.2	7-9	Cone et al. 2005
M. pseudokoi	Common shiner (*Notropis cornutus)*	gills, skin	13.5 (11.5-14.0)	6.5 (6.0-7.58)	6.5 (6.0-7.5)	2.5 (2.0-3.0)	6-7	Li and Desser, 1985
M. longisporus	Common carp *(Cyprinus carpio heamatopterus L.)*	gills	15.7 (15.5-16.5)	6.7 (6-8)	8.5	2.5	9 (8-10)	Nie and Li, 1992
M. koi	Common carp *(Cyprinus carpio)*	gills	15.4(14.5-16.5)	8.3(7.1-9.0)	10.1(9.0-10.9)	3.1(2.5-3.5)	10 (9-11)	Camus and Griffin, 2010
M. toyami	Common carp (*Cyprinus carpio)*	gills	14.3(13.5-15.8)	5.5(4.5-6.3)	Large capsule:5.8(5.0-6.8) Small capsule: 3.4(2.7-4.5)	Large capsule: 3.5(2.3-4.5) Small capsule: 0.8(0.5-1.4)	2-3	Yokoyama and Ogawa, 2015

Table 2.5: Summary of myxozoan species diversity in samples of Fathead Minnows collected from ponds in southern Alberta.

Species	Number of fish	Site found	Tissue site
Myxobolus hendricksoni	1	Bathing Lake	Brain
Myxobolus hyborhynchi	3	Bathing Lake, Coulee Creek stormwater pond	Visceral mesenteries
Unicauda magna	2	Spruce Coulee reservoir, Coulee Creek stormwater pond	Fin buds
Myxobolus rasmusseni n. sp.	5	University Pond, Spruce Coulee reservoir, Coulee Creek stormwater pond	Head, eyes, mouth, mandibles, pectoral girdle
Myxobolus sp.	1	University Pond	Pseudocyst in kidney

Figure 2.1: A Fathead Minnow infected with *Myxobolus rasmusseni* n. sp. surfacing in University Pond, southern Alberta, Canada. The black arrow indicates inflamed host tissue (lesions) in the ocular cavity. This host tissues contains myxospores of the myxozoan *M. rasmusseni*.

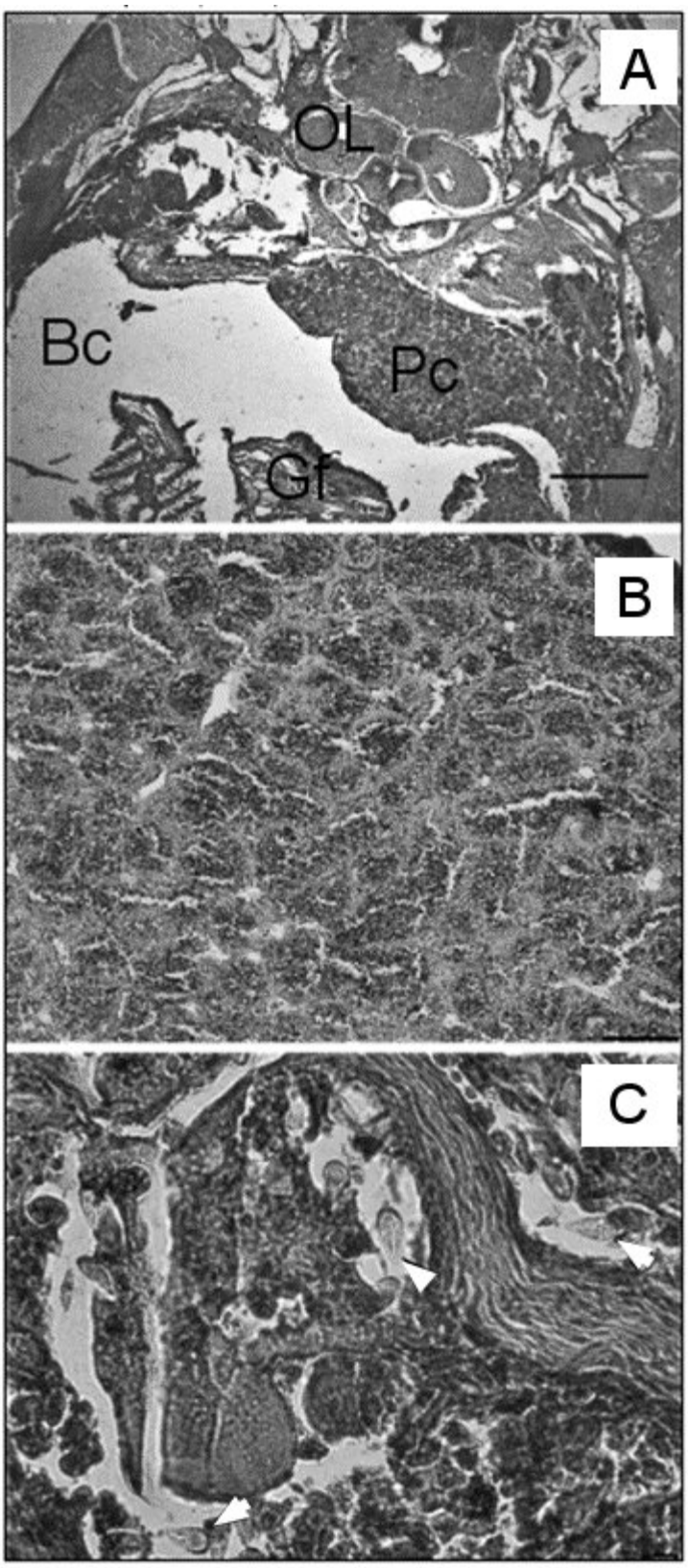

A
OL
Bc
Pc
Gf
B
C

Figure 2.2: (A-C) Sequential images of a coronal section through the head of an infected Fathead Minnow that contained pseudocysts of *Myxobolus rasmusseni* n. sp. developing in the oral cavity. (A) Landscape coronal view of the head taken at 10X magnification; labelled structures include: OL: Optic lobe; Bc: Buccal cavity; Gf: Gill filament; Pc: Pseudocyst. (Scale bar = 1000 μm). (B) Image of pseudocyst tissue from the oral cavity taken at 20X magnification (Scale bar = 100 μm). (C) Image of pseudocyst tissue taken at 100X magnification (Scale bar = 10 μm). Individual myxospores of *Myxobolus rasmusseni* n. sp. are marked with white arrowheads. 4

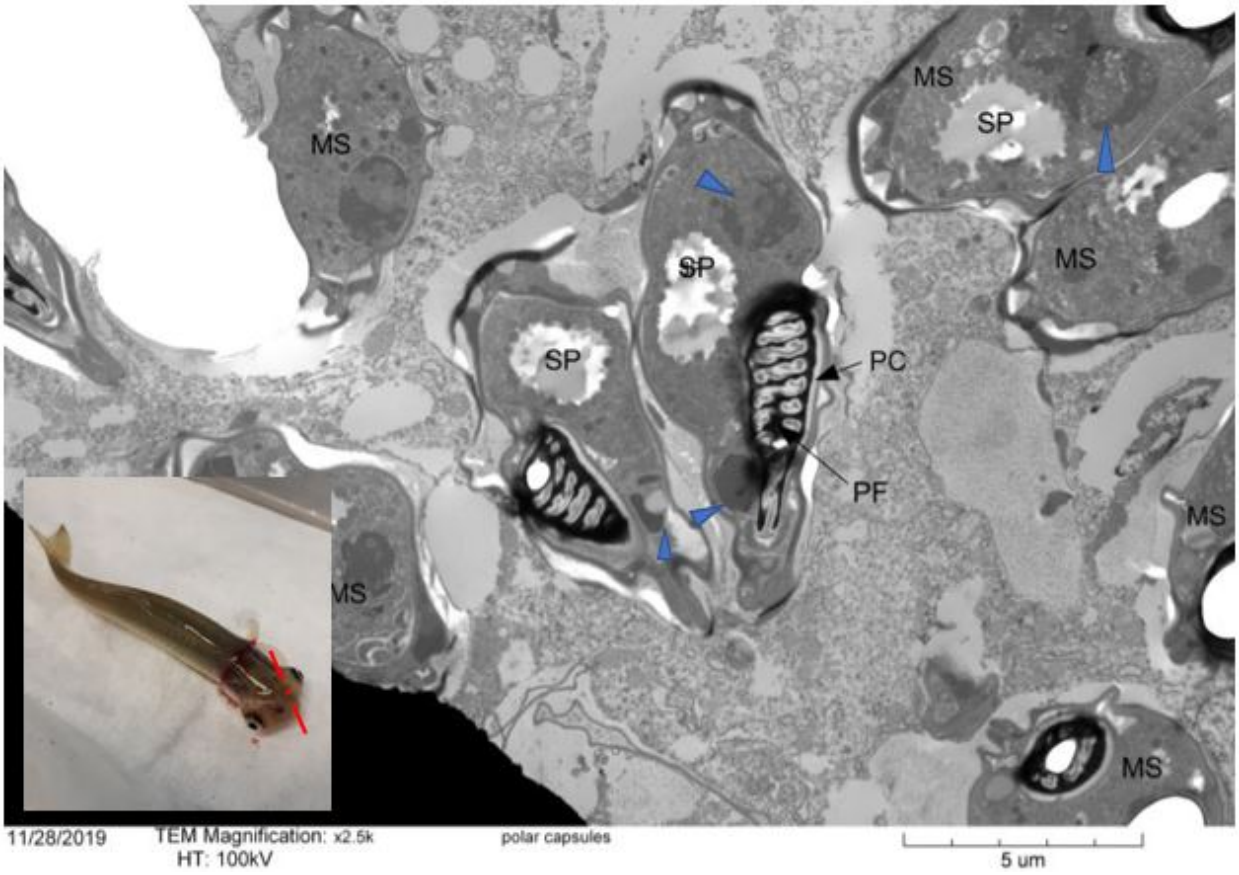


Figure 2.3: Transmission electron image of mature myxospores of *Myxobolus rasmusseni* n. sp. from the ocular cavity of Fathead Minnows. MS: mature myxospore; PC: polar capsules; PF: Polar filament, SP: sporoplasm. Arrowheads indicate nuclei. 2500X magnification. (Scale bar = 5 μm). Inset image in bottom left represents the location of the tissue excised and processed for TEM as shown by the dashed red line.

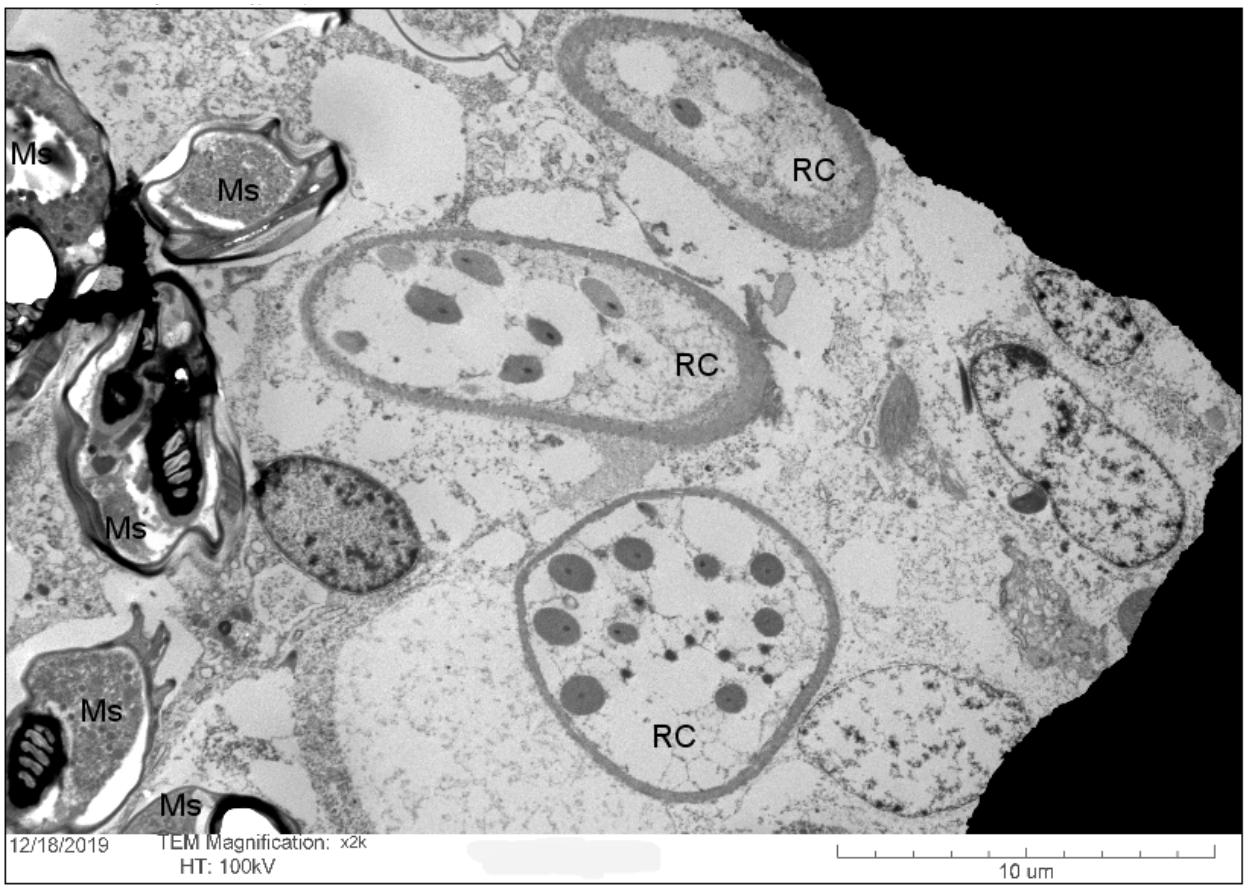


Figure 2.4: Transmission electron image of host immune cells associated with development of *Myxobolus rasmusseni* n. sp. within the ocular cavity of Fathead Minnows. RC: rodlet cell; Ms: mature myxospore. x2000 magnification, Scale bar = 10µm.

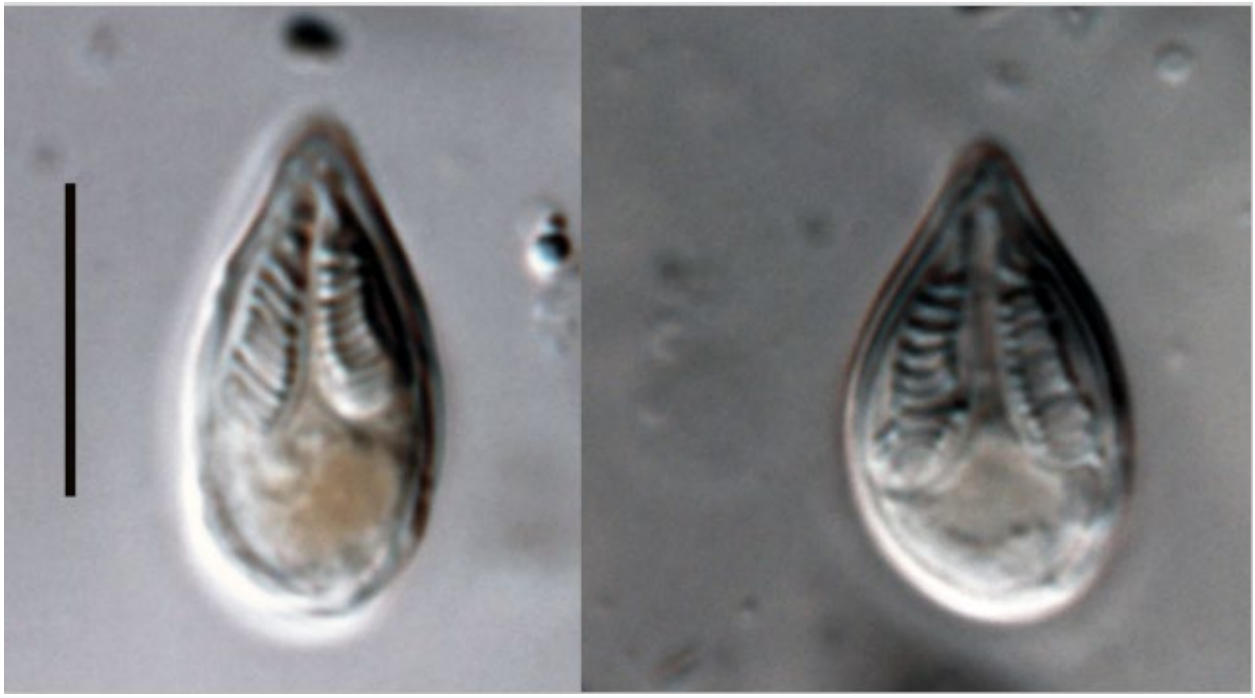
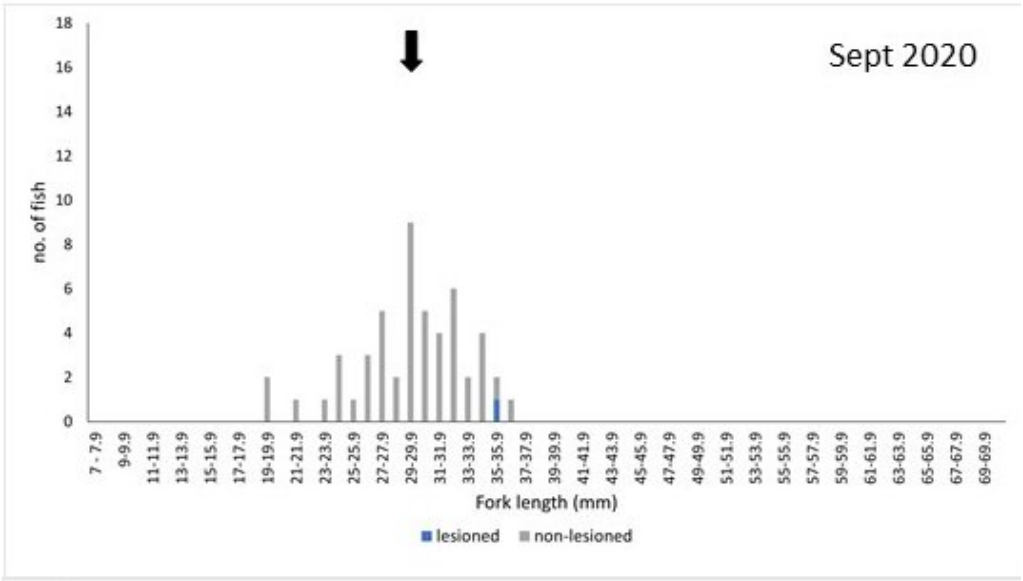
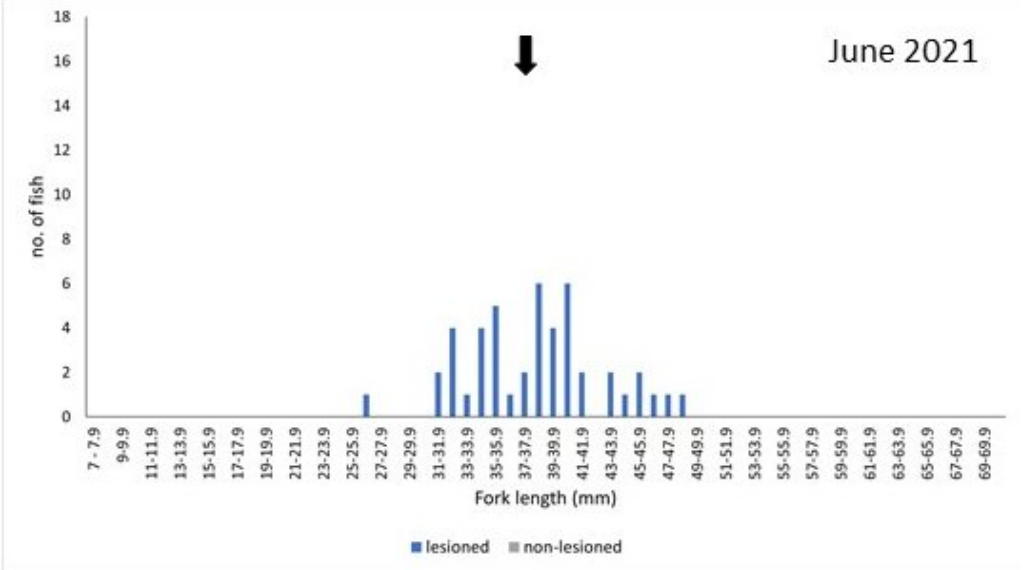


Figure 2.5: Image of mature myxospores of *Myxobolus rasmusseni* n. sp. from the ocular cavity of infected Fathead Minnow at 100X magnification and embedded in 1% agar. (Scale bar = 10µm).


Sept 2020
no. of fish
Fork length (mm)
lesioned
non-lesioned


June 2021
no. of fish
Fork length (mm)
lesioned
non-lesioned

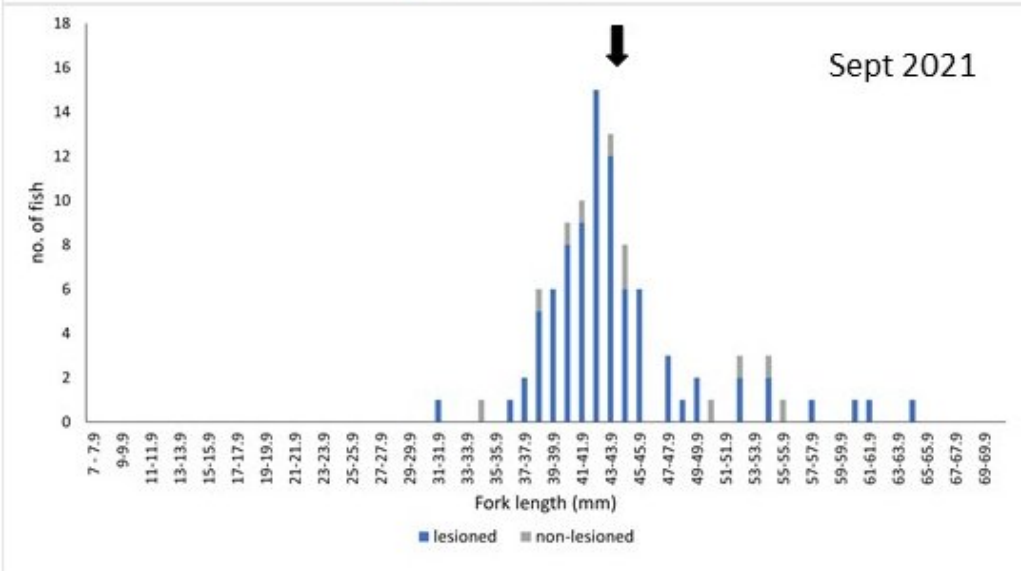
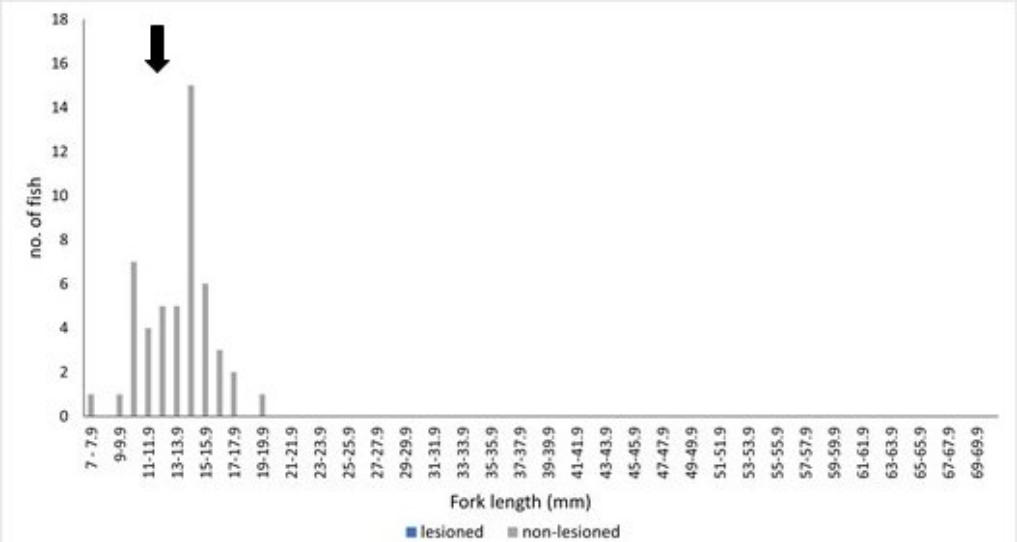
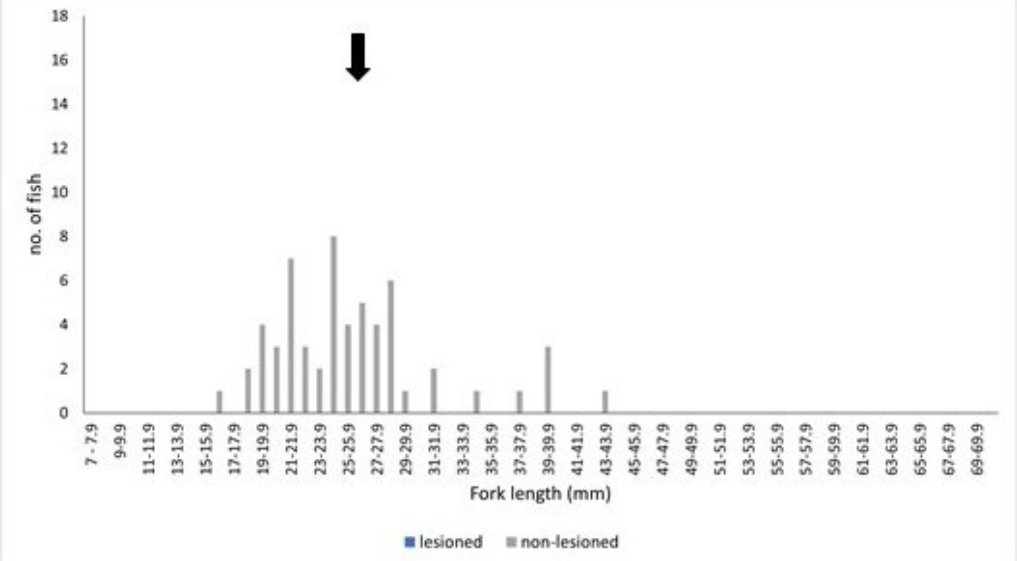

Sept 2021
no. of fish
Fork length (mm)
lesioned
non-lesioned

Figure 2.6A: Size-length frequency distributions of 4-, 12-, and 16- mo. old Fathead Minnows sampled from Coalhurst Stormwater Pond, southern Alberta, Canada. Minnows with visually detectable lesions caused by developing *Myxobolus rasmusseni* n. sp. is indicated by blue hatching. Non-lesioned minnows are indicated by grey hatching. The vertical arrow indicates mean host length.


no. of fish
Fork length (mm)
lesioned
non-lesioned


no. of fish
Fork length (mm)
lesioned
non-lesioned

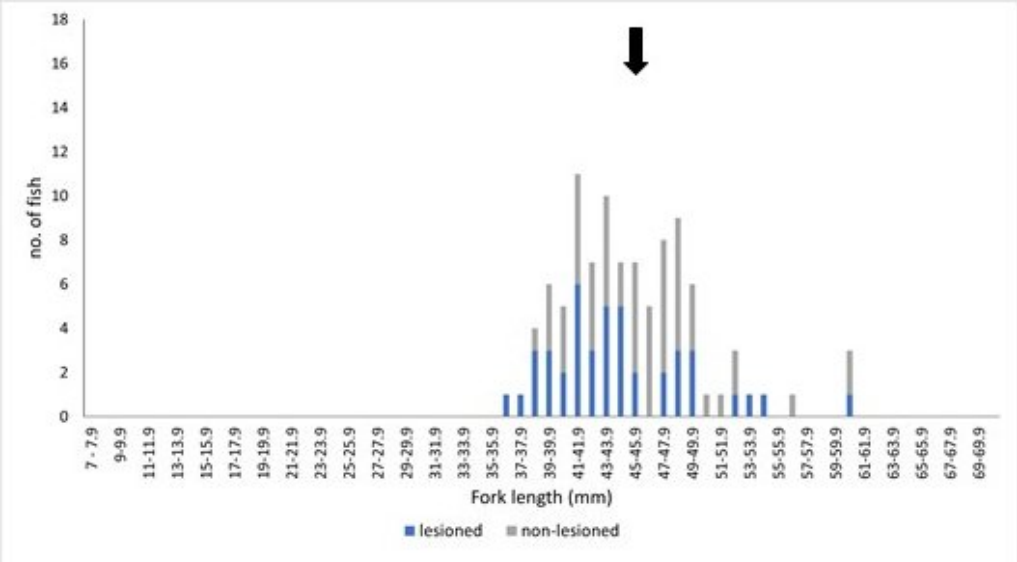

no. of fish
Fork length (mm)
lesioned
non-lesioned

Figure 2.6B: Size-length frequency distributions of 4-, 12-, and 16- mo. old Fathead Minnows sampled from McQuillan Reservoir, southern Alberta, Canada. Minnows with visually detectable lesions caused by developing *Myxobolus rasmusseni* n. sp. is indicated by blue hatching. Non-lesioned minnows are indicated by grey hatching. The vertical arrow indicates mean host length.

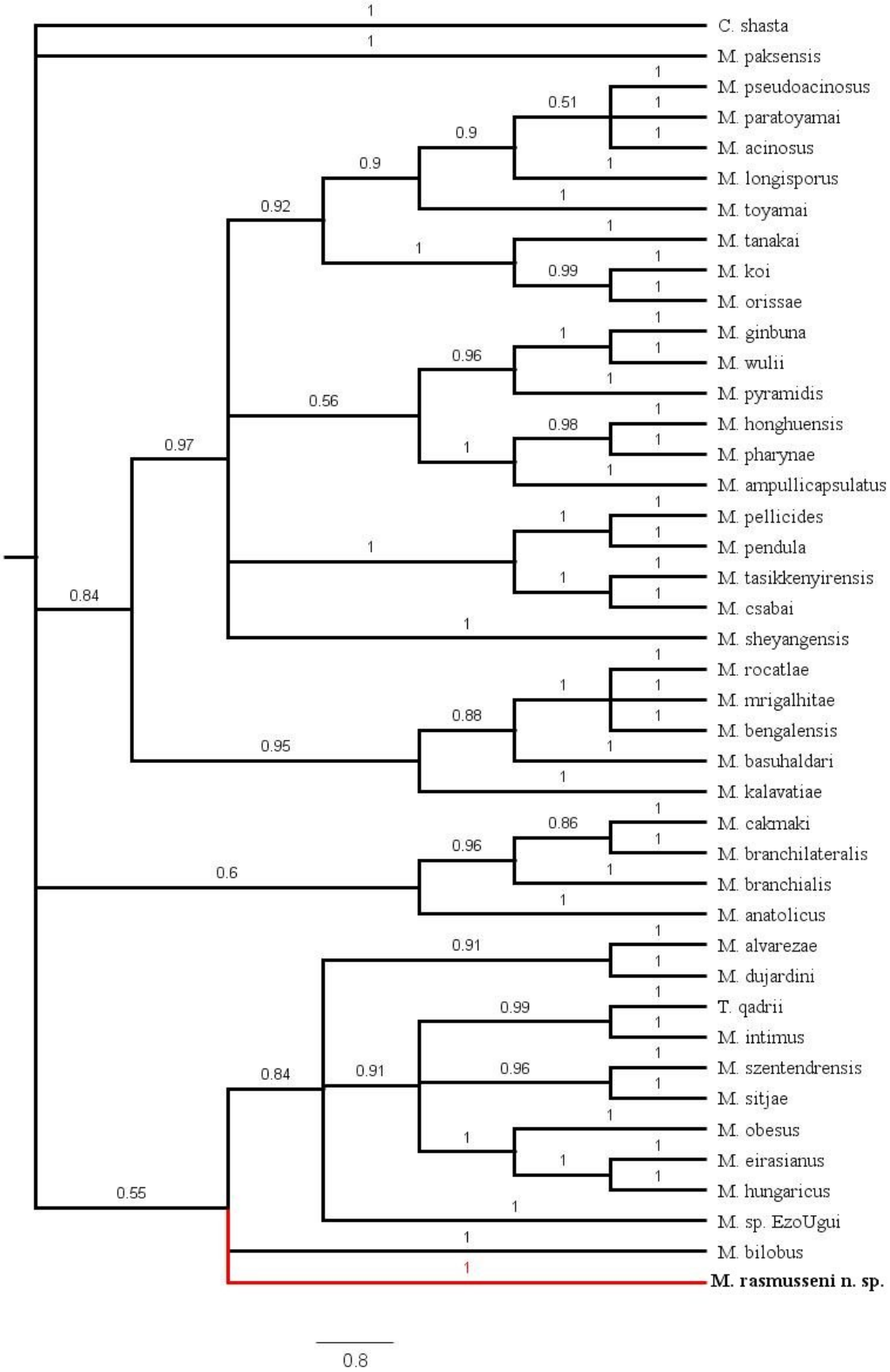

C. shasta
M. paksensis
M. pseudoacinosus
M. paratoyamai
M. acinosus
M. longisporus
M. toyamai
M. tanakai
M. koi
M. orissae
M. ginbuna
M. wulii
M. pyramidis
M. honghuensis
M. pharynae
M. ampullicapsulatus
M. pellicides
M. pendula
M. tasikkenyirensis
M. csabai
M. sheyangensis
M. rocatlae
M. mrigalhitae
M. bengalensis
M. basuhaldari
M. kalavatiae
M. cakmaki
M. branchilateralis
M. branchialis
M. anatolicus
M. alvarezae
M. dujardini
T. qadrii
M. intimus
M. szentendrensis
M. sitjae
M. obesus
M. eirasianus
M. hungaricus
M. sp. EzoUgui
M. bilobus
M. rasmusseni n. sp.
0.8

Figure 2.7: Phylogenetic tree produced by Bayesian analyses of aligned partial 18S rDNA gene sequences of *Myxobolus rasmusseni* n. sp. and other species of myxobolids infecting cyprinid fishes throughout Canada, Europe, and Asia. The tree is rooted with *Ceratomyxa shasta* (AF001579). Nodes are denoted with the posterior probabilities generated by Bayesian analyses.

CHAPTER 3: EFFECTS OF AN EMERGING MYXOZOAN MYXOBOLUS SP. ON VISION, CHEMOSENSATION, SWIMMING PERFORMANCE, AND SURVIVAL OF FATHEAD MINNOWS, PIMEPHALES PROMELAS

3.1 Abstract

Fish rely on various sensory systems and physiological behaviours to navigate through their aquatic ecosystems. Infection with the emerging myxozoan *Myxobolus* sp. causes lesions to form around the eyes and nose and large swellings of the opercula and pectoral girdle of Fathead Minnow. These lesions can potentially cause impairments to vision, olfaction, and overall performance, including survival. I evaluated visual acuity, olfactory acuity, and endurance swimming performance using an optomotor response apparatus, electro-olfactography, and swim flume assays in lesion-bearing, non-lesion bearing, and uninfected Fathead Minnows. I then conducted an outdoor mesocosm experiment comparing survival of lesion-bearing and non-lesion bearing minnows. The results showed that lesion bearing minnows had poorer olfactory response and poorer swimming endurance compared to non-lesion bearing and uninfected minnows. Lesion bearing minnows also had poorer survival under semi-natural conditions in outdoor mesocosms. Overall, the lesions caused by the obligate development of *Myxobolus* sp. are pathologic in individual Fathead Minnows and they reduce host survival. These negative consequences of myxozoan development may reduce the size of populations of this important forage fish.

3.2 Introduction

A fish's ability to sense its' environment and respond appropriately is integral to its success in foraging, predator aversion, survival, and reproduction (Binning et al.,

2017). Fish accomplish these key tasks through integration of numerous complex sensory systems that handle, for example, photoreception, chemoreception, mechanoreception, and electroreception. These systems are highly specialized for fish to perceive their surroundings and allow them to mount appropriate physiological or behavioural responses to various stimuli present within the water column. Among these stimuli are those linked to stressors such as predation, competition, and disease (Caputi, 2005). Parasites and diseases, for example, are stressors that are well-recognized for their impact on host responses, especially behaviours that influence habitat choice, foraging efficiency, predator aversion, sexual reproduction, mate-choice and swimming performance. Yet there are few cases in which the mechanisms underlying the complex effects of parasites and disease on individual hosts have been demonstrated (reviews in Barber et al., 2000; Goater et al. 2014). Parasites that invade the host's sensory tissues, in particular, such as the central nervous system and eyes, can damage and impair normal host function, which can then alter key host behaviours and ultimately lead to reduced survival of infected hosts (Lari et al., 2017; Lari & Pyle, 2017; Seppälä et al., 2004, 2005; Shirakashi & Goater, 2005).

Myxozoans are a clade of microscopic endoparasites within the Phylum Cnidaria (Goater et al., 2014; Okamura et al., 2015). Most species of myxozoans are obligate parasites of fish (Lom & Dykova, 2006; Okamura et al., 2015). Although there are over 2400 described species of myxozoan (Alama-Bermejo & Holzer, 2021), there remains a poor understanding of their impacts on individual hosts beyond those described in anecdotal reports involving tissue pathology and immunopathological responses (Atkinson et al., 2015; Fontes et al., 2015; Okamura et al., 2015). Even fewer empirical

studies have taken the next step and investigated potential myxozoan-induced impairments to host behaviour, host performance, or host survival. The few exceptions tend to involve myxozoans of economically important fish species such as *Myxobolus cerebralis, Ceratonova shasta, Tetracapsuloides bryosalmonae,* and *Kudoa thrysites* of salmonids and *Henneguya ictalurid* of channel catfish (Fontes et al., 2015). Where concern is with pathogens of wild populations of fish, the research focus tends to be driven by investigations of farmed species of fish, especially those raised in net pens where they share the water column with wild fishes and/or parasite vectors (Chapman et al., 2021; Lafferty & Hofmann, 2016). Even rarer are empirical population-level studies designed to compare patterns of host survival in myxozoan-infected versus uninfected samples of fish (Fontes et al., 2015; Okamura et al., 2015). Our poor understanding of the effects of myxozoan infection on individual hosts, and by extension, on populations of hosts is an important shortcoming, particularly for cases involving myxozoan emergence within a new species of host.

The myxozoan, *Myxobolus sp.*, is common in populations of Fathead Minnows (*Pimephales promelas*) in southern Alberta (Chapter 2). Although samples of minnows have been systematically evaluated for the presence and abundance of up to 12 species of endoparasite since 1997 (Ahn, 2019; Sandland, 1999; Sandland et al., 2001), *Myxobolus* sp. had never been observed in these populations prior to 2017 (reviewed in Chapter 2). Infection with this species has also not been reported in host surveys completed in other regions within its broad geographical range (Wisenden et al., 2012). Fathead Minnows harbouring the distinctive lesions characteristic of *Myxobolus* sp. infection (see Fig. 1.1 and Fig. 2.1) have been observed each year in 6 populations in southern Alberta, Canada,

with prevalences ranging from 8 – 96% (Chapter 2). Within individual fish, the infection is associated with the development of large white lesions, known as pseudocysts, that are composed of host immune cells and thousands of mature myxospores (Fig. 3.1). The lesions tend to be highly site- and tissue-specific, developing within the head region in the ocular, oral, and sinus cavities. The lesions often lead to complete or partial oblation of the eyes, mouth, gill apparatus, and nasal cavities. The lesions also commonly develop on the opercula and along the pectoral girdle (Fig. 3.1).

My anecdotal observations indicate that the overall condition of Fathead Minnows that have well-developed lesions on the head is poor. Lesion-bearing minnows tend to swim slowly and erratically near the water surface and are easily collected with a small dip net or by hand. Intuitively, lesion-associated ablations of the oral, opercular, and or nasal regions (Fig. 3.1) of heavily infected Fathead Minnows is likely to interfere with normal feeding, respiration, and olfaction, respectively. The purpose of this study is to compare the performance of Fathead Minnows harbouring lesions associated with the development of *Myxobolus* sp. pseudocysts to the performance of non-lesion bearing minnows collected concurrently from the same population. I further compare host performance between samples of lesion and non-lesion bearing minnows with age- and size-matched controls reared under transmission-free conditions. Given the apparent site-specificity of lesions to the regions behind the eyes, the nasal cavities, and the opercula (Chapter 2), my focus is on key performance indicators associated with visual and olfactory acuity and with overall swimming performance. A further aim is to assess the effect of lesions on the short-term (2 wk) survival of minnows within outdoor mesocosms.

3.3 Material and Methods

3.3.1 Source of hosts

Fathead Minnows were collected using Gee traps and dip nets in August, September, and October 2020, and transported to the Aquatic Research Facility (ARF) at the University of Lethbridge. Methods used to collect and process minnows are described in Chapter 2. Since collections occurred after the 2020 breeding season, I assumed that all minnows that were sampled by the Gee traps were 12- 15 months old and were born in June/July 2019. All minnows collected for the visual acuity, olfactory acuity and swimming performance tests were from Stirling Children's Pond in Stirling, in southern Alberta (49.500691 N, -112.537370 W).

Samples of minnows were separated into two categories based upon the presence of the characteristic lesions that are putatively caused by host responses to pseudocysts of developing *Myxobolus sp.* (Chapter 2). These lesions are characterized by inflammation of the head region, the ocular cavity, and the oral and sinus cavities. Inflammation of the head region in particular is associated with a 'bulging' of area immediately adjacent to the eyes (Chapter 2). Pseudocysts also occur along the pectoral girdle and can develop into large lesions that may impede proper movement and positioning of the pectoral fins (Fig. 3.1).

A total of 90 minnows was collected for the swimming performance, visual acuity, and olfactory acuity tests. Each of the 3 independent assays included 30 minnows. From this total of 30 minnows, the first 15 within a trap set that showed obvious lesions of the head region were selected as 'lesion-bearing' fish. An additional 15 were selected from the same trap set in which no lesions were visually detectable. Minnows were

acclimated in the laboratory for a maximum of 24 h or overnight as there tended to be significant losses of lesion bearing minnows in the laboratory as time passed, this is owing likely to the poor overall condition of lesion bearing minnows and stress. Both lesion bearing and non-lesion bearing minnows were transported and maintained in the same manner.

I cannot discount the possibility that field-collected minnows that did not have obvious lesions were never-the-less infected with early developmental stages of *Myxobolus* sp. that could influence host performance. To address this possibility, I included an additional sample of naïve controls for comparison to the samples of field-collected fish. These minnows came from a cohort collected as eggs from Stirling Children's Pond in 2019. These eggs were transported in aerated coolers still attached to the rock substrate they had been deposited on and were hatched in outdoor mesocosms. This cohort was raised under transmission-free conditions in the 1000 L outdoor mesocosms on the University of Lethbridge campus (Pearson & Goater, 2009; Stumbo et al., 2012) and then transported in October 2019 to the ARF where they were subsequently raised in six, 9 L aquaria (20 cm high, 30 cm length, 21 cm wide) under standard laboratory conditions (20 ◦C, *ad libitum* daily feeding with fish flakes).

For the assessment of *Myxobolus* sp.- induced effects on host survival, juvenile Fathead Minnows (ca. 12 mo. old) were collected using dip nets from Coulee Creek Stormwater Pond in July 2020 (n=360, coordinates and site description in Chapter 2). Fish were approximately size matched by eye and separated into two groups based upon the visual detection of *Myxobolus* sp. – induced lesions (Fig. 3.1).

3.3.2 Visual Acuity

Visual acuity was assessed for individual minnows placed into an optomotor testing apparatus. This method is standard for testing the visual component of the rheotaxis response in fish (Bak-Coleman et al., 2015; Jones, 1963; Krauss & Neumeyer, 2003; Rock et al., 1965; Rock & Smith, 1986). Methods followed those used to test visual acuity of Fathead Minnows infected with a brain-encysting trematode (Shirakashi & Goater, 2001, 2005). In brief, individual minnows were placed into a stationary 30 cm X 30 cm (diameter X height) glass cylindrical tank. This tank was surrounded by an outer screen that could be rotated around the stationary tank in a direction and speed that was controlled by a motor and transformer (Shirakashi & Goater, 2005). The screen had 25 mm alternating black and white bands. The optomotor response was assessed as the proportion of time that a minnow followed in the direction as the spinning drum (Neave, 1984). Water in the stationary glass tanks was kept at room temperature, had no flow, and was aerated for one minute between trials. Individual minnows were randomly selected from the holding tanks and placed in the optomotor apparatus for an initial, 6 min acclimation period (Shirakashi & Goater, 2005), after which video-monitoring was started. Monitoring involved a Samsung A50 smartphone suspended 30 cm above the apparatus. Prior to turning-on the spinning apparatus, fish were video- monitored for an additional 6 minutes. The first 2 min included a period when the camera was on, but not recording. Over the next 4 min, fish were video- recorded to evaluate general levels of activity. The screen then began to rotate at 15 rpm in a randomly- selected direction (clockwise or counter- clockwise). The minnows were acclimated for 2 min after which a 4 min period began where general activity was monitored. After the 4 min the direction

of the screen rotation was randomly selected and rotated in that direction 2 min. Finally, after 2 min the rotation of the screen was switched for another 2 min. In total, minnows were assessed for a total of 8 minutes (Shirakashi & Goater, 2001). Minnows were euthanized in 1g/l TMS-222 immediately after the end of each trial and were measured for fork length (mm), standard length (mm) and wet weight (g). The video for each fish was processed manually to measure following time and then with EthoVision 15 (Noldus et al., 2001), a software program that tracks movement, to measure general activity. Following time was calculated as the duration that each minnow swam in the direction of the screen rotation, divided by 4 min (total time) minus latency time (the time it takes a fish to reorient after the screen direction was reversed). General activity was quantified using EthoVision 15 which obtained measurements for total distance swam (cm), average velocity of swimming (cm/s), and the duration of time spent in motion (%) during the four- min period of pre-spinning.

3.3.3 Olfactory acuity

Fathead Minnows with well-developed pseudocysts typically had whiteish lesions located on one or more of the ocular, oral and sinus cavities, and the opercula (Chapter 2). The presence of distinct lesions in and on the ocular and sinus cavities was especially common. Lesions associated with the ocular cavities often showed protrusion of the olfactory rosette sometimes distending past the opening of the olfactory chamber (Fig 3.1b). These pseudocyst-induced morphological alterations to the olfactory chamber led me to test for effects on olfactory acuity. The olfactory performance of uninfected, lesion bearing, and non-lesion bearing minnows was compared by assessing differences in mean electro-olfactography (EOG) responses. I followed Scott & Scott-Johnson (2002) by

placing a recording electrode onto the olfactory epithelium of anaesthetized minnows and a grounding electrode on the skin between the minnows nose and the eye. The negative electrical potential change (mV) of olfactory receptor neurons (ORNs) was recorded before and after exposure to an odorant. These methods for the assessment of olfactory acuity have been applied specifically to Fathead Minnows in prior studies in our laboratory (Lari et al., 2017). For each group I tested 12 minnows due to losses in the lesion bearing group.

Samples of uninfected, lesion bearing, and non-bearing minnows were collected as described above. Wild collected minnows were acclimated to the laboratory for 24 h before individual fish were randomly selected from the treatment groups and anaesthetized in a solution of 120mg/L of MS-222 and 310 mg/L of sodium bicarbonate until the opercula and mouth of the fish had ceased pumping. The naris, or nostril covering, was then removed to expose the olfactory chamber and the fish was secured on the EOG apparatus (Lari et al., 2017). The fish remained anaesthetized by the gill perfusion solution that contained 75 mg/L of MS-222 and 180 mg/L of sodium bicarbonate. The olfactory chamber was continuously perfused with processed water from the Aquatic Research Facility (ARF) warm water system which provides dechlorinated water. One recording electrode probe was placed on the 3rd lamellae of the olfactory rosette and a second grounding electrode was placed on the epidermis between the nostril and the eye of the fish. Fish were acclimated to the rig for 5 min following the beginning of the trial, during which cue was absent. During this time adjustments were made, if necessary, to ensure that the electrodes were receiving electrical signals. L-arginine at a concentration of 10^{-4} M (Lari et al., 2017), or a blank which consisted of

water from the same ARF processed warm water system was randomly delivered to the olfactory rosette every 2 min until at least three successful responses to each cue were recorded. Following the completion of each trial, the fish was removed from the EOG apparatus, euthanized in a 1g/L solution of MS-222, and their weight and fork length measured.

3.3.4 Swimming Performance

I compared sustained swimming performance of uninfected, lesion bearing, and non-lesion bearing minnows following the methods of Hammer (1995). Swimming performance trials were completed in two open surface swim flumes following methods similar to Goertzen et al. (2012). The flumes were recirculating units with four individual lanes per unit. The dimensions (cm) of each lane were 80 (length) x 12 (depth) x 14 (width). The water in each unit was maintained at a temperature between 21.0 ◦C and 22.5◦C. The first and last two lanes in each unit shared the same pump and filter, respectively. The average individual water velocities per pump and per increment increase (2, 4, 6, 8, 10 cm/s) were determined by adding red food dye to the water at the upstream end and timing it until it reached the downstream end at 80 cm.

Uninfected, lesion bearing, and non lesion bearing minnows were collected as described above. Wild collected minnows were acclimated to the laboratory for 24 h before individual fish were randomly assigned to a flume channel. Acclimation to the flume occurred over a 5 min interval. Each flume was then set to increase water velocity 2 cm/s every 2 min until a final flume water velocity of 10 cm/s was reached. Once maximum water velocity was reached, fish were continually exercised until the fish fatigued. Fatigue was determined as the time when fish became pinned against the back

grating of the flume and could not be coaxed to recorrect and continue swimming for more than 3 s (Goertzen et al., 2012). Alternatively, the tests were ended if the fish did not fatigue within 6 h (Hammer, 1995). The total time to fatigue was recorded for each fish. Minnows from the lesion group and the non-lesion group were euthanized following the end of their trials and frozen. The uninfected fish were photographed and returned to their 9L housing unit in the Aquatic Research Facility. Fish that did not orient swimming towards the water flow were removed and replaced with another random fish from the respective treatment group.

3.3.5 Survival

One year old (n = 360) from Coulee Creek Stormwater Pond were transported to the University of Lethbridge and placed at random in groups of 30 into one of 12, 1200 L outdoor mesocosms. Each mesocosm contained only 'lesion-bearing' or only 'non-lesion bearing' minnows. The mesocosms had been set-up 30 d prior following methods described for the maintenance of Fathead Minnows (Stumbo et al., 2012). Prior to release into each mesocosm, individual fish were photographed and measured for fork length using FIJI software. The mesocosms were visually assessed daily for fish mortalities and fed every other day with a 400 ml solution of concentrated zooplankton collected from a nearby fishless pond. The mesocosms were maintained for two weeks, after which they were drained, and the surviving minnows were photographed, and fork length measured using FIJI.

3.3.6 Statistical Analyses

All data from the visual acuity, olfactory acuity, and swimming performance tests were evaluated for normality with Shapiro-Wilks and Levene's tests. Differences in

following time between the three groups were tested with ANCOVA with fork length (mm) as a covariate. Pairwise comparisons used Tukey-Kramer HSD tests. Differences among the 3 groups in the total distance travelled (prior to the drum spinning) were first Levene's median tested prior to analyses then Kruskal-Wallis and post hoc Nemenyi tested. The proportion of time spent moving and the average velocity (cm/s) data were also Kruskal-Wallis and post hoc Nemenyi tested.

The raw EOG responses were blank corrected for each individual fish (Lari et al., 2017; Lari & Pyle, 2017), which obtained the difference (mV) between the L-arginine response and the blank response. Then the data were Kruskal-Wallis and post-hoc Nemenyi tested to identify significant differences between the mean responses of uninfected, lesion bearing, and non-lesion bearing minnows.

Swimming performance data were recorded as time to fatigue (min) within the flumes and evaluated with GLM to determine if size (fork length; mm) should be considered in the analyses. These data could not be transformed to meet the assumptions of normality. Data were Kruskal-Wallis and post hoc Nemenyi tested to evaluate differences in swim performance between uninfected, lesion bearing, and non-lesion bearing minnows.

Survival data within each of the 12 mesocosms was collected as the number of surviving fish after 14 days and was analyzed as the mean proportional survival per treatment (uninfected vs. infected) using a z-test (Mishra et al. 2019). The data pertaining to daily mortalities during the two-week period within a mesocosm were analyzed using the Kaplan Meier test (Jager et al., 2008).

3.4 Results

3.4.1 Performance in the optomotor apparatus

The 45 minnows that were assessed in the optomotor apparatus spent between 62% and 65% of their time following in the direction, and at the same speed, as the spinning drum. Overall, there were no differences in the average time spent following the drum between uninfected, lesion bearing, and non-lesion bearing minnows after controlling for host size [ANCOVA; $F(2,41) = 0.055$, $p = 0.978$]. There were also no significant differences between the 3 groups in the total distance the fish travelled in the optomotor apparatus [Kruskal-Wallis; $H(2,45) = 0.427$, $p = 0.807$], or in their average velocity of movement (cm/s) [Kruskal- Wallis; $H(2,44) = 2.57$, $p = 0.277$]. In contrast, there was a significant difference between the three groups in the mean proportion of time that minnows spent swimming within the optomotor drum [Kruskal- Wallis; $H(2, 45) = 10.5$, $p < 0.01$]. On average, lesion-bearing minnows spent 48.0 ± 7.5 % of the 4 min period in motion, whereas the non-lesion bearing minnows and laboratory-reared control minnows spent a mean proportion of 22.9 ± 7.4 % and 15.2 ± 3.8 % in motion, respectively (Fig. 3.2a).

3.4.2 Olfactory Acuity

Fathead Minnows that were infected with *Myxobolus sp.* had weaker mean EOG responses than both the non-lesion bearing minnows and the uninfected minnows (Fig. 3.2b). The average response amplitude of lesion-bearing Fathead Minnows was approximately a third of the response amplitude of the non-lesion bearing and uninfected fish (Fig. 3.2b). Overall, there was a significant difference in mean amplitudes (mV) responses to L-arginine delivery between lesion bearing minnows and the other groups,

but no significant difference in EOG responses between non lesion bearing and uninfected minnows [H (2, 36) = 6.83, p = 0.03]. The lesion bearing minnows had significantly lower mean EOG amplitude responses (mV) at 35.1±10.4 mV than either of the other groups. The non-lesion bearing, and uninfected minnows had similar mean response amplitudes at 93.1±10.6 mV and 98.3±23.6 mV, respectively (Fig. 3.2b).

3.4.3 Swimming Performance

Lesion bearing minnows fatigued faster in the fixed velocity swimming experiment than both non-lesion bearing and uninfected minnows (Fig. 3.2c). Overall, lesion bearing minnows spent, on average, 125.5 ± 24.0 min swimming in the flume before reaching fatigue. In contrast, the non-lesion bearing minnows spent a mean of 310 ± 26.1 min swimming before reaching fatigue. However, 'time to fatigue' data was highly variable. One lesion-bearing minnow, for example, reached the six-hour endpoint of the experiment and therefore did not reach fatigue at all, whereas another infected minnow fatigued at 6:23 (min: sec), only reaching a maximum water velocity of 7.9 cm/s. Ten of the 15 non-lesion bearing minnows reached the six-hour end point and did not fatigue. The uninfected minnow group spent a mean of 270.9 ± 26.3 minutes of swimming before reaching fatigue. Lesion bearing minnows disproportionately fatigued within the first three hours with 66.7% of minnows fatiguing in this timeframe compared to 13.3% and 33.3% of non-lesion bearing and uninfected minnows, respectively. Lesion bearing Fathead Minnows fatigued significantly faster than both the non-lesion bearing minnows and the uninfected Fathead Minnows [H (2, 44) = 18.4 p <0.0001]. There were significant differences in swimming performances between the lesion bearing group and both the non-lesion bearing and uninfected pairwise group associations. The wild-

collected uninfected Fathead Minnows fatigued later than both the laboratory-reared control minnows and the infected minnows (Fig. 3.2c).

3.4.4 Survival

Mean survival of lesion bearing fish in the mesocosms at 14 days was significantly lower, 11.7±2.8 %, than mean survival of non-lesion bearing fish at 48.7±4.3 % (z-test, z = -6.01, p = <0.001). In total, 77 of 180 non-lesion bearing minnows survived the 14-day period whereas 21 of 180 lesion bearing minnows survived.

The results of the Kaplan Meier survival analyses showed that there was a significant difference in survival between the non-lesion bearing and lesion bearing groups during the 14-day experimental period ($X^2 = 54.7$, $p = 1.40 \times 10^{-13}$). Both the non-lesion bearing and lesion bearing fish experienced mortalities in the first two days of the experiment. However, the lesion bearing group continued to experience a greater number of mortalities than the non-lesion bearing fish (Fig. 3.3).

3.5 Discussion

The results of this study indicate that individual Fathead Minnows infected with *Myxobolus* sp.- induced lesions have reduced olfactory acuity, fatigued more rapidly, and were hyperactive compared to uninfected minnows and non-lesion bearing minnows. The results of the mesocosm experiment indicated that reduced performance of individuals was associated with reduced survival under semi-natural conditions. These results are consistent with the growing body of evidence that supports an association between intra-host development of myxozoans, ultimately leading to the expression of distinctive, myxospore-packed lesions on the host (Chapter 2), and reduced host performance. The myxozaon *Parvicapsula pseudobranchicola* also causes distinctive lesions and

exophthalamia, cataract, and hemorrhaging of the eyes of sea trout, leading to abnormal swimming behaviour (Hansen et al., 2015). These effects of infection are similar to the altered swimming of Fathead Minnows infected with *Myxobolus* sp., including surfacing and lethargic, disjointed swimming patterns. Nylund et al. (2018) indicate that clinical symptoms of parvicapsulosis include abnormal swimming behaviours and that these may be explained by vision loss caused by the accumulation of developing parasite stages in the choroidea of the eyes where they may physically obstruct vision or blood supply to the eyes. Observations indicating vision impairment, reduced host survival, and reduction in foraging rates are similar to my general observations involving Fathead Minnows infected with *Myxobolus* sp.

Lesion bearing Fathead Minnows were hyperactive compared to non-lesion bearing and uninfected minnows. While lesion-bearing minnows swam at the same average speed and travelled the same overall distance during the 4-min trial in the optomotor drum, those with lesions spent a significantly greater proportion of time actively swimming. These results support my anecdotal observations indicating that lesion-bearing minnows tended to swim persistently near the surface of the water, at a consistent average speed, with occasional short bursts of activity. The slow swimming pattern displayed by the *Myxobolus* sp. infected minnows is similar to the pattern of swimming described for three-spined stickleback infected with the cestode *Schistocephalus solidus* (Barber et al., 2004). In contrast, non-lesion bearing, and uninfected minnows typically remained stationary on the bottom of the drum, followed by brief swimming bouts where they would circle the drum. Parasite-induced hyperactivity has been reported for sticklebacks infected with larval stages of the cestode

Schistocephalus solidus (Barber et al., 2004; Barber & Scharsack, 2010; Blake et al., 2006) and the microsporidian *Glugea anomola* (Petkova et al., 2018), and for the larval stages of the trematode *Ornithodiplostomum ptychocheilus* (Shirakashi & Goater, 2005). Similarly, McElroy et al. (2015) found hyperactivity in fish infected with the muscle-dwelling myxozoan, *Kudoa inornata,* where infected fish maintained their activity longer in continuous acceleration swimming challenges than uninfected fish. The authors explained this result by the significant positive association between gait transition speed and myxospore density as well as evidence from other studies that amino acid liberation from host myofibrils by the proteolytic activity of myxozoan parasites (Funk et al., 2008; Patashnik et al., 1982) may increase bioavailability of energy for the host's other unaffected muscles (McElroy et al., 2015).

The persistent and hyperactive swimming behaviour demonstrated by lesion bearing minnows infected with *Myxobolus* sp. may be a side-effect of parasite development within key host tissue. Lesion development may lead to a suite of direct and indirect changes in host morphology that interfere with a fish's positioning within the water column or its ability to streamline. Evidence from histological sectioning of lesioned tissue (Chapter 2) indicates that the host epidermis that lines pseudocyst tissue is thin and lacks typical components such as goblet cells.

Alternatively, hyperactivity may result from a minnow's requirement to compensate for loss of oxygen that may be caused by the obstruction of respiratory structures, such as lesion development on or around the opercula. Gill ventilation is critical to respiration and fish will modify behaviour, such as the rate of bucco-opercular movements, to regulate the partial pressure of oxygen to maintain a specific range within

the gill tissue. Failure to maintain this optimal partial pressure range is associated with increased oxidative stress and damage to adjacent tissues (Martins et al., 2012). Considering that large lesions are typically associated with the mouth and opercular regions of the host, it is possible that the observed histopathology reduces a fish's capacity to effectively regulate oxygen consumption. Although lesion bearing minnows sustain movement or are hyperactive, this constant motion appears to be lethargic and laboured. This observation would also help explain the reduction that was observed in the time it took for lesion-bearing minnows to fatigue compared to non-lesioned and uninfected minnows.

The finding that uninfected minnows fatigued sooner than the non-lesion bearing minnows in the swimming performance assay was surprising. This result may be due to the difference in rearing conditions between non-lesion bearing minnows collected from a natural pond and those maintained under laboratory conditions. The results of numerous studies have demonstrated that wild fish tend to outperform captive reared fish in nature. Significant research has focussed on improving captive rearing conditions to create better post release outcomes for stocking programs, where wild fish generally outcompete new hatchery releases (Bergendahl et al., 2017; Johnsson et al., 2014). The uninfected minnows used in my study were reared in a 9L aquaria for over 1 year and the outdoor mesocosms lacked exposure to predators and other stressors (e.g., water currents, wind) that may influence an individual's ability to optimize swim performance. Therefore, the wild-caught, non-lesion bearing minnows were likely better able to recognize and respond to sustained water velocity in the swim flume than the uninfected minnows.

Fathead Minnows infected with *Myxobolus* sp. also had impaired olfactory acuity. This is the first study to demonstrate myxozoan-induced impairment of host olfaction. Lesion bearing Fathead Minnows had significantly reduced mean EOG responses to L-arginine than the non-lesion bearing and uninfected minnows. The development of parasite pseudocyst tissues within the nasal chamber may cause physical damage to the olfactory lamellae as well as physical blockage of the olfactory chamber, which could explain the inability to respond to chemo-odorants. Lari et al (2017) demonstrated reduced olfactory acuity in Fathead Minnows that were infected with the monogenean, *Dactylogyrus olfactorius.* This parasite does not invade minnow tissues but resides within the nasal cavity and physically obstructs the surface of lamellae, disrupting olfaction. The authors suggest that the capacity for *Dactylogyrus olfactorius*-infected hosts to locate food, mates, identify conspecifics, and avoid predation suffers accordingly. Similarly, a monogenean parasite infecting the nasal cavity of rainbow trout was also found to impair the olfactory acuity of infected trout and cause physical damage or 'pitting' to the olfactory epithelium (Lari & Pyle, 2017). My previous histological investigations of *Myxobolus* sp. in the ocular cavity of infected Fathead Minnows show that the pseudocyst tissue, which causes the characteristic exophthalmia and swellings of the head region, are comprised of various host immune cells including mast cells, macrophages, eosinophils and rodlet cells, together with thousands of mature myxospores (Chapter 2). Aggressive host immune responses have been implicated to result from the development of other myxozoans. For example, *Tetracapsuloides bryosalmonae* induced disease, PKX, is caused by aggressive host tissue inflammation to the renal interstitial tissues, and subsequently causes increased mortality in salmonid fishes (Lom & Dykova, 2006). My

results suggest that the physical manifestation of infection in Fathead Minnows caused by *Myxobolus* sp. is likely responsible for reduced host performance and abnormal host behaviours.

Fathead Minnows infected with *Myxobolus* sp. suffered higher mortality compared to uninfected minnows. These results are consistent with the effects of other myxozoans, such as *Myxobolus cerebralis* and *Tetracapsuloides bryosalmonae,* which cause severe pathology, and are associated with well-known population declines (Bartholomew & Reno, 2002; Lom & Dykova, 2006; Nehring & Walker, 1996). Although both the lesioned and non-lesioned fish experienced an initial die off in the first few days within the mesocosms, the rates of daily mortalities observed in the lesioned fish were typically greater than that of those in the non-lesioned mesocosms. Unfortunately, water turbidity in the mesocosms made it difficult to obtain an accurate count of daily mortalities. Therefore, the magnitude of the difference in daily mortalities and overall survival between the infected and uninfected populations is likely conservative. The cryptic nature of fish mortality in natural and semi-natural populations makes the assessment of survival challenging, particularly when disease, predation, and scavenging rapidly removes individuals (Chapman et al., 2021). Initially, some of the mortalities in the non-lesioned mesocosms involved fish in which lesions were not detectable during the time of capture. In these fish, lesions developed within approximately one-week. Overall, the results of the mesocosm study supports the idea that pathology caused by *Myxobolus* sp. is severe and detrimental to both host body condition, host behaviour, and ultimately to host survival.

3.6 Literature Cited

Ahn, S. 2019. *Effects of host community structure on parasite transmission and disease risk.* [Master of Science]. University of Lethbridge.

Alama-Bermejo, G., & Holzer, A. S. 2021. Advances and discoveries in myxozoan genomics. Trends in Parasitology 37: 552–568.

Atkinson, S. D., Bartosova-Sojkova, P., Whipps, C. M., & Bartholomew, J. L. 2015. Approaches for characterising myxozoan species. In In B. Okamura, A. Gruhl, & J.L. Bartholomew (Eds.), *Myxozoan Evolution, Ecology, and Development* (pp. 111–124). Springer International Publishing.

Bak-Coleman, J., Smith, D., & Coombs, S. 2015. Going with, then against the flow: evidence against the optomotor hypothesis of fish rheotaxis. Animal Behaviour 107: 7–17.

Barber, I., & Scharsack, J. P. 2010. The three-spined stickleback-*Schistocephalus solidus* system: an experimental model for investigating host-parasite interactions in fish. Parasitology 137: 411–424.

Barber, I., Walker, P., & Svensson, P. A. 2004. Behavioural responses to simulated avian predation in female three spined sticklebacks: the effect of experimental *Schistocephalus solidus* infections. Behaviour 141: 1425–1440.

Blake, R. W., Kwok, P. Y. L., & Chan, K. H. S. 2006. Effects of two parasites, *Schistocephalus solidus* (Cestoda) and *Bunodera* spp. (Trematoda), on the escape fast-start performance of three-spined sticklebacks. Journal of Fish Biology 69: 1345–1355.

Chapman, J. M., Kelly, L. A., Teffer, A. K., Miller, K. M., & Cooke, S. J. 2021. Disease ecology of wild fish: opportunities and challenges for linking infection metrics with behaviour, condition, and survival. Canadian Journal of Fisheries and Aquatic Sciences 78: 995–1007.

Fontes, I., Hallett, S. L., & Mo, T. A. 2015. Comparative epidemiology of myxozoan diseases. In B. Okamura, A. Gruhl, & J. L. Bartholomew (Eds.), In *Myxozoan Evolution, Ecology and Development* (pp. 317–342). Springer International Publishing.

Funk, V. A., Olafson, R. W., Raap, M., Smith, D., Aitken, L., Haddow, J. D., Wang, D., Dawson-Coates, J. A., Burke, R. D., & Miller, K. M. 2008. Identification, characterization and deduced amino acid sequence of the dominant protease from *Kudoa paniformis* and *K. thrysites*: a unique cytoplasmic cysteine protease. Comparative Biochemistry and Physiology 149: 477–489.

Goater, C. P., Goater, T. M., & Esch, G. W. 2014. *Parasitism: The diversity and ecology of animal parasites, 2nd edition* (2nd ed.). Cambridge University Press.

Hansen, H., Poppe, T. T., Markussen, T., & Karlsbakk, E. 2015. Seatrout (*Salmo trutta*) is a natural host for *Parvicapsula pseudobranchicola* (Myxozoa, Myxosporea), an important pathogen of farmed Atlantic salmon (*Salmo salar*). *Parasites & Vectors 8*: 218-222.

Jager, K. J., Van Dijk, P. C., Zoccali, C., & Dekker, F. W. 2008. The analysis of survival data: the Kaplan-Meier method. Kidney International 74: 560–565.

Jones, F. R. H. 1963. The reaction of fish to moving backgrounds. Journal of Experimental Biology 40: 437–446.

Krauss, A., & Neumeyer, C. 2003. Wavelength dependence of the optomotor response in zebrafish (*Danio rerio*). Vision Research 43: 1273–1282.

Lafferty, K. D., & Hofmann, E. E. 2016. Marine disease impacts, diagnosis, forecasting, management and policy. Philosophical Transactions of the Royal Society B: Biological Sciences 371: 1-3.

Lari, E., Goater, C. P., Cone, D. K., & Pyle, G. G. 2017. Parasites and a host's sense of smell: reduced chemosensory performance of fathead minnows (*Pimephales promelas*) infected with a monogenean parasite. Journal of Animal Ecology 86: 434–441.

Lari, E., & Pyle, G. G. 2017. *Gyrodactylus salmonis* infection impairs the olfactory system of rainbow trout. Journal of Fish Diseases 40: 1279–1284.

Lom, J., & Dykova, I. 2006. Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. Folia Parasitologica 53: 1–36.

McElroy, E., George, A., & de Buron, I. 2015. The muscle dwelling myxozoan, *Kudoa inornata*, enhances swimming performance in the spotted seatrout, *Cynoscion nebulosus*. Parasitology Research 114: 2451–2457.

Neave, D. A. 1984. The development of visual acuity in larval plaice (*Pleuronectes platessa* L.) and turbot (*Scophthalmusmaximus* L.). Journal of Experimental Marine Biology and Ecology 78: 167–175.

Okamura, B., Gruhl, A., & Bartholomew, J. L. 2015. An introduction to myxozoan evolution, ecology and development. In B. Okamura, A. Gruhl, & J. L. Bartholomew (Eds.), In *Myxozoan Evolution, Ecology and Development* (pp. 1–22). Springer International Publishing.

Patashnik, M., Groninger, H. S., Barneti, H., Kudo, G., & Koury, B. 1982. Pacific whiting, *Merluccius productus*: I. abnormal muscle texture caused by myxosporidian-induced proteolysis. Marine Fisheries Review 44: 1-12.

Pearson, K. J., & Goater, C. P. 2009. Effects of predaceous and nonpredaceous introduced fish on the survival, growth, and antipredation behaviours of long-toed salamanders. Canadian Journal of Zoology 87: 948–955.

Petkova, I., Abbey-Lee, R. N., & Løvlie, H. 2018. Parasite infection and host personality: glugea-infected three-spined sticklebacks are more social. Behavioral Ecology and Sociobiology 72: 173-182.

Rock, I., & Smith, D. 1986. The optomotor response and induced motion of the self. Perception 15: 497–502.

Rock, I., Tauber, E. S., & Heller, D. P. 1965. Perception of stroboscopic movement: evidence for its innate basis. Science 147: 1050–1052.

Sandland, G. J. 1999. *Interactions between a brain-encysting trematode and its intermediate host, the fathead minnow*. [Master of Science]. University of Lethbridge.

Sandland, G. J., Goater, C. P., & Danylchuk, A. J. 2001. Population dynamics of *Ornithodiplostomum ptychocheilus* metacercariae in fathead minnows (*Pimephales promelas*) from four northern-Alberta lakes. Journal of Parasitology 87: 744–748.

Seppälä, O., Karvonen, A., & Tellervo Valtonen, E. 2004. Parasite-induced change in host behaviour and susceptibility to predation in an eye fluke–fish interaction. Animal Behaviour 68: 257–263.

Seppälä, O., Karvonen, A., & Valtonen, E. T. 2005. Impaired crypsis of fish infected with a trophically transmitted parasite. Animal Behaviour 70: 895–900.

Shirakashi, S., & Goater, C. P. 2005. Chronology of parasite-induced alteration of fish behaviour: effects of parasite maturation and host experience. Parasitology 130: 177–183.

Stumbo, A. D., James, C. T., Goater, C. P., & Wisenden, B. D. 2012. Shoaling as an antiparasite defence in minnows (*Pimephales promelas*) exposed to trematode cercariae. Journal of Animal Ecology 81: 1319–1326.

Wisenden, B. D., Martinez-Marquez, J. Y., Gracia, E. S., & McEwen, D. C. 2012. High intensity and prevalence of two species of trematode metacercariae in the fathead minnow (*Pimephales promelas*) with no compromise of minnow anti-predator competence. Journal of Parasitology 98: 722–727.

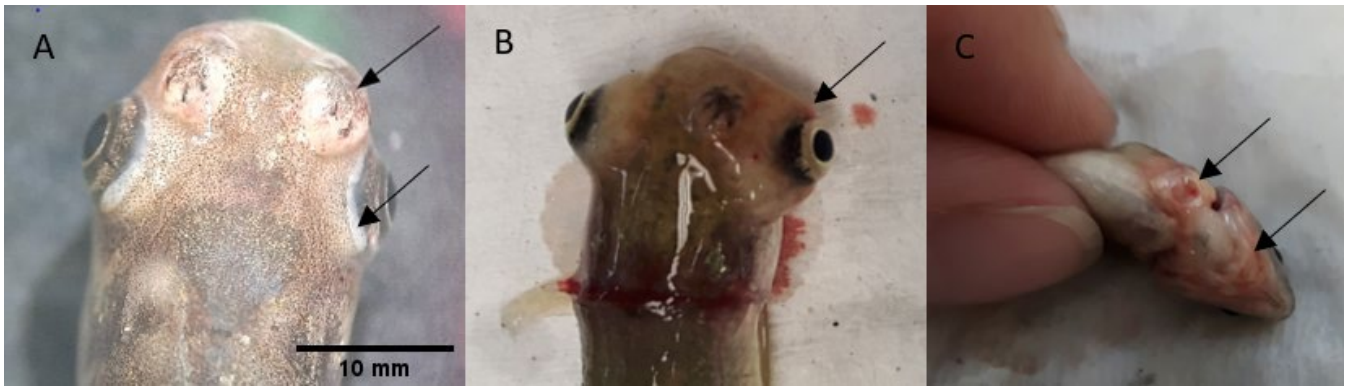
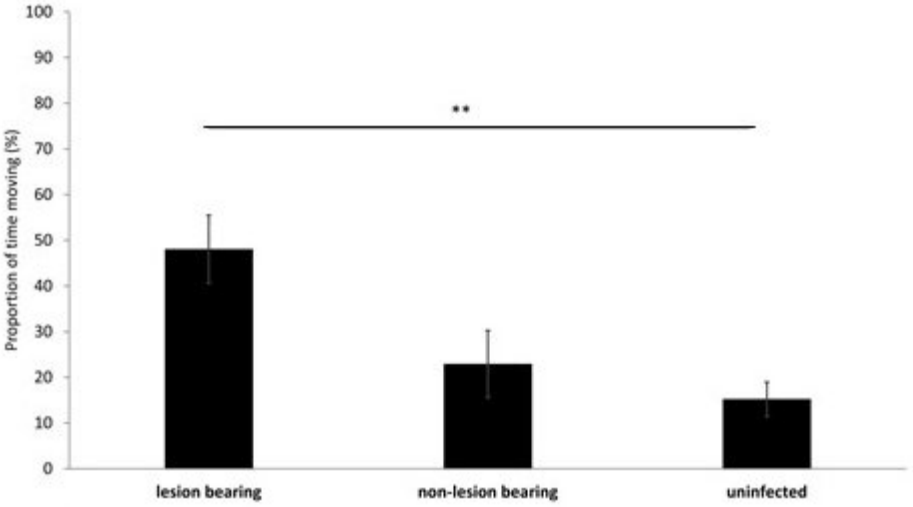
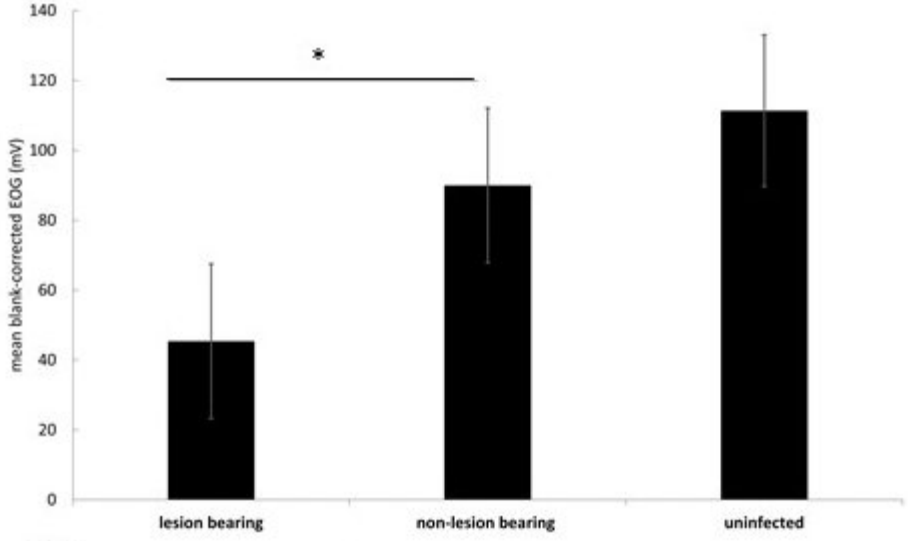


Figure 3.1. Images of the large, distinctive lesions associated with the development of *Myxobolus* sp. pseudocysts in the heads of Fathead Minnows. A. Lesions on both olfactory rosettes (top arrow). This fish has relatively small lesions behind both eyes (lower arrow). B. Severe lesions (arrow) immediately adjacent to, and encircling, the ocular cavity. C. Ventral surface of the head region indicating extensive lesion on pectoral girdle (upper arrow) and lower mandible (lower arrow).


Proportion of time moving (%)
100
90
80
70
60
50
40
30
20
10
0
**
lesion bearing
non-lesion bearing
uninfected


mean blank-corrected EOG (mV)
140
120
100
80
60
40
20
0
*
lesion bearing
non-lesion bearing
uninfected

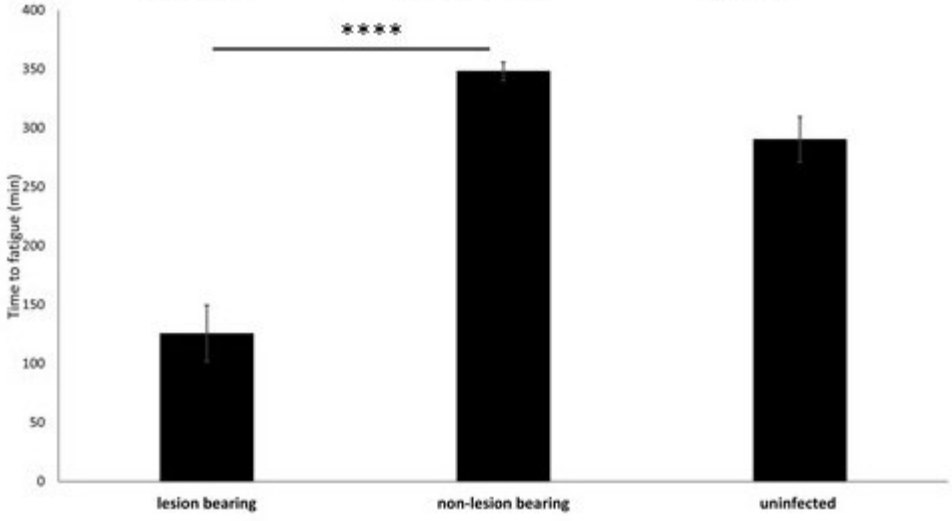

Time to fatigue (min)
400
350
300
250
200
150
100
50
0
****
lesion bearing
non-lesion bearing
uninfected

Figure 3.2: Effects of *Myxobolus* sp. on the physiological performance of Fathead Minnows. A) Proportion of time spent moving in the general activity evaluation of lesion-bearing, non-lesion bearing, and uninfected Fathead Minnows. B) Mean EOG responses for lesion bearing (infected), non-lesion bearing (uninfected), and uninfected wild Fathead Minnows. C) Mean time to fatigue, calculated as time spent swimming divided by individual fork length (mm) of lesion-bearing, non-lesion bearing, and uninfected Fathead Minnows.

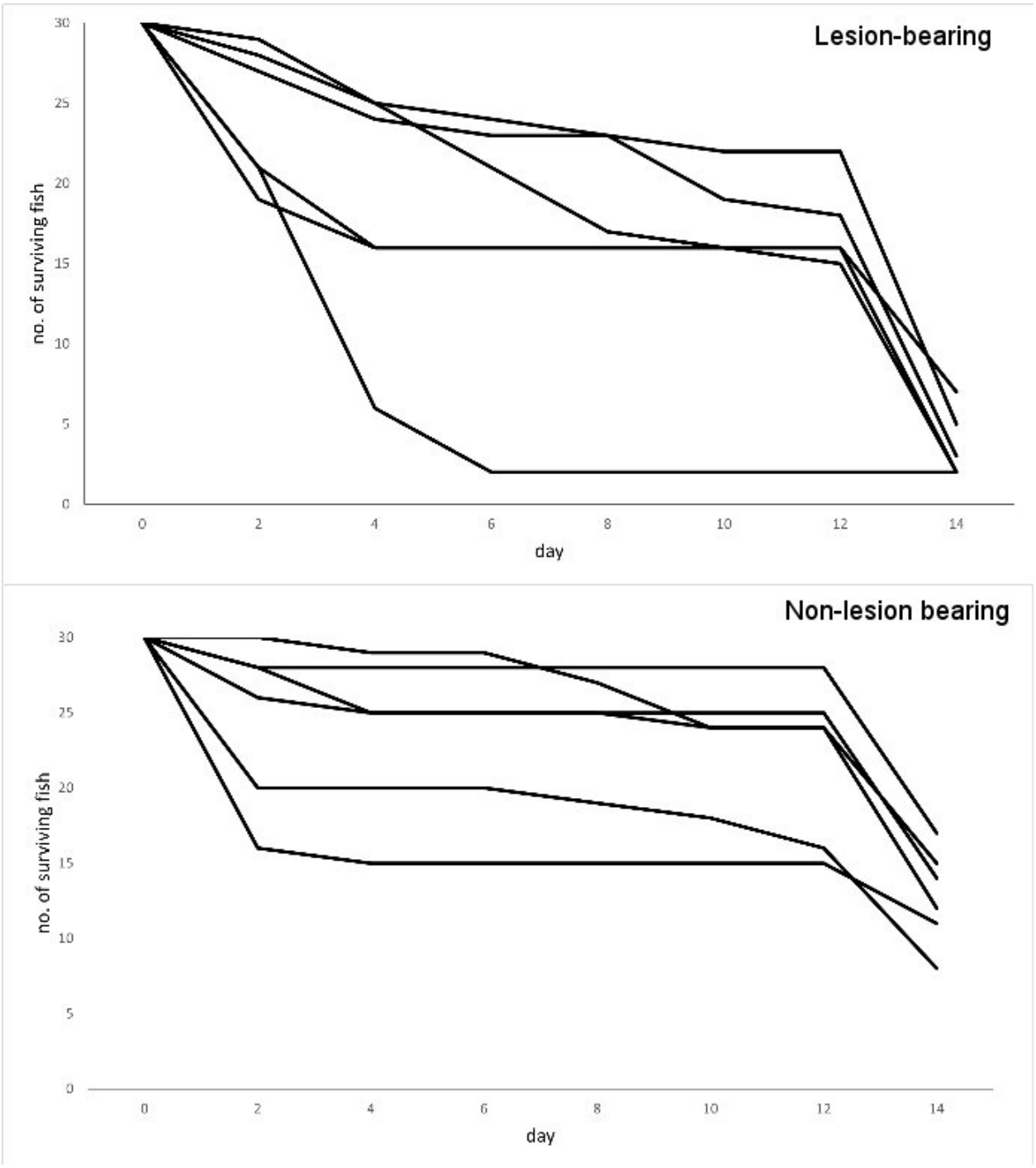


Figure 3.3. Estimated daily survival over 14 days of lesion bearing (right) vs non-lesion bearing (left) Fathead Minnows collected from Coulee creek stormwater pond in July

2019 and then reared in six outdoor mesocosms. Each line represents estimated survival within each mesocosm.

CHAPTER 4: GENERAL DISCUSSION

My thesis aimed to characterize a new species of myxozoan emerging in Fathead Minnows in southern Alberta and to describe its effects on individual hosts. My results in Chapter 2 provide multiple lines of evidence that *Myxobolus* sp. is indeed a new species of myxozoan and one that is likely emerging in several populations of Fathead Minnow in this region. In that chapter, I describe this new species and I characterize the oligochaete, *Tubifex tubifex,* as its definitive host. In, Chapter 3, I combined the results of physiological and behavioural assays to provide evidence that the distinctive lesions, first introduced in the species description provided by Chapter 2, influences the capacity of lesion-bearing minnows to respond to external stimuli. Overall, by demonstrating differences in performance between lesion bearing and non-lesion bearing minnows and documenting a reduction in survival between lesioned and non-lesioned minnows, I suggest that *Myxobolus* sp. is a pathological agent of disease and is emerging in Fathead Minnow populations in southern Alberta.

The results of Chapter 2 provide the first description of the diverse assemblage of myxozoans in Fathead Minnows in western Canada and is the first to document concurrent infections within individual fish. Small-bodied forage fish, like Fathead Minnows, are known to harbour multiple species of myxozoans (Ahn, 2019; Sandland et al., 2001; Wisenden et al., 2012). In Eastern Canada, spot-tail and common shiner can be infected with 4-9 different myxozoan species, respectively (Marcogliese & Cone, 2001, 2021). In each of these shiner species maximum myxozoan infracommunity richness was 3 and 4, respectively, within a single host. The results from my histological data (Chapter 2) show that myxozoan richness in individual Fathead Minnows and in populations of

Fathead Minnows in this region are similar to the rich communities in other cyprinids that have been described using similar methods (Minchew, 1981; Cone & Frasca, 2002; Mitchell et al., 1985). These results demonstrate a level of myxozoan biodiversity that has previously not been documented in any other cyprinids in western Canada.

Another important result from Chapter 2 is the demonstration that *Tubifex tubifex* is the final host of *Myxobolus* sp. This revelation places *Myxobolus* sp. among the comparatively few myxozoan life cycles in which both required hosts have been confirmed (Fontes et al., 2015). Even well-known and intensively studied myxozoan species, such as *Enteromyxxum leei,* still have unresolved life cycles (Fontes et al., 2015; Yanagida et al., 2006). Furthermore, my finding that there are at least two species of myxozoan TAMs released from the oligochaetes from University Pond demonstrates the incredible colonization ability of myxozoans. Indeed, the only known intermediate hosts of *Dicauda athernoidi,* the only other myxozoan recovered from oligochaetes in University Pond, emerald shiner and mimic shiner (Loch et al., 2017), are not (and have never been) present at that site. Emerald shiners are found in the Oldman River, which is geographically distinct yet adjacent to University Pond. This means that *Dicauda athernoidi* likely originates from the feeding and movement of fish-eating birds moving between the Oldman River and University Pond.

Although, the two species of hosts required to complete life cycle of *Myxobolus* sp. are now known, follow-up studies should aim to complete transmission through all the life stages under laboratory conditions. Laboratory studies could also help determine the complex course of development of each life cycle stage in each host. Should this 'next-step' be completed, the *Myxobolus* sp./Fathead Minnow/*Tubifex tubifex* system

would be among the very select few myxozoans for which the entire life cycle could be completed in the laboratory. The gold standard (Eszterbauer et al., 2015) could then be used to answer countless questions that remain regarding features such as routes of TAM entry, migration to the target tissues, and subsequent intra-host development of *Myxobolus* sp. in each host. In short, one key implication of the results of Chapters 2 and 3 is the demonstrated ease with which the *Myxobolus* sp./Fathead Minnow/*Tubifex tubifex* system interaction could develop into a model system that parallels the well-known *Myxobolus cerebralis*/trout interaction (El-Matbouli et al., 1995; El-Matbouli & Hoffmann, 1998). Overall, the results from my thesis provide important life cycle information and illustrates the complexities of myxozoan life cycles and identifies clear knowledge gaps in *Myxobolus* sp. development that may now be addressed in future laboratory studies.

Emerging infectious diseases of wildlife have garnered increased attention in recent years, particularly by epidemiologists, conservation biologists, and global change biologists. Myxozoan infections of fish have not been prominent in this increased attention, despite the fact that *M. cerebralis* has emerged in salmonid populations around the world, including Alberta (James et al., 2021). Emerging infectious diseases of wildlife have been recognized as a major threat to the conservation of biodiversity globally (Daszak, 2000; Daszak et al., 2001; James et al., 2021; Thompson et al., 2009). The results described in chapters 2 and 3, provide evidence suggesting that *Myxobolus* sp. is a novel emerging disease within several Fathead Minnow populations in southern Alberta. This result inspires key questions regarding its origin. One speculative possibility is that it arose from a host-shift from a fish species that is sympatric with Fathead Minnows in

southern Alberta. The results of studies involving the transmission and phylogeny of the cyprinid-infecting myxozoan *M. pseudodispar* demonstrate that a series of host shifts allowed it to exploit at least four distinct cyprinid hosts. Interestingly, isolates of *M. pseudodispar* from different hosts show comparatively large genetic differences, a result that suggests the potential for active speciation (Forro & Eszterbauer, 2016). Stenoxenic species of myxozoan, such as *M. cerebralis,* can infect multiple species of fish (Americus et al., 2021; Fetherman et al., 2011; Gilbert & Granath, 2003; Koel et al., 2006; Kotob et al., 2017), and have been reported to infect oligochates in addition to *Tubifex tubifex* (Ksepka et al., 2021). Thus, the conditions for a host shift (Agosta et al., 2010) from an original cyprinid host into Fathead Minnows are likely met within fish communities in ponds in southern Alberta. Follow-up studies involving host surveys that include sympatric cyprinids would be useful. Ideally, these studies would incorporate similar histological and molecular methods used in this thesis. Relying solely on the detection of characteristic lesions in other potential hosts would be inconclusive if patterns of lesion development were host species specific.

An alternative explanation for the origin of *Myxobolus* sp. in Fathead Minnow is the possibility that there was an anthropogenic role in its dissemination. Human activity has been implicated as a driver of *M. cerebralis* dissemination in Alaskan watersheds (Arsan & Bartholomew, 2008; Bartholomew & Reno, 2002), and indeed anthropogenic influences more generally have been implicated as significant drivers of infectious disease emergence (Daszak et al., 2001). Cyprinids are common baitfish used for recreational fishing and are often infected with various species of parasite (Purdy, 2011). The transport of baitfish by recreational fisherman could lead to introduced fish species

that are not naturally sympatric with wild Fathead Minnow. In Alberta, the introduction of Fathead Minnows as a food source to support recreational trout fisheries is common. The anthropogenic transportation of muddy substrate between adjacent waterbodies could also play a role in the movement of infected *T. tubifex* or *Myxobolus* myxospores between water bodies. Overall, my results in Chapter 2 are concerning from a conservation viewpoint because even a very small (1 Ha), recently constructed, and artificial wetland has been colonized by at least two species of myxozoan in a very rapid period of time, one of which is emerging within a new host. This colonization history emphasizes the incredible dispersal ability of myxozoans and perhaps also emphasizes the potential for high rates of emergence within a new species of host in new locations.

Importantly, the results from Chapter 3 support the notion that the lesions caused by *Myxobolus* sp. are pathogenic. Lesioned fish had poorer endurance and olfaction performance and thus are likely less capable of effectively locating food, mates, and avoiding predators (Lari et al., 2017). Additionally, lesioned minnows showed higher mortality than non-lesioned minnows. Somewhat unexpectedly, lesioned minnows displayed hyperactivity, spending a disproportionate time actively swimming compared to non-lesioned minnows. Hyperactivity is often a general indicator of stress in fish (Martins et al., 2012). Despite the obvious bulging of the eyes due to parasite development within the ocular cavity, the results of the optomotor assay found no significant reduction in visual acuity of lesioned minnows. This result is surprising considering the nature of the lesions. It is especially surprising given my observations that I could easily catch lesioned minnows by hand. One explanation for this result is that the crude designations that I applied to individual minnows as “lesion bearing” or “non-

lesion bearing" underestimated the real extent of host pathology. The dose and intensity of myxozoan infections have been recognized in other species as determinants of pathology or adverse outcomes for infected hosts (Hedrick et al., 2001; Ryce et al., 2004; Wise et al., 2008). Similarly, increased pathology caused by other myxozoans is generally associated with exposure to greater doses of triactinomyxons (Hedrick et al., 1999; McGurk et al., 2006). It is therefore likely that the magnitude of *Myxobolus* sp.-related pathology is dose dependent. Development of a score related to the age or extent of lesions in individual fish would be a logical direction for future studies as it may give insight into severity progression of lesions and ultimately indicate at what point lesions become lethal.

An intriguing alternative explanation for potential mechanisms of myxozoan-induced effects on behaviour and performance is that *Myxobolus* sp. drives the alterations to facilitate rates of myxospore transmission into the next host. Thus, one possibility is that the structure and locations of the lesions themselves facilitate the release of myxospores, even prior to the death of the host. Anecdotally, the consistency of the lesioned tissues, especially in fish with advanced pathology, are pus-like; sloughing of mucus and myxospores is easily observed upon minimal contact. Pre-death liberation of mature myxospores has been demonstrated for *M. artus* of common carp. In this case, mature myxospores are phagocytosed by host macrophages and expelled from the host via the kidneys, gills, skin and intestine (Ogawa et al., 1992; Yokoyama et al., 1996). It is conceivable that the location and structure of the lesions in Fathead Minnows may function similarly. A second scenario is that the lesions may accelerate the death of the host, either through pathological impairment of normal functions (e.g., swimming) or by

increasing rates of predation. Other parasites alter host behaviours to increase or accelerate transmission to the next host. Killifish infected with larval trematodes often display flashing and other characteristic behaviours that uninfected killifish do not. These behaviours make infected fish more conspicuous to bird predators and subsequently increase bird predation (Lafferty & Morris, 1996). Similarly, the eye fluke *Diplostomum spathaceum* induces cataract formation in Rainbow trout that impairs vision. These infected hosts are more vulnerable to predation, especially during the period of time when parasites are at their full maturity and infective to the bird host (Seppälä et al., 2005a, 2005b). Surfacing behaviour is also demonstrated by sea trout infected with the myxozoan *Parvicapsula pseudobranchiola* which also cause pathology to the eyes, and it is assumed that this parasite causes impairment to sea trout vision which likely help facilitate predation (Nylund et al., 2018). Thus, it is conceivable that the hyperactive and surfacing behaviour demonstrated by lesion-bearing Fathead Minnows infected with *Myxobolus* sp. could facilitate predation by bird, fish, and invertebrate predators that could then liberate infective myxospores. This explanation is tantalizing because the lesions, especially those that encircle the eyes, are extremely conspicuous, at least to human observers.

Furthermore, these results provide tantalising preliminary data for formal tests of population-level effects. For the sample of fish from the 2020 cohort in Coalhurst Stormwater Pond for example, the high prevalence of lesions in 18-mo old minnows begs the question of whether lesion-induced pathology will result in extensive mortality, either over winter or in April/May after ice-off. If so, I can predict a loss of the 2020 cohort during the 2022 breeding season. This scenario is testable with further sampling in this

and other high-prevalence sites. In contrast, I may expect to see a higher recovery of lesion-bearing adults in the June 2022 sample in fish from McQuillan Reservoir. Fathead Minnow population sizes, reproduction, spawning and ultimately survival is influenced by external factors such as anoxia-based winter-kill, predation by piscivorous birds, fish, and invertebrates (Danylchuk & Tonn, 2006; Divino & Tonn, 2007; Jung & Tonn, 2011). Therefore, the emergence of pathogenic *Myxobolus* sp. in Fathead Minnow populations in Alberta, combined with these other external stressors, and additional stressors such as climate change, could lead to important alterations within aquatic food webs in southern Alberta.

4.1 Literature Cited

Agosta, S. J., Janz, N., & Brooks, D. R. 2010. How specialists can be generalists: resolving the "parasite paradox' and implications for emerging infectious disease. Zoologia 27: 151–162.

Ahn, S. 2019. *Effects of host community structure on parasite transmission and disease risk.* [Master of Science]. University of Lethbridge.

Americus, B., Schisler, G. J., Bartholomew, J. L., & Atkinson, S. D. 2021. *Myxobolus cerebralis* causes presporogonic mortality in juvenile mountain whitefish. Journal of Aquatic Animal Health 33: 116–122.

Arsan, E. L., & Bartholomew, J. L. 2008. Potential for dissemination of the nonnative salmonid parasite *Myxobolus cerebralis* in Alaska. Journal of Aquatic Animal Health 20: 136–149.

Bartholomew, J. L., & Reno, P. W. 2002. The history and dissemination of whirling disease. American Fishereies Society Symposium 26:1-26.

Cone, D. K., & Frasca, S. 2002. Revised diagnosis, site of development, and lesions due to *Myxobolus hyborhynchi* (Myxozoa) parasitizing fathead minnow in Minnesota. Journal of Aquatic Animal Health 14: 209–215.

Danylchuk, A. J., & Tonn, W. M. 2006. Natural disturbance and life history: consequences of winterkill on fathead minnow in boreal lakes. Journal of Fish Biology 68: 681–694.

Daszak, P. 2000. Emerging infectious diseases of wildlife: threats to biodiversity and human health. Science 287: 443–449.

Daszak, P., Cunningham, A. A., & Hyatt, A. D. 2001. Anthropogenic environmental change and the emergence of infectious diseases in wildlife. Acta Tropica 78: 103–116.

Divino, J. N., & Tonn, W. M. 2007. Effects of reproductive timing and hatch date on fathead minnow recruitment. Ecology of Freshwater Fish 16: 165–176.

El-Matbouli, M., & Hoffmann, R. W. (1998). Light and electron microscopic studies on the chronological development of *Myxobolus cerebralis* to the actinosporean stage in *Tubifex tubifex*. International Journal for Parasitology 28: 195–217.

El-Matbouli, M., Hoffmann, R. W., & Mandok, C. 1995. Light and electron microscopic observations on the route of the triactinomyxon-sporoplasm of *Myxobolus cerebralis* from epidermis into rainbow trout cartilage. Journal of Fish Biology 46: 919–935.

Eszterbauer, E., Atkinson, S. D., Diamant, A., Morris, D. J., EL-Matbouli, M., & Hartikainen, H. 2015. Myxozoan Life Cycles: Practical Approaches and Insights. In B. Okamura, A. Gruhl, & J. L. Bartholomew (Eds.), *Myxozoan Evolution, Ecology and Development*. (pp. 175-200). Springer International Publishing.

Fetherman, E. R., Winkelman, D. L., Schisler, G. J., & Myrick, C. A. 2011. The effects of *Myxobolus cerebralis* on the physiological performance of whirling disease resistant and susceptible strains of rainbow trout. Journal of Aquatic Animal Health 23: 169–177.

Fontes, I., Hallett, S. L., & Mo, T. A. 2015. Comparative epidemiology of myxozoan diseases. In B. Okamura, A. Gruhl, & J. L. Bartholomew (Eds.), In *Myxozoan evolution, ecology and development* (pp. 317–342). Springer International Publishing.

Forro, B., & Eszterbauer, E. 2016. Correlation between host specificity and genetic diversity for the muscle-dwelling fish parasite *Myxobolus pseudodispar*: examples of myxozoan host-shift? Folia Parasitologica 63: 19-27.

Gilbert, M. A., & Granath, W. O. 2003. Whirling disease of salmonid fish: life cycle, biology, and disease. Journal of Parasitology 89: 658–667.

Hedrick, R. P., McDowell, T. S., Gay, M., Marty, G. D., Georgiadis, M. P., & MacConnell, E. 1999. Comparative susceptibility of rainbow trout *Oncorhynchus mykiss* and brown trout *Salmo trutta* to *Myxobolus cerebralis*, the cause of salmonid whirling disease. Diseases of Aquatic Organisms 37: 173-183.

Hedrick, R. P., McDowell, T. S., Mukkatira, K., Georgiadis, M. P., & MacConnell, E. 2001. Susceptibility of three species of anadromous salmonids to experimentally induced infections with *Myxobolus cerebralis*, the causative agent of whirling disease. Journal of Aquatic Animal Health 13: 43–50.

James, C. T., Veillard, M. F., Martens, A. M., Pila, E. A., Turnbull, A., Hanington, P., Luek, A., Alexander, J., & Nehring, R. B. 2021. Whirling disease in the Crowsnest River: an emerging threat to wild salmonids in Alberta. Canadian Journal of Fisheries and Aquatic Sciences 78: 1–14.

Jung, J. A., & Tonn, W. M. 2011. Alarm substances elicit limited population-level responses in fathead minnow. Ecology of Freshwater Fish 20: 220–230.

Koel, T. M., Mahony, D. L., Kinnan, K. L., Rasmussen, C., Hudson, C. J., Murcia, S., & Kerans, B. L. 2006. *Myxobolus cerebralis* in native cutthroat trout of the Yellowstone Lake ecosystem. Journal of Aquatic Animal Health 18: 157–175.

Kotob, M. H., Gorgoglione, B., Kumar, G., Abdelzaher, M., Saleh, M., & El-Matbouli, M. 2017. The impact of *Tetracapsuloides bryosalmonae* and *Myxobolus cerebralis* co-infections on pathology in rainbow trout. Parasites & Vectors 10: 442-456.

Ksepka, S. P., Rash, J. M., Cai, W., & Bullard, S. A. 2021. Detection of *Myxobolus cerebralis* (Bivalvulida: Myxobolidae) in two non-*Tubifex tubifex* oligochaetes in the southeastern USA. Diseases of Aquatic Organisms 143: 51–56.

Lafferty, K. D., & Morris, A. K. 1996. Altered behavior of parasitized killifish increases susceptibility to predation by bird final hosts. Ecology 77: 1390–1397.

Lari, E., Goater, C. P., Cone, D. K., & Pyle, G. G. 2017. Parasites and a host's sense of smell: reduced chemosensory performance of fathead minnows (*Pimephales promelas*) infected with a monogenean parasite. Journal of Animal Ecology 86: 434–441.

Loch, T. P., Rosser, T. G., Baumgartner, W. A., Boontai, T., Faisal, M., & Griffin, M. J. 2017. New host record and molecular characterization of *Dicauda atherinoidi* Hoffman & Walker (Bivalvulida: Myxobolidae): a parasite of the emerald shiner *Notropis atherinoides* Rafinesque, 1818 and mimic shiner *Notropis vollucellus* Cope, 1865. Journal of Fish Diseases 40: 1405-1415.

Marcogliese, D. J., & Cone, D. K. 2001. Myxozoan communities parasitizing Notropis hudsonius (Cyprinidae) at selected localities on the St. Lawrence River, Quebec: possible effects of urban effluents. Journal of Parasitology 87: 951–956.

Marcogliese, D. J., & Cone, D. K. 2021. Myxozoan communities in two cyprinid fishes from mesotrophic and eutrophic rivers. Journal of Parasitology 107: 39–47.

Martins, C. I. M., Galhardo, L., Noble, C., Damsgård, B., Spedicato, M. T., Zupa, W., Beauchaud, M., Kulczykowska, E., Massabuau, J.-C., Carter, T., Planellas, S. R., & Kristiansen, T. 2012. Behavioural indicators of welfare in farmed fish. Fish Physiology and Biochemistry 38: 17–41.

McGurk, C., Morris, D. J., Auchinachie, N. A., & Adams, A. 2006. Development of *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea) in bryozoan hosts (as examined by light microscopy) and quantitation of infective dose to rainbow trout (*Oncorhynchus mykiss*). Veterinary Parasitology 135: 249–257.

Minchew, C. D. 1981. *Unicauda magna* sp. n. (Protozoa: Myxozoa): a new myxozoan from the fin tissues of the fathead minnow, *Pimphales promelas* Rafinesque. Journal of Fish Diseases 4: 513–518.

Mitchell, L. G., Seymour, C. L., & Gamble, J. M. 1985. Light and electron microscopy of *Myxobolus hendricksoni* sp. nov. (Myxozoa: Myxobolidae) infecting the brain of

the fathead minnow, *Pimephales promelas* Rafinesque. Journal of Fish Diseases 8: 75–89.

Nylund, A., Hansen, H., Brevik, Ø. J., Hustoft, H., Markussen, T., Plarre, H., & Karlsbakk, E. 2018. Infection dynamics and tissue tropism of *Parvicapsula pseudobranchicola* (Myxozoa: Myxosporea) in farmed Atlantic salmon (*Salmo salar*). Parasites & Vectors 11: 17-30.

Ogawa, K., Delgahapitiya, K., Furuta, T., & Wakabayashi, H. 1992. Histological studies on the host response to *Myxobolus artus* Akhmerov, 1960 (Myxozoa: Myxobolidae) infection in the skeletal muscle of carp, *Cyprinus carpio* L. Journal of Fish Biology 41: 363–371.

Pan, T., Gladen, K., Duncan, E. C., Cotner, S., Cotner, J. B., McEwen, D. C., & Wisenden, B. D. 2016. Bold, Sedentary Fathead Minnows Have More Parasites. Zebrafish 13: 248–255.

Purdy, A. M. 2011. *Parasite survey of fathead minnows, golden shiners, and white suckers used as bait in Wisconsin*. [Master of Science). University of Wisconsin-La Crosse.

Robinson, C. L. K., & Tonn, W. M. 1989. Influence of environmental factors and piscivory in structuring fish assemblages of small Alberta lakes. Canadian Journal of Fisheries and Aquatic Sciences 46: 81–89.

Ryce, E. K. N., Zale, A. V., & MacConnell, E. 2004. Effects of fish age and parasite dose on the development of whirling disease in rainbow trout. Diseases of Aquatic Organisms 59: 225-233.

Sandland, G. J., Goater, C. P., & Danylchuk, A. J. 2001. Population dynamics of *Ornithodiplostomum ptychocheilus* metacercariae in fathead minnows (*Pimephales promelas*) from four northern-Alberta lakes. Journal of Parasitology 87: 744–748.

Seppälä, O., Karvonen, A., & Valtonen, E. T. 2005a. Impaired crypsis of fish infected with a trophically transmitted parasite. Animal Behaviour 70: 895–900.

Seppälä, O., Karvonen, A., & Valtonen, E. T. 2005b. Manipulation of fish host by eye flukes in relation to cataract formation and parasite infectivity. Animal Behaviour 70: 889–894.

Shirakashi, S., & Goater, C. P. 2001. Brain-encysting parasites affect visually-mediated behaviours of fathead minnows. Écoscience 8: 289–293.

Thompson, R. C. A., Kutz, S., & Smith, A. 2009. Parasite zoonoses and wildlife: emerging issues. International Journal of Environmental Research and Public Health 6: 678–693.

Wise, D. J., Griffin, M. J., Terhune, J. S., Pote, L. M., & Khoo, L. H. 2008. Induction and evaluation of proliferative gill disease in channel catfish fingerlings. Journal of Aquatic Animal Health 20: 236–244.

Wisenden, B. D., Martinez-Marquez, J. Y., Gracia, E. S., & McEwen, D. C. 2012. High intensity and prevalence of two species of trematode metacercariae in the fathead minnow (*Pimephales promelas*) with no compromise of minnow anti-predator competence. Journal of Parasitology 98: 722–727.

Yanagida, T., Sameshima, M., Nasu, H., Yokoyama, H., & Ogawa, K. 2006. Temperature effects on the development of *Enteromyxum* spp. (Myxozoa) in experimentally infected tiger puffer, *Takifugu rubripes* (Temminck & Schlegel). Journal of Fish Diseases 29: 561–567.

Yokoyama, H., Danjo, T., Ogawa, K., Arima, T., & Wakabayashi, H. 1996. Hemorrhagic anemia of carp associated with spore discharge of *Myxobolus artus* (Myxozoa: Myxosporea). *Gyobyō Kenkyu*, *31*: 19–23.