INVESTIGATING INFECTIOUS DISEASES AS CONTRIBUTORS TO DECLINING RECRUITMENT IN GREAT LAKES LAKE WHITEFISH (*COREGONUS CLUPEAFORMIS*)

By

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ABSTRACT

INVESTIGATING INFECTIOUS DISEASES AS CONTRIBUTORS TO DECLINING RECRUITMENT IN GREAT LAKES LAKE WHITEFISH (*COREGONUS CLUPEAFORMIS*)

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Lake whitefish (*Coregonus clupeaformis*; LWF) is an economically and ecologically important indigenous Great Lakes (GL) fish species. Recently, declines in lake whitefish abundance, growth, and early life stage recruitment have generated substantial concern. Although numerous studies have attempted to elucidate the factors behind these declines, the cause(s) remain undetermined. To investigate the potential role(s) of infectious diseases in these declines, I collected wild adult and juvenile GL-LWF over two years from multiple sites within Lakes Superior, Michigan, and Huron, and performed extensive fish health analyses. For the first time, I recovered *Flavobacterium psychrophilum* from systemically infected adult GL-LWF and likewise isolated *Carnobacterium maltaromaticum* from gonadal tissues of infected adult GL-LWF, highlighting potential health risks to their offspring. In this context, I reared juvenile GL-LWF under laboratory conditions for *in vivo* challenge experiments, but before experiments commenced, juvenile GL-LWF showed clinical signs of disease with mortality quickly following. Upon diagnostic investigation, I discovered that affected fish were systemically infected with *C. maltaromaticum*. To further investigate effects that infectious microbes have on the health and survival of juvenile GL-LWF, *in vivo* challenge experiments with *Aeromonas salmonicida* subsp. *salmonicida* and Viral Hemorrhagic Septicemia Virus (VHSV) were performed, revealing high susceptibility to both fish pathogens. Collectively, my results highlight multiple fish pathogens as potential contributors to reduced GL-LWF health and survival.

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Introduction

Lake whitefish (*Coregonus clupeaformis*) is an important indigenous salmonid species within the Great Lakes basin that plays an integral role in cycling energy through the food web (Mohr and Nalepa 2005) and supports a highly valued commercial fishery (Ebener et al. 2008). The Great Lakes lake whitefish population abundance has fluctuated over time, reaching an all-time low in the 1960s (Baldwin et al. 1979, Ebener 1997) followed by a successful recovery resulting from improvements in fishery management, sea lamprey (*Petromyzon marinus)* population control, and successful lake whitefish reproduction (Ebener 1997). More recently, lake whitefish populations have also experienced declines in adult abundance and growth that has been hypothesized to be partially attributable to the decline in their primary food source, *Diporeia* spp., resulting from the establishment of *Dreissena* spp. to the Great Lakes (Pothoven et al. 2001, Mohr and Nalepa 2005).

Since the early 2000s and concomitant with declines in population abundance and growth, juvenile lake whitefish recruitment has substantially declined throughout many sites in the four lower Great Lakes (reviewed in Ebener et al. 2021). Several abiotic and biotic factors, including climate effects, water temperature, ice cover, spawning and nursery habitat changes, and food web shifts, have been hypothesized as contributing to declines in recruitment, although currently no definitive driving factors have been deduced (Ebener et al. 2021).

Infectious disease as a potential factor in Great Lakes lake whitefish recruitment has not been extensively investigated despite being a known cause of significant mortality in some wild fish populations (Lafferty et al. 2015), including those residing within the Great Lakes Basin (Holey et al. 1998, Faisal et al. 2012). Of note, multiple microbial pathogens that cause significant

diseases in a range of fishes have been detected in wild adult Great Lakes lake whitefish populations, including Viral Hemorrhagic Septicemia Virus (VHSV), *Renibacterium salmoninarum*, *Carnobacterium maltaromaticum*, and *Aeromonas salmonicida* subsp. *salmonicida* (reviewed in Loch and Faisal 2011). Likewise, these same microbial pathogens were found in a subset of adult Great Lakes lake whitefish with clinical signs of disease

In the context of risks associated with recruitment, microbial pathogens detected in wild adult Great Lakes lake whitefish have the potential to transmitted from infected parents to offspring via the reproductive tissues and/or fluids (Starliper et al. 1992, Cipriano et al. 2001, Austin and Austin 2007, OIE 2021) or be transmitted *intra ova* (Evelyn et al. 1984, Evelyn et al. 1986, Wiens et al. 2011). Furthermore, these pathogens have also been linked to substantial mortality in young fish in other salmonid species, which is important given that juvenile lake whitefish recruitment is poor in some part of the Great Lakes.

Unfortunately, the effects infectious diseases have on Great Lakes lake whitefish populations in general, and early life stage/juvenile lake whitefish survival specifically, are completely unknown. If pathogens are present within the reproductive tissues of adult lake whitefish, is there potential for the pathogen to be transmitted from the infected parent to their offspring? Likewise, if the offspring become infected by those same pathogens, can they persist into later early life stages? Additionally, if juvenile lake whitefish are infected with these same pathogens, can they induce disease and/or mortality? Above all else, the effects of these pathogens on the health and survival of juvenile Great Lakes lake whitefish, and the potential for these pathogens to be linked as a factor contributing to poor recruitment, are not fully understood.

1. Study objectives

This study focused on investigating infectious diseases as possible contributing factors to declining Great Lakes lake whitefish recruitment. To elucidate the risk that microbial infections of adult Great Lakes lake whitefish potentially pose to juvenile recruitment, wild adult lake whitefish were collected from five sites within Lakes Superior, Michigan, and Huron during 2018 and 2019. Wild juvenile lake whitefish were collected from sandy beaches adjacent to adult spawning locations within those same lakes from May – July 2019, and in June of 2021. Paired sites within Lakes Michigan and Huron were selected based on historical and recent data on juvenile lake whitefish recruitment and to compare findings from good and poor recruitment sites. Fish were collected from only one site within Lake Superior, as lake whitefish have had persistently good/stable recruitment within this lake. The overarching objectives of this study were to: 1) assess disease status in wild adult spawning Great Lakes lake whitefish; 2) assess disease status in wild juvenile Great Lakes lake whitefish; and 3) determine the pathogenicity of multiple microbial pathogens in juvenile Great Lakes lake whitefish under controlled laboratory conditions.

This thesis consists of five chapters. The first chapter is a thorough review of literature pertaining to the thesis' topic. The second chapter describes the first isolation of *Flavobacterium psychrophilum*, etiological agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), from adult Great Lakes lake whitefish. The third chapter details the widespread presence of systemic *C. maltaromaticum* infections in Great Lakes lake whitefish, including within the reproductive tissues of infected fish, and details a natural disease outbreak involving the same bacterium in juvenile Great Lakes lake whitefish reared under laboratory

conditions. The fourth chapter involves experimental challenges investigating virulence and effects of Viral Hemorrhagic Septicemia Virus and *Aeromonas salmonicida* subsp. *salmonicida* on the health and survivability of juvenile Great Lakes lake whitefish. Finally, the fifth chapter will present my conclusions and potential directions for future research.

Chapter 1:

Literature Review

1. Lake whitefish in the Great Lakes Basin of North America

Lake whitefish (*Coregonus clupeaformis*) within the family Salmonidae, are ecologically and economically important indigenous fish within the Great Lakes. Lake whitefish arrived in North America and into the Great Lakes from northern Europe and Asia via the Arctic Ocean, where populations survived on the edges of glacial ice sheets for thousands of years (Franzin and Clayton 1977, Bernatchez and Dobson 1991). Before the establishment of the Great Lakes commercial fishery, aboriginal people heavily relied on lake whitefish for subsistence due to their abundance and had developed gillnet fisheries targeting lake whitefish among other species in waters nearshore (Ebener et al. 2008). Not only are lake whitefish culturally important, but they also play a key role in cycling energy from the lower to upper trophic levels in the Great Lakes food web (Mohr and Nalepa 2005), which simultaneously supports a highly valuable commercial fishery (Ebener et al. 2008). As of 2020, lake whitefish composed approximately 90% of the harvest and 95% of sales within Michigan commercial fisheries (Michigan Sea Grant – Commercial Fishing).

2. Historical and contemporary declines of Great Lakes lake whitefish population abundance and growth

Beginning in the mid-1800s and primarily due to a lack of regulation on commercial harvests, the abundance of Great Lakes lake whitefish declined substantially; technological advancements in fishing equipment in the early 1900s exacerbated these declines (Ebener 1997). Further declines in lake whitefish populations occurred during this time due to timber harvesting, which negatively impacted the habitats used by lake whitefish for reproduction, feeding, and rearing of young fish (Ebener 1997). The introduction of non-native species, such as alewife

(*Alosa pseudoharengus),* rainbow smelt (*Osmerus mordax*), white perch (*Morone americana*), and the invasion of sea lamprey in the 1930s and 1940s resulted in predation on and competition with lake whitefish populations; by the 1960s, lake whitefish population abundance was at an all-time low (Ebener 1997, Mohr and Nalepa 2005). Fortunately, with improved management practices, sea lamprey population control, commercial fishery restrictions, and successful lake whitefish reproduction, population abundance recovered (Ebener 1997).

Despite the abundance rebound that occurred in the late 1990s, lake whitefish abundance and growth in the four lower Great Lakes have been declining since the early 2000s. The decrease of *Diporeia* spp., a benthic amphipod that historically served as a primary food source of lake whitefish, resulted in changes in feeding habits of lake whitefish to organisms of lower nutritional value or are less abundant, which have been linked to declines in adult lake whitefish growth (Pothoven et al. 2001). The establishment of the zebra mussel (*Dreissena polymorpha*) and quagga mussel (*D. bugensis*), which are voracious filter-feeders (May and Marsden 1992, Carlton 2008), in the Great Lakes resulted in a loss of food sources relating to mussel filtering (Mohr and Nalepa 2005).

3. Declining juvenile lake whitefish recruitment

Concomitant with declines in adult lake whitefish abundance and growth during the early 2000s were notable reductions in the survival of lake whitefish early life stages. This survival of the early life stages often referred to as "recruitment," refers to the process of young fish surviving to become part of the adult population that is subsequently harvested (Ebener et al. 2021). However, a more contemporary definition of recruitment is thought of as a two-stage

process; the first being survival of early life stages (e.g., eggs and larvae), which suffer high mortality rates (>99%) that affect their recruitment during their first year of life; and second, after year class abundance is established in the first year or two of life, juvenile fish suffer lower mortality rates (<15-30%) similar to that of adults, where their recruitment is a function of growth rate and movement to fishing grounds (Ebener et al. 2021).

Since the early 2000s, both early life stage and juvenile lake whitefish recruitment have consistently declined in many sites throughout the four lower Great Lakes (Mohr and Nalepa 2005, Ebener et al. 2008, Brenden et al. 2010, Lenart and Caroffino 2016, Ebener et al. 2021), whereas Lake Superior lake whitefish recruitment has remained stable (Ebener et al. 2021). The declines in Great Lakes lake whitefish recruitment around the basin is concerning because of their significant contribution to the ecology of the Great Lakes Basin and to the Great Lakes fishery, thus elucidating the factors behind these declines has become a major focal point of Great Lakes fisheries managers and researchers alike.

4. Possible factors contributing to recruitment declines

4.1. Abiotic factors

Several abiotic aspects of Great Lakes lake whitefish recruitment dynamics have been examined, including lake whitefish spawning and nursery habitat, climate effects, water temperature, ice coverage, and Great Lakes water levels (Ebener et al. 2021). Lake whitefish deposit gametes and fertilized embryos in nearshore high energy zones in water <7 meters deep (Hart 1930, Hoagman 1973, Freeberg et al. 1990, Roseman et al. 2007, McKenna and Johnson 2009). Once hatched, larvae are transported by winds and currents or their own locomotion to

shallower (e.g., <1 meter) water nursery habitat that provides protection from strong winds and currents (Hart 1930, Hoagman 1973). These shallow nursery habitats are crucial to the protection of lake whitefish larvae, and variation in the availability of these areas contribute to differences in lake whitefish recruitment within the Great Lakes (Ebener et al. 2021).

Concerns revolving around the changes of Great Lakes lake whitefish nursery habitats have grown, especially considering large scale climate effects that extends to the Great Lakes (Ebener et al. 2021). The El Niño Southern Oscillation (ENSO) and North Atlantic Oscillation (NAO) are the primary climate factors of the Great Lakes basin (Ebener et al. 2021). Changes in the pressure gradient of the ENSO and NAO produce massive variation in mean wind speed, moisture, heat, and intensity and frequency of winter storms in the Great Lakes (Hurrell and Loon 1997, Hurrell et al. 2001). Subsequently, these variations in climate have effects on water temperature, ice coverage, and currents that affect the survival, dispersal, and growth of lake whitefish (Ebener et al. 2021). Cold water temperatures are important for reproductive success and survival of lake whitefish. Early studies found that lake whitefish embryos reared on colder water temperatures were larger at time of hatching compared to embryos reared at higher temperatures (Hall 1925, Price 1940). Unfortunately, the Great Lakes water temperatures have been increasing over time (Mason et al. 2016), which has the potential to increase natural mortality and accelerate hatching rates of lake whitefish (Berlin et al. 1977). Thus, recruitment variability in Great Lakes lake whitefish is positively correlated with increased temperature and proximity to southern geographic distribution (Ebener et al. 2021).

Other abiotic factors that have been hypothesized as contributing to declines in Great Lakes lake whitefish recruitment is ice coverage and water levels. Ice coverage over lake whitefish nursery habitats protect embryos from strong winds and currents, and extended coverage may enhance embryo incubation and growth of larval lake whitefish that enhance survival of early life stages (Eckmann and Pusch 1991, Næsje and Jonsson 1988). Concerningly, there is evidence for continuous declines in ice coverage and a continual increase in summer surface water temperatures in all of the Great Lakes (Mason et al. 2016, Ebener et al. 2021). Additionally, water levels of the Great Lakes could be a contributing factor behind recruitment declines. High water levels are believed to increase nutrient concentration, which give larval fish additional prey and coverage from predators (Casselman and Lewis 1996). In contrast, lower water levels should experience opposite effects so that nutrients and coverage in nearshore areas are reduced. There is also potential of decreasing water levels to reduce biomass of zooplankton within the nursery habitat, thereby increasing starvation mortality (Ebener et al. 2021).

4.2. Biotic factors

Alterations in multiple biotic factors affecting Great Lakes lake whitefish recruitment have also been hypothesized, including changes in the food web across the Great Lakes, and the establishment of dreissenid mussels (Ebener et al. 2021). The food webs of the four lower Great Lakes have changed significantly over the last two decades (Ives et al. 2018). The establishment of invasive dreissenid mussels (e.g., *Dreissena polymorpha* and *D. bugensis*) is believed to be a primary driver of these food web shifts, particularly as they relate to changes in lake whitefish habitat and food resources (Ebener et al. 2021). In Lakes Michigan and Huron, abundance of native benthic invertebrates (*Diporeia* and *Mysis*), which are rich in energy and nutrient-dense,

have decreased to where lake whitefish now primarily feed upon dreissenid mussels, round goby (*Neogobius melanostomus*), young of the year alewife and rainbow smelt, ninespine stickleback (*Pungitius pungitius*), and sculpins (Cottidae; Pothoven and Madenjian 2013, He et al. 2015, Madenjian et al. 2015).

Trophic transfer of energy (TTE) is a measure of food web efficiency and can be measured from a balanced summation of biomass produced by different species and their diets to organize biomass into trophic levels (Stewart et al. 2018). On average, TTE across the Great Lakes was 8.9%, meaning that 8.9% of energy is transferred from lower trophic levels (e.g., prey fish, larger zooplankton, and benthic organisms) to higher trophic levels (predatory fish) in the Great Lakes (Stewart et al. 2018). The range of TTE across the Great Lakes is 3.4 – 12.7% (Stewart et al. 2018). In Lake Huron, TTE has decreased from approximately 8% to 3.5%, and in Lake Michigan, though TTE has remained more constant compared to Lake Huron, has also been on the lower (6.5%), whereas TTE in Lake Superior has remained high at 12.7% (Stewart et al. 2018). Low TTE suggests that the food web is not properly functioning and resources near the bottom of the food web are not effectively being transferred, in contrast to high TTE, where greater fish biomass can be reached. This suggests that the food webs of Lakes Michigan and Huron may be disrupted enough that they can no longer produce the level of fishery production as they once did (Stewart et al. 2018, Ebener et al. 2021). Such drastic changes within the Great Lakes food web, particularly in the primary food source of lake whitefish, can certainly cause stress in the species, and subsequently, may disrupt other physiological factors that could negatively affect fish health.

4.3. Infectious diseases in the Great Lakes Basin

Infections caused by viruses, bacteria, fungi, and parasites that can cause disease in wild fish are present within the Great Lakes basin, some of which can have substantial negative effects on fish populations. For example, Viral Hemorrhagic Septicemia Virus (VHSV) spread throughout the Great Lakes and caused mortality in a range of wild fishes, including lake whitefish (see below; Elsayed et al. 2006, Faisal et al. 2012). This virus belongs to the order *Mononegavirales*, family *Rhabdoviridae*, genus *Novirhabdovirus*, and species *Piscine novirhabdovirus* (Walker et al. 2018, Batts et al. 2020), and is a World Organization for Animal Health (OIE) reportable fish pathogen (OIE 2021). The virus has a single, negative stranded ribonucleic acid (RNA) genome with 11,184 nucleotides and consists of 6 genes in the order 3'-N-P-M-G-NV-L-5' (Schütze et al. 1999). Nucleotide sequencing of the G and N genes have dichotomized VHSV into four distinct genotypes, I-IV (Snow et al. 2004, Einer-Jensen et al. 2004). Genotypes I-III occur primarily in Europe, whereas Genotype IV is predominately found in North America, Japan, and Korea (Stone et al. 1997, Shütze et al. 1999, Nishizawa et al. 2002). In 2003, VHSV was isolated from kidney and spleen tissues of an adult muskellunge (*Esox masquinongy*) collected from Lake St. Clair, Michigan (Elsayed et al. 2006). Following phylogenetic analysis, the recovered Great Lakes isolate was determined to belong to genotype IV, where it was distinct from other strains and thus placed into a new sublineage, VHSV-IVb (Elsayed et al. 2006). This same VHSV genotype has also been detected in *Diporeia* spp. (i.e., a primary food source of Great Lakes lake whitefish) in Lake Huron (Faisal et al., 2011). Of note, Great Lakes lake herring (*Coregonus artedi*), belonging to the same genus as *C. clupeaformis*, is highly susceptible to VHSV-IVb (Weeks et al., 2011).

Some bacterial infectious diseases have also had negative impacts on fish populations within the Great Lakes. *Renibacterium salmoninarum*, which means "kidney bacterium of salmonids", is a small Gram-positive, non-motile, diplobacillus that is the causative agent of bacterial kidney disease (Sanders and Fryer 1980). In the Great Lakes Basin, this bacterium was first detected in brook trout (*Salvelinus fontinalis*) in 1955 and is believed to have been introduced along with the introduction of salmonids hailing from the Pacific Northwest, USA (Allison 1958). Since then, *R. salmoninarum* has become widespread in Great Lakes fishes and is believed to have been a leading factor behind substantial losses of Chinook salmon (*Oncorhynchus tshawytscha*) populations in Lake Michigan in the late 1980's—early 1990's (Holey et al. 1998, Faisal and Hnath 2005, Faisal and Eissa 2009). BKD caused mass mortalities in Chinook salmon in Lake Michigan that reduced their population by 50%, which was the result of elevated chinook salmonid densities in Lake Michigan, heavy infections of parasites within the species, and a decrease in their primary food source (Holey et al. 1998, Hansen and Holey 2001). Of note, *R. salmoninarum* has also been detected in Great Lakes sea lamprey (*Petromyzon marinus*; Eissa et al. 2004), a fish parasite known to feed heavily upon lake whitefish (Jensen 1976, Spangler and Collins 1980).

Aeromonas salmonicida is another serious infectious disease present in the Great Lakes basin. This bacterium was first described in 1890 among German trout hatcheries and was believed to only infect salmonid fish (Emmerich and Weibel 1890). Since then, it is widely known that the bacterium can infect all species of salmon, trout, charr, grayling, and some non-salmonid fish, and extends its geographic range worldwide (Cipriano and Bullock 2001). *Aeromonas salmonicida* subsp. *salmonicida* is a Gram-negative, nonmotile coccobacilli that is

cytochrome oxidase and catalase positive, and produces a brown pigment on TSA followed by incubation after 48 hours at 22°C (Austin and Austin 2007). Since the initial description of *Aeromonas salmonicida*, five subspecies have been described, including *A. salmonicida* subspecies *salmonicida*, *achromogenes*, *masoucida*, *smithia,* and *pectinolytica* (Holt et al. 2000, Pavan et al. 2000). In particular, *A. salmonicida* subsp. *salmonicida* is the etiological agent of the devastating fish disease known as furunculosis, whereas the other subspecies produce atypical forms of the disease (Cipriano and Bullock 2001). *Aeromonas salmonicida* subsp. *salmonicida* causes acute mortality and severe septicemia in susceptible salmonid hosts, while peracute infections primarily affect fingerlings, in which fish darken in color and die without development of clinical signs of disease (Cipriano and Bullock 2001). Other typical disease signs of furunculosis include exophthalmia, external and internal hemorrhage, splenomegaly, focal necrosis of parenchymatous tissue, erratic swimming and lethargic behavior, and in chronic forms of the disease, fish may display furuncle-like lesions on the dermis where ulcers may extend into the muscle (Cipriano and Bullock 2001). It is notable that *A. salmonicida* subsp. *salmonicida* has been recovered from apparently healthy fish, thus suggesting that infected individuals may act as a reservoir for the bacterium (Hiney et al. 1997).

Aeromonas salmonicida subsp. *salmonicida* is currently recognized as being relatively widespread in the Great Lakes basin. Indeed, the bacterium has been detected from wild adult Chinook salmon returning to spawn in the Lake Michigan watershed (Loch et al. 2012). Another study found the bacterium yet again in adult *Oncorhynchus* spp. returning to spawn, including Chinook salmon (*O. tshawytscha*), coho salmon (*O. kisutch*), Hinchenbrook coho salmon (*O. kisutch*), Atlantic salmon (*Salmo salar*), and steelhead trout (*O. mykiss*; Diamanka et al. 2013).

Aeromonas salmonicida subsp. *salmonicida* was also detected in the invasive fish-parasitic sea lamprey, where interestingly, isolates recovered from sea lamprey were found to be pathogenic to rainbow trout in experimental studies (Diamanka et al. 2014).

Lactobacilli (within the family *Lactobacillaceae*) are ubiquitous within normal intestinal microbiota of fish (Ringo and Gatesoupe 1998, Gonzalez et al. 2000, Balcazar et al. 2007). However, lactobacilli have also been associated with negative health affects in fish (Rucker et al. 1953, Ross and Toth 1974, Cone 1982). Of these, *Carnobacterium maltaromaticum* (Mora et al. 2003) has been linked to diseases in fish, especially those in the spawning and post-spawning phases (Evelyn and McDermott 1961, Herman et al. 1985, Starliper et al. 1992, Austin and Austin 2007). Additionally, *C. maltaromaticum* has a penchant for the gonadal tissues of infected salmonids (Starliper et a. 1992, Austin and Austin 2007). The bacterium is a gram-positive bacillus that is non-spore forming, non-motile, with colonies being white and round with entire margins (Hiu et al. 1984).

In the Great Lakes, *C. maltaromaticum* infections were detected in feral and captive *Oncorhynchus* spp. during the fall spawning seasons (September – November) of 2005 to 2008 (Loch et al. 2011). Of note, the bacterium was present in both diseased and apparently healthy fish, with clinical signs of disease including bilateral exophthalmia, petechial hemorrhage, musculature ulcerations, nephrocalcinosis, and most commonly were visceral congestion and opacity/thickening of the swim bladder wall (Loch et al. 2011).

Another group of serious bacterial pathogens affecting Great Lakes fishes are members of the genus *Flavobacterium*, including *Flavobacterium branchiophilum*, *F. columnare*, and *F. psychrophilum,* which are the etiological agents of bacterial gill disease, columnaris disease, and bacterial cold water disease, respectively (reviewed in Loch and Faisal, 2017). As a group, flavobacterial fish pathogens have caused more mortality in Michigan hatcheries than all other pathogens combined (Van Vliet et al. 2015) and have also been linked to systemic infections and/or disease in a plethora of wild and feral Great Lakes fishes (Loch et al. 2013; Van Vliet et al. 2016; Faisal et al. 2016; Knupp et al 2019). Of note, some flavobacterial variants have host species preference, such as *F. columnare* (LaFrentz et al. 2018) and *F. psychrophilum* (Knupp et al. 2019, Knupp et al. 2021).

5. Infectious diseases and Great Lakes lake whitefish

Infectious diseases as a contributing factor in declining Great Lakes lake whitefish recruitment has not been thoroughly investigated. However, a multitude of infectious diseases have been detected in adult Great Lakes lake whitefish populations, as reviewed most recently by Loch and Faisal (2011). Below, I highlight some particularly pertinent microbial pathogens that in the context of risks associated with poor recruitment can be either directly transmitted, have the potential to be transmitted from infected parent to offspring, or are linked to early life stage mortalities in other salmonid species.

5.1. Viral Hemorrhagic Septicemia Virus

Since its emergence in the Great Lakes Basin, VHSV-IVb has been reported from Great Lakes lake whitefish at least four times. In 2005, VHSV-IVb was isolated from a single lake

whitefish in Lake Huron that displayed severe splenomegaly and internal hemorrhage (Loch and Faisal 2011). During surveillance in Lake Huron, VHSV-IVb was isolated from lake whitefish collected near Cheboygan, which exhibited splenic congestion and severe dermal hemorrhages (Thompson et al. 2011). This virus was again detected in Great Lakes lake whitefish that were collected following a mortality event in Thunder Bay (Lake Huron), infected fish again presented with hemorrhagic lesions that were consistent with VHS (Thompson et al. 2011). Viral Hemorrhagic Septicemia Virus-IVb has also been isolated from lake whitefish collected from Green Bay, Lake Michigan (Wisconsin DNR News Release May 24, 2007; reviewed in Faisal et al. 2012). Since then, it is common to observe lake whitefish collected from Lakes Superior, Michigan, and Huron during May and June of each year and as waters begin to warm, with internal hemorrhaging, which is a clinical sign of VHSV (Ebener et al. 2021).

Of particular note for VHSV, this virus has been detected within the gonads of other salmonid fish within the Great Lakes basin (Al-Hussinee and Lumsden 2011, Faisal et al. 2012) and is known to be shed in the reproductive fluids of infected spawning fish (OIE Manual of Diagnostic Tests for Aquatic Animals 2021). Just as concerning is that VHSV-IVb experimentally-infected juvenile lake herring (*C. artedi*) were found to be highly susceptible to clinical disease, with cumulative mortality ranging from 37% to 53% from days 3 – 6 post-infection (Weeks et al. 2011). Unfortunately, whether VHSV can infect the reproductive tissues of adult lake whitefish and how virulent this virus may be to juvenile lake whitefish remains completely unknown.

5.2. *Renibacterium salmoninarum*

In the late 1990s and as a result of mortality events in other fish species associated with bacterial kidney disease, a study was conducted to assess the prevalence of *R. salmoninarum* in two commercially valuable Great Lakes coregonid species; the lake whitefish and bloater (*Coregonus hoyi*) in Lakes Superior, Michigan, and Huron (Jonas et al. 2002). In 1997 in Lake Michigan, *R. salmoninarum* was detected using a field Enzyme Linked Immunosorbent Assay (ELISA) in Great Lakes lake whitefish at a prevalence of 32%, and in the following two years (1998-1999) within the same lake, the bacterium was detected yet again in lake whitefish ranging from 2—6%, though no evidence of overt clinical signs of bacterial kidney disease were observed (Jonas et al. 2002). Of note, lake whitefish collected from Lake Superior during this study tested negative for *R. salmoninarum*. Importantly, this was the first study to document the presence of *R. salmoninarum* infections in Great Lakes lake whitefish.

To further investigate the prevalence of *R. salmoninarum* in adult Great Lakes lake whitefish, fish were collected from four lake whitefish stocks in northern Lakes Michigan and Huron between 2003—2006. A quantitative enzyme-linked immunosorbent assay (Q-ELISA) was deployed, which measures the amount of *R. salmoninarum* p57 antigen in the analyzed tissues (Pascho and Mulcahy 1987, Austin and Austin 2007). *R. salmoninarum* antigens were detected in all four lake whitefish stocks during 2003—2006 sampling periods with a prevalence of 62.31% overall (Faisal et al. 2010). Lake Michigan had a higher prevalence of *R. salmoninarum* (62.46%) than Lake Huron (62.15%), and between both lakes, female fish had a higher prevalence (64.32%) than in males (59.45%). Beyond high prevalence of *R. salmoninarum* and of equal importance, multiple clinical abnormalities were present in adult lake whitefish

including renal congestion (44%), friable consistency (21%), swelling (4%), pallor (3%), and white to yellow nodules in the parenchyma (~0.5%), which suggests that infection of *R. salmoninarum* may have led to overt clinical signs of disease (Faisal et al. 2010). In addition, the study investigated differences between infection prevalence by season and found that prevalence was generally highest in the fall around the time of lake whitefish spawning (Faisal et al. 2010). Detecting such a high prevalence of *R. salmoninarum* and with the highest prevalence during adult lake whitefish spawning is concerning because in addition to horizontal transmission, the bacterium is known to be vertically transmitted *intra ova* and via the reproductive fluids of infected parents (Evelyn et al. 1984, Evelyn et al. 1986, Wiens 2011).

5.3. *Carnobacterium maltaromaticum*

Between the fall of 2003 and summer of 2006, *Carnobacterium maltaromaticum*-like isolates were first recovered from Great Lakes lake whitefish collected from northern Lakes Michigan and Huron (Loch et al. 2008). Bacterial isolates were recovered from kidney tissue and swim bladder exudate of fish that also showed signs of clinical disease, including severe splenomegaly, hyperemia and friability within the kidney, pallor and mottling of the liver, and congestion of the testes of male fish (Loch et al. 2008). Additionally, 2 fish had the presence of mucoid exudate within the swim bladder lumen, along with hemorrhage and general opacity and thickening of the swim bladder wall.

The prevalence of *C. maltaromaticum*-like infections was relatively low, being 2.2% in Lake Michigan and 1.4% in Lake Huron, and infection prevalence varied by seasons, where infections were highest during the winter (i.e., post-spawning) season (Loch et al. 2008). In this context, the

bacterium has a penchant for the reproductive tissues of other salmonids (Starliper et al. 1992, Austin and Austin 2007), posing a potential risk for parent to offspring transmission. To date, whether *C. maltaromaticum* can infect the gonadal tissues of adult lake whitefish, or what effects this bacterium has on juvenile lake whitefish have not been thoroughly investigated.

5.4. *Aeromonas salmonicida* subspecies *salmonicida*

From 2003—2006, adult Great Lakes lake whitefish were collected from sites within Lakes Michigan (Naubinway and Big Bay de Noc) and Huron (Detour Village and Cheboygan), revealing systemic *A. salmonicida* subsp. *salmonicida* infections in fish originating from Naubinway and Detour Village (Loch and Faisal 2010). The bacterium was isolated from kidney tissue of four infected fish, and clinical signs associated with infection included external and internal hemorrhaging, exophthalmia, splenomegaly, splenic and renal congestion, and fibrinous adhesions of the liver and spleen, suggesting that severe disease can ensue upon infection of *A. salmonicida* subsp. *salmonicida* (Loch and Faisal 2010). The overall prevalence of infection within lake whitefish from the four sites in Lakes Michigan and Huron was 0.3%, and infections were detected in winter, spring, and summer seasons (Loch and Faisal 2010).

Although prevalence of *A. salmonicida* subsp. *salmonicida* infection in lake whitefish was low, the significance of isolating the bacterium from this Great Lakes fish is not understated. This bacterium can be transmitted from infected broodstock to their offspring by being shed in high numbers with the reproductive fluids (Cipriano et al. 2001), which is alarming given that the bacterium has caused significant mortalities in salmonid fry and fingerlings (McCarthy and Roberts 1980, Drinan 1985). However, there are currently no reports of the bacterium being

isolated from the reproductive fluids of adult lake whitefish. Furthermore, virulence and effects of *A. salmonicida* subsp. *salmonicida* in juvenile lake whitefish is completely unknown.

5.5. *Flavobacterium* species

From 2003 – 2010, a large study was undertaken to elucidate the diversity of *Flavobacterium* and *Chryseobacterium* spp. that were recovered during fish health inspections and disease epizootics in the state of Michigan (Loch et al. 2013). During this study, *F. chilense* was recovered from the kidneys of wild lake whitefish collected in Lake Michigan (Loch et al. 2013). Although found, little is known about the effects this bacterium has on lake whitefish. However, another bacterium within the same genus, *F. psychrophilum,* is widespread within the Great Lakes basin and has been detected from other salmonids (Van Vliet et al. 2016) that are known to cohabitate with lake whitefish. Furthermore, *F. psychrophilum* can be transmitted inside of the egg (Cipriano 2005) and is known to cause substantial mortality in young fish (Cipriano and Holt 2005). In this context, investigating *Flavobacterium* spp. as a potential risk for juvenile lake whitefish recruitment is warranted.

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Chapter 2:

First Isolation of *Flavobacterium psychrophilum*, Causative Agent of Bacterial Coldwater Disease, from Wild Adult Great Lakes Lake Whitefish (*Coregonus clupeaformis*)

1. Abstract

Lake whitefish (*Coregonus clupeaformis*; Family Salmonidae) are economically and ecologically important indigenous Great Lakes (GL) fish. However, recent declines in GL lake whitefish abundance, growth, and early life stage recruitment have generated much concern. Although numerous studies have attempted to elucidate the factors behind these declines, the potential role(s) of infectious diseases has not been thoroughly investigated. As part of a two-year study investigating infectious diseases in both wild adult and juvenile GL lake whitefish populations, fish were collected from sites within Lakes Superior, Michigan, and Huron with historically good and recently poor recruitment histories during 2018 and 2019. Herein, we describe the first report of *Flavobacterium psychrophilum,* etiological agent of Bacterial Coldwater Disease (BCWD) and Rainbow Trout Fry Syndrome (RTFS), isolated from systemically infected adult GL lake whitefish. Isolates were yellow-orange and were Gram-negative, filamentous bacilli that were oxidase and catalase positive, and produced a flexirubin pigment when immersed in 3% potassium hydroxide. Isolate identity was confirmed via *F. psychrophilum*-specific PCR, followed by multilocus sequence typing, which revealed three new singleton sequence types (STs) that are distinct from all previously described STs, including all known genotypes within the Great Lakes basin. Out of 600 total adult GL lake whitefish sampled, prevalence of *F. psychrophilum* infections was 3.3% and 1.7% in Lakes Superior and Michigan, respectively. Clinical examination of infected fish revealed external and internal hemorrhage, visceral pallor and swelling, and parasitic infections within the ventricle of the heart and swim bladder lumen. Packed cell volumes (PCV) in *F. psychrophilum*-infected fish (n=3) were 32.0, 34.0, and 56.0 compared to the overall mean PCV of 44.1 from uninfected fish. The visceral fat index (VFI) of infected fish was a mean of 3.0, compared to the overall mean VFI of

1.4 in uninfected fish. Findings highlight the potential for *F. psychrophilum* to cause systemic infections in adult GL lake whitefish. Given previous observations of *F. psychrophilum* being a significant mortality source for other salmonid species in both hatchery and wild settings, our detection of the bacterium in adult GL lake whitefish, albeit at a low prevalence, is an issue of potential management concern and additional study is warranted to determine its potential role in declining lake whitefish recruitment.

2. **Introduction**

The lake whitefish (*Coregonus clupeaformis*; Family *Salmonidae*) is a culturally and ecologically invaluable native Great Lakes fish species that cycles energy through the food-web (Mohr and Nalepa, 2005) and supports a highly valuable commercial fishery (Ebener et al. 2008, Ebener et al. 2021). Although Great Lakes lake whitefish recovered from substantial abundance declines that occurred in the 1950s – 1960s (Ebener 1997), declines in body condition and growth in the late 1990's have persisted to present (Schneeberger et al. 2005, Mohr and Ebener 2007, Hoyle 2005, Lenart and Caroffino 2016, 2017). Similarly, declines in early life-stage recruitment and survival of juvenile Great Lakes lake whitefish were observed in many sites throughout the four lower Great Lakes in the late 1990's – early 2000's and have persisted to present day (Mohr and Nalepa 2005, Ebener et al. 2008, Brendan et al. 2010, Lenart and Caroffino 2016, 2017; Ebener et al. 2021).

A multitude of abiotic and biotic factors have been hypothesized as potentially contributing to and/or driving Great Lakes lake whitefish recruitment declines, including changes in physical, spawning, and nursery habitats, large-scale climate effects, movement and inter-mixing of lake

whitefish stocks and declining commercial yields, changes in growth, condition, and energetics, and changes in Great Lakes food webs (reviewed in Ebener et al. 2021). Despite numerous studies aiming to uncover the root of these declines, recruitment remains poor in many sites throughout the Great Lakes, with the exception of Lake Superior where recruitment has remained consistent (Mohr and Nalepa 2005, Ebener et al. 2008, Lenart and Caroffino 2016, 2017; Bence et al. 2019, Ebener et al. 2021).

Infectious diseases can negatively affect wild fish populations (Holey et al. 1998, Faisal et al. 2012, Lafferty et al. 2015). Indeed, several microbial pathogens that cause systemic disease and mortality in other salmonid species have been detected in adult lake whitefish stocks collected from Lakes Michigan and Huron, including Viral Hemorrhagic Septicemia Virus, *Renibacterium salmoninarum*, *Carnobacterium maltaromaticum*, and *Aeromonas salmonicida* subsp. *salmonicida* (Jonas et al. 2002, reviewed in Loch and Faisal 2011). At least some of these microbial pathogens have been detected in the reproductive fluid of infective hosts and can cam be transmitted vertically from infected parents to offspring. For example, *R. salmoninarum* was detected within the reproductive fluids of other infected salmonid species (Evelyn et al. 1984, 1986; Wiens 2011) and was found to be transmitted vertically from infected parent to offspring *intra ova* (Pascho et al. 2002, Elliott et al. 2014). *R. salmoninarum* infections have also been linked to early life stage mortality in other salmonid species (Maule et al. 1996, Mesa et al. 2000).

Another bacterial fish pathogen well-recognized for vertical transmission in salmonids is *Flavobacterium psychrophilum* (Phylum *Bacteroidetes*; Family *Flavobacteriaceae*), causative

agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS; Borg 1948, Holt 1987). As the latter name implies, *F. psychrophilum* can cause substantial early life stage mortality in other salmonids, where survivors can shed high loads of the bacterium (Madetoja et al. 2000), including during spawning (Taylor 2004). In the Great Lakes, systemic *F. psychrophilum* infections are prevalent in both wild and hatchery reared Great Lakes salmonids, with prevalence as high as 86.7% in spawning Chinook salmon in the Lake Michigan watershed (Van Vliet et al. 2015).

Despite its widespread prevalence in wild, feral, and hatchery-reared Great Lakes salmonids, *F. psychrophilum* has never been isolated from Great Lakes lake whitefish. However, during the course of a study aiming to elucidate the potential role infectious diseases may be playing in poor lake whitefish recruitment, this bacterium was recovered from spawning phase Great Lakes lake whitefish. Herein, we report the first isolation of *F. psychrophilum* from systemically infected adult Great Lakes lake whitefish, a particularly noteworthy finding given the devastating effects *F. psychrophilum* has had on other young salmonid species, which could potentially have negative health effects on the survival and recruitment of young lake whitefish in the Great Lakes.

3. Materials and Methods

3.1. Collection of adult lake whitefish

In the fall of 2018 and 2019, adult lake whitefish were collected from three sites (n=60 fish per site) associated with historically good/stable (i.e., stable biomass and abundance) recruitment (Figure 1; Mohr and Nalepa 2005, Rennie 2014, Fera et al. 2015, Lenart and Caroffino 2017,

Ebener et al. 2021): Whitefish Bay (Lake Superior), Menominee River (Lake Michigan) and Saginaw Bay (Lake Huron). Additionally, adult lake whitefish were collected from two sites with historically poor/low recruitment (i.e., overall less biomass and abundance, along with evidence of few early-life stage lake whitefish): Baileys Harbor (Lake Michigan) and Alpena (Lake Huron); Mohr and Nalepa 2005, Rennie 2014, Fera et al. 2015, Lenart and Caroffino 2017, Ebener et al. 2021).

Fish were collected via commercial trap nets at each site. To reduce sampling bias, attempts were made to collect 30 males and 30 females from each site across three size classes: <450 cm, 450-550 cm, and >550 cm. Collected fish were immediately placed into aerated live wells on fishing vessels and then transferred into live wells supplied with compressed oxygen for transportation back to the Michigan State University - Aquatic Animal Health Laboratory (MSU-AAHL). Upon arrival, live lake whitefish were euthanized using 250 mg/L of MS-222 (Tricaine methanesulfonate; Syndel, Ferndale, WA) buffered with 500 mg/L of sodium bicarbonate (Millipore Sigma, St. Louis, MO). All euthanasia was conducted in accordance with the Michigan State University – Institutional Animal Care and Use Committee (AUF 202100272).

3.2. Clinical examination of adult lake whitefish

Following euthanasia of adult fish, blood was collected via venipuncture of caudal vertebral vessel(s) using sterile needles and 5 ml sterile syringes (Beckton, Dickinson and Company, Franklin Lakes, NJ). Morphometric data (e.g., length and weight) was collected from each individual lake whitefish and thorough external and internal clinical examinations performed.

During the examination, the visceral fat index (VFI) of each fish was also estimated and tissues for bacteriological analyses were collected (see below).

3.3. Packed cell volume

The packed cell volume (PCV) of each fish was measured by immediately transferring a portion of collected un-heparinized whole blood into glass capillary tubes (Fisher Scientific, Pittsburg, PA), which were centrifuged for two minutes in a StatSpin CritSpin Microhematocrit Centrifuge (Iris Sample Processing), after which PCV measurements were recorded using the provided card-style reader (product #HR05).

3.4. Statistical analyses of packed cell volume and visceral fat index

A Kruskal-Wallis test was used to determine whether median PCV values of adult lake whitefish were equal among collection locations. For VFI values, which are ordinal variables, a one-way permutation test of independence was used to determine whether values were equal among the collection locations. For both tests, if the null hypothesis of no differences among collection locations was rejected, follow-up pairwise tests were undertaken to determine which collection locations may have differences in PCVs or VFIs. For PCV, follow-up analyses consisted of Dunn's (1964) multiple comparison tests. For VFIs, follow-up analyses consisted of pairwise permutation tests of independence. For both follow-up analyses, Bonferroni corrections were used to protect the Type-1 error rate of the tests. All statistical testing was conducted in R (4.1.2 GUI 1.77 High Sierra build 8007) using the Fisheries Stock Analysis (FSA; Ogle et al. 2021), coin (Hothorn et al. 2006), and rcompanion (Mangiafico 2021) packages.

3.5. Collection of wild juvenile lake whitefish

From May—July of 2019, and in June of 2021, juvenile Great Lakes lake whitefish were collected from sandy beaches adjacent to the wild adult spawning locations (Figure 1) using a 45.7 m long x 1.8 m tall seine with a 1.8 m x 1.8 m x 1.8 m bag in the center, constructed with 0.3 cm mesh. Fish were collected from Whitefish Bay (Lake Superior), Baileys Harbor (Lake Michigan), Marinette (Lake Michigan), Alpena (Lake Huron), and Saginaw Bay (Lake Huron). Although an attempt was made to collect fish from Menominee Bay (Lake Michigan) in 2019, collections were not successful due to unfavorable water conditions. Multiple seine hauls were performed at each location. Juvenile lake whitefish were identified by the presence of two dorsal fins including one adipose fin, subterminal mouth, clear fins, and greenish-brown backs with silver sides (Michigan Department of Natural Resources, 2021). Collected individuals were transferred to a cooler supplied with dissolved oxygen via air pumps for transport back to the MSU-AAHL. Upon arrival, juvenile fish were euthanized as described above.

3.6. Clinical examination of wild juvenile lake whitefish

Following euthanasia of wild juvenile Great Lakes lake whitefish, morphometric data were collected, gross examinations performed, and kidney tissues were collected for bacterial isolation. Due to the small size of collected individuals, blood was not collected.

3.7. Bacteriological analyses of adult and juvenile lake whitefish

During gross clinical examination, fish external surfaces were disinfected with 70% ethanol prior to the coelom being opened with sterile scissors (one pair per fish) in a laminar flow hood. Tissue from the reproductive organs (adult fish only) and kidneys of each fish were collected

using either sterile disposable 10 µl (adults) or 1 µl (juvenile) loops and then inoculated directly onto tryptone soya agar (Fisher Scientific, Pittsburg, PA), as well as Hsu-Shotts medium (HSU; Bullock et al. 1986) and tryptone yeast extract salts agar (TYES; Holt 1987) that were both supplemented with 4 mg liter^{-1} of neomycin sulfate. During year 2 sampling, slight modifications to TYES preparation were made, as ongoing medium-optimization experiments suggested potential for improved bacterial recovery; however, further experimentation revealed no significant differences in bacterial recovery between the two media preparation methods. Briefly, tryptone, yeast extract, $MgSO_4 \cdot 7H_2O$, and $CaCl_2 \cdot 2H2O$ were mixed into 250 mL water and adjusted to a pH of 7.2, filter sterilized using UltraCruz® Filter Flasks, polyethersulfone (PES; 0.22 µm), and then added to a previously autoclaved and cooled (i.e., 55°C) agar/water suspension. Following inoculation of tissues onto the three media, primary cultures were incubated at 22°C (TSA and HSU) and 15°C (TYES) for ≤7 days and checked intermittently for visible bacterial growth. Any resultant, yellow-pigmented bacterial growth present on HSU and/or TYES was sub-cultured onto fresh analogous media and subsequently checked for purity. Once verified pure, all isolates were supplemented with glycerol and cryopreserved at -80°C for future identification and analyses. To initially characterize recovered yellow-pigmented bacteria, 24-hour old cultures incubated at 15°C and/or 22°C were biochemically and morphologically characterized for oxidase (BD BBL™ DrySlide™, Becton, Dickinson and Company, USA) and catalase (hydrogen peroxide solution, 3%; Millipore Sigma, St. Louis, MO) activity, presence of flexirubin-type pigments via 3% potassium hydroxide (Reichenbach et al. 1974), the string test (AFS-FHS 2016), and Gram-stain reactions (reagents from Remel, San Diego, CA). All translucent yellow pigmented bacterial isolates that were recovered on TYES at 15°C, were

Gram-negative, oxidase and catalase positive, and produced a flexirubin-type pigment were selected for further molecular analyses.

3.8. Molecular identification

Bacterial genomic DNA was extracted from 7-day-old bacterial cultures using the DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol for Gram-negative bacteria. Nucleic acids were then quantified using the Quant-iT DS DNA Assay Kit and a Qubit fluorometer (Life Technologies, Grand Island, NY) and diluted to 20 ng/µl using nuclease-free water (Fisher Scientific, Pittsburgh, PA). Yellow-pigmented bacteria suspected of being *F. psychrophilum* were assayed using the *F. psychrophilum*-specific endpoint PCR assay of Toyama et al. (1994) as previously described (Van Vliet et al. 2015). The template for negative control reactions consisted of nuclease-free water, whereas positive control template was derived from a previously sequence-confirmed *F. psychrophilum* isolate. Resultant PCR products were electrophoresed in a 1.5% agarose gel for 30 minutes (100 V) and then visualized under UV transillumination. The presence of a ~1,088 bp-sized amplicon was considered confirmatory for bacterial identification as *F. psychrophilum* (Toyama et al. 1994).

3.9. Multilocus sequence typing of *F. psychrophilum* and data analysis

Multilocus sequence typing (MLST) is a well-established method for characterizing the strain diversity of bacterial pathogens, including *F. psychrophilum* (Nicolas et al. 2008, Knupp et al. 2019). Indeed, MLST-based analyses of *F. psychrophilum* have revealed important data of genetic diversity and how it correlates with geographic distribution, host specificity, and virulence. As *F. psychrophilum* had never been isolated from Great Lakes lake whitefish prior to

this study and to elucidate the relatedness of the newly recovered isolates to those that are widespread in the Great Lakes basin (Van Vliet et al. 2016, Knupp et al. 2019) MLST was utilized.

The partial sequences of seven genes (*trpB, gyrB, dnaK, fumC, murG, tuf,* and *atpA*; Nicolas et al. 2008) were PCR amplified, after which the resulting products were electrophoresed and the appropriate size of the amplicon verified as previously described (Knupp et al. 2019). Next, amplicons were purified using ExoSAP-IT (Fisher Scientific, Pittsburg, PA) and bidirectionally sequenced at the Michigan State University - Research Technology Support Facility using the same primers used for PCR amplification of each housekeeping genes. The quality of chromatograms was verified using an in-house script as previously described (Nicolas et al. 2008) prior to allele and sequence type assignment. GoeBURST (www.phyloviz.net/goeburst; Francisco et al. 2009) was used to visualize phylogenetic relationships, where sequence types (STs) were dichotomized into clonal complexes or singletons based on locus variations in their allelic profiles (Feil et al. 2004). All 1,545 *F. psychrophilum* isolates present in the pubMLST database (https://pubmlst.org/fpsychrophilum/; Jolley et al. 2018) were included in the analysis.

4. Results

During the course of this study, 600 wild adult lake whitefish were collected from five sites in Lakes Superior, Michigan, and Huron (Figure 1; Table 1), as were 436 wild juvenile Great Lakes lake whitefish (Figure 1; Table 2). The overall mean length and weight of collected adult lake whitefish was 51.7 ± 0.2 cm and 1382.4 ± 22.4 g, with some variation noted by site (Table

1). The overall mean length and weight of collected juvenile lake whitefish was 4.3 ± 0.1 cm and 0.9 ± 0.0 g (Table 2).

To elucidate the potential role that bacterial infections may be playing in reduced recruitment of Great Lakes lake whitefish, tissues for bacterial isolation were collected from the collected adult and juvenile lake whitefish. Following approximately 7 days of incubation on TYES at 15°C, two kidney cultures derived from adult lake whitefish collected from Whitefish Bay (Lake Superior) and one kidney culture derived from an adult LWF collected from Menominee (Lake Michigan) yielded yellow-orange, semi-translucent, low convex colonies with slightly undulate margins. In all three cases, these isolates yielded one colony forming unit (CFU) per 10 µl of kidney inoculum.

Following subculture of these three yellow-pigmented bacterial isolates, analyses revealed they were Gram-negative, filamentous bacilli that were positive for cytochrome oxidase and catalase activities and produced a flexirubin-type pigment when immersed in 3% potassium hydroxide. Based on these initial results, the bacteria were suspected as belonging to the genus *Flavobacterium* and thus were subjected to further molecular characterization.

Using the *F. psychrophilum*-specific endpoint PCR assay of Toyama et al. (1994), all three isolates produced amplicons of approximately 1,088 base pairs, confirming their identity as *F. psychrophilum*. To determine the *F. psychrophilum* genotype, which in some cases has been linked to host specificity (Knupp et al. 2019, Fujiwara-Nagata et al. 2013, Van Vliet et al. 2016, Knupp et al. 2021), geographic distribution (Fujiwara-Nagata et al. 2013, Avendaño-Herrera et

al. 2014, Van Vliet et al. 2016, Knupp et al. 2019, Li et al. 2021), and virulence (Knupp et al. 2021, Li et al. 2021), the isolates recovered from adult Great Lakes lake whitefish were further characterized using MLST. All three *F. psychrophilum* isolates collected from Great Lakes lake whitefish fell into three new MLST singleton STs (e.g., ST374, ST377, and ST378) that were distinct from all other described STs, including all known genotypes within the Great Lakes basin (Figure 2).

The prevalence of systemic *F. psychrophilum* infections in adult Great Lakes lake whitefish ranged from 0 – 3.3% across the five sampled sites by year (Table 1); however, the bacterium was never detected in the gonads of any adult fish, nor from any of the 436 wild juvenile lake whitefish. Although *F. psychrophilum* was recovered from lake whitefish collected from both Lakes Superior and Michigan, the bacterium was detected exclusively in fish collected from sites associated with historically good recruitment (Table 1).

External signs of disease that were observed in *F. psychrophilum*-infected adult lake whitefish included bilateral ocular hemorrhage, multifocal to coalescing hemorrhage along the ventrum, and hemorrhage within the caudal fin. Internally, splenic congestion and swelling, hepatic pallor and friability, renal congestion, heart pallor, and hyperemia of the swim bladder vasculature were noted. In one case, diffuse petechial hemorrhage within the swim bladder and congestion of the ovaries were also observed (Figure 3). This same fish also harbored approximately 5 *Cystidicola* sp. within the swim bladder lumen. In all cases, *F. psychrophilum*-infected fish harbored encysted metacercariae (presumptive *Tetracotyle* sp.) within the ventricle

of the heart. No other bacteria, viruses, or parasites were detected in *F. psychrophilum* infected fish (data not shown).

Overall, the mean packed cell volume (PCV) of adult Great Lakes lake whitefish that were analyzed in this study was 44.1 (Table 1). By lake, the mean PCV of fish collected from Lakes Superior, Michigan, and Huron ranged from 40.2, 54.4, and 46.0, respectively (Table 1). Some variation in PCV by collection site also occurred, whereby the mean PCV in good and poor recruitment sites was 45.5 and 42.0, respectively. The null hypothesis of no difference in median PCV values of uninfected adult lake whitefish among the sampling locations was rejected in both 2018 (χ^2=31.2, df=4, P < 0.001) and 2019 (χ^2=20.0, df=4, P < 0.001). We were unable to reject the null hypothesis of no difference in median PCV values for uninfected and infected adult fish among sampling locations in 2018 and 2019 (χ^2=4.6, df=3, P = 0.200). Based on pairwise comparisons for 2018, Whitefish Bay median PCVs were significantly different from all other locations except for Menominee (Table 3). In 2019, Baileys Harbor and Menominee median PCVs were significantly different from all other locations (Table 4). Because PCV can vary by sex, comparisons by sex were also made. Overall, the mean PCV of male and female Great Lakes lake whitefish was 46.2 and 41.2, respectively. In Whitefish Bay (Lake Superior), mean male PCVs ranged from 39.8 – 44.1 during 2018-2019, and mean female PCVs ranged from 37.3 – 40.5. In Lake Michigan, male PCVs were 41.8 – 51.2, and female were 37.0 – 39.6 in Baileys Harbor, whereas in Menominee, male PCVs were 48.2 – 53.7 and female were 47.0 – 53.3. Lastly, in Lake Huron, male PCVs in Alpena ranged from 43.2 – 45.0, and female were 36.6 – 39.4; whereas in Saginaw Bay, male PCVs ranged from 45.9 – 49.0, and female were 38.0 – 43.0. The PCVs of *F. psychrophilum*-infected fish collected from Whitefish Bay (Lake Superior;

female) were 32.0 and 34.0 compared to the mean PCV of 41.9 from uninfected fish collected from the same site (Table 1). The PCV of the *F. psychrophilum*-infected fish (male) from Menominee (Lake Michigan) was 56.0 compared to the mean PCV of 54.4 from uninfected fish (Table 1).

The visceral fat index (VFI) of Great Lakes lake whitefish ranged from 0 (lowest possible index) to 4 (highest possible index) throughout this study, with an overall mean of 1.4 (Table 1). Mean VFI varied by collection site, with fish collected from Menominee having the highest mean VFI across both years and Whitefish Bay having the lowest (Table 1). Mean VFI did not vary substantially among fish that were collected from good (1.5) vs poor (1.4) recruitment sites (Table 1). Interestingly, the mean VFI of *F. psychrophilum* infected fish was 3 compared to means of 1.8 and 1.5 in Whitefish Bay and Menominee, respectively. The null hypothesis of no differences in VFI values for uninfected fish among sampling located was rejected for 2018 ($P < 0.001$) but not in 2019 ($P = 0.059$). For 2018, VFIs in uninfected fish from Baileys Harbor were significantly different from all other locations (Table 5). When testing differences in VFIs between infected and uninfected across 2018 and 2019, the null hypothesis of no overall difference was rejected ($P < 0.001$; Table 6).

5. Discussion

Herein, *Flavobacterium psychrophilum*, the cause of substantial salmonid mortality around the globe (Starliper 2011), was recovered from the kidneys of systemically infected adult Great Lakes lake whitefish, marking the first time to our knowledge this notorious bacterial pathogen of salmonids has been isolated from *C. clupeaformis* populations in the Great Lakes. Indeed, this

bacterium was recovered from lake whitefish collected from both Lakes Superior and Michigan that concurrently showed gross signs of systemic disease (e.g., hemorrhage, visceral pallor and swelling, etc.). Although it is not possible to attribute the observed disease signs to the *F. psychrophilum* infections that were detected in the affected fish, similar disease signs are frequently reported in other fish species suffering from BCWD (Bernardet et al. 1988, Starliper 2011).

Only one previous study, which utilized molecular pyrosequencing techniques, has detected *F. psychrophilum* in *C. clupeaformis*, which were collected from Cliff, Indian, East, and Webster lakes within the St. John River drainage, Québec (Canada) and Maine (United States; Sevellec et al. 2014). Importantly, pyrosequencing is a sensitive means of detecting genetic traces of bacteria (Ronaghi 2001), which may or may not be indicative of a true infection status (Nocker et al. 2010). Thus, this study provides important evidence that Great Lakes lake whitefish are susceptible hosts for *F. psychrophilum* and can suffer from systemic infections as they approach spawning. Only one other study has documented that *F. psychrophilum* may be capable of infecting fish within the genus *Coregonus;* Lorenzen et al. (1997) recovered a phenotypically similar bacterium from skin lesions of muksun (*C. muksun*) in Finland, but no further description of disease was given.

Although the prevalence of *F. psychrophilum* infection was relatively low in adult lake whitefish stocks that were analyzed in this study (Table 1), the discovery of these infections in spawning lake whitefish is potentially significant for several reasons. First, during spawning, lake whitefish congregate in large numbers in shallow water shoals (Becker 1983), thus

providing an opportunity for horizontal transmission of *F. psychrophilum* within spawning aggregations. Indeed, *F. psychrophilum* can be transmitted either directly (fish-to-fish) or indirectly (through the water column; Madetoja et al. 2000). Possibly most importantly, *F. psychrophilum* is known to be vertically transmitted in other salmonids from infected parents to offspring via reproductive fluids and within infected eggs (Cipriano et al. 1995, Rangdale et al. 1996, Taylor 2004), thereby potentially leading to substantial mortality in the early life stages. For example, Decostere et al. found that clinical disease signs and mortality was more severe in rainbow trout (*Oncorhynchus mykiss*) that were 10 weeks old compared to rainbow trout of 15 weeks old (Decostere et al. 2001), and there is also evidence that the bacterium can be associated with mortality of Atlantic salmon (*Salmo salar*) eggs (Cipriano 2015). Although *F. psychrophilum* was not detected in the gonads of any lake whitefish in the current study, the bacterium was recovered from the kidneys. The kidney excretory function of fish is such that bacteria can shed with urine, thus contacting eggs (Perry 2011). Thus, in conjunction with poor recruitment of juvenile Great Lakes lake whitefish, the presence of *F. psychrophilum* in systemically infected adult fish during spawning creates a pathway for the bacterium to be transmitted from infected parent to offspring, thus potentially resulting in additional infections, disease, and/or mortality.

Following morphological and molecular analyses that confirmed the identity of the recovered bacterium as *F. psychrophilum*, MLST revealed that the Great Lakes lake whitefish *F. psychrophilum* isolates each belonged to newly identified singleton STs that are distinct from the >1,500 isolates that were genotyped using the same MSLT scheme and originated from five different continents (Nicolas et al. 2008, Siekoula-Nguedia et al. 2012, Apablaza et al. 2013,

Strepparava et al. 2013, Nilsen et al. 2014, Fujiwara-Nagata et al. 2013, Van Vliet et al. 2016, Knupp et al. 2019, Einarsdottir et al. 2020, Sebastião et al. 2020). This "distinctness" from all other MLST-genotyped *F. psychrophilum* isolates is notable for several reasons. First and based on currently available data, this finding may suggest that the range of other Great Lakes salmonids that are susceptible to *F. psychrophilum* and frequently suffer from systemic infections and/or BCWD outbreaks (Loch et al. 2013, Van Vliet et al. 2016, Knupp et al. 2019) are not the likely transmission source for the detected Great Lakes lake whitefish infections and vice versa. Indeed, at least two studies have shown that some *F. psychrophilum* MLST genotypes show strong preference for a particular host species (Van Vliet et al. 2016, Knupp et al. 2021). Whether the *F. psychrophilum* strains recovered in this study represent variants with a host preference for lake whitefish remains to be determined. Moreover, the prospect of lake whitefish hatchery propagation within the Great Lakes basin is being increasingly considered (Bence et al. 2019, Ebener et al. 2021), and if pursued, the risk of *F. psychrophilum* transmission from infected broodstock to hatchery stocks is a concern that hatchery managers need be aware. Additionally, all *F. psychrophilum* isolates in this study have been cryopreserved and can now serve as a resource for developing BCWD prevention and control strategies, including the development of autogenous vaccines, should lake whitefish hatchery propagation become an emphasis.

In the context of good versus poor recruitment locations of this study, we detected *F. psychrophilum* infections exclusively in spawning aggregations of adult lake whitefish that have a history of good recruitment (Table 1), leaving the role of *F. psychrophilum* in declining recruitment unknown. However, complex interactions between a pathogen, host, and the

environment must occur for pathogens to cause disease (Hedrick 1998). If fish in some sites are healthier, for example, fish collected from Whitefish Bay had higher VFIs (Table 1), which is indicative of good nutritional status (Adams 1999), it may be that fish are capable of surviving with infections and are able to be caught. In contrast, it is possible that *F. psychrophilum* could have caused disease and mortality in fish from other sites, leading to a low probability of detection. Again, the role of *F. psychrophilum* as a contributing factor in poor recruitment remains unknown but given that *F. psychrophilum* has the capacity to kill early life stages in other salmonids warrants further investigation along with studies on the virulence of isolates recovered from this study.

Although not a primary goal of this study, findings from this study have contributed to potentially establishing baseline hematological (e.g., PCV) values for Great Lakes lake whitefish. A handful of toxicological and dietary studies in hatchery-reared adult lake whitefish have reported PCVs of *C. clupeaformis* to range from 36.7 – 42.3 (Pedlar et al. 2002), to 36.8 – 43.3. (Cooley et al. 2000), to 38.5 – 40.1 (Ptashynski et al. 2002). In this study, lake whitefish overall PCV was 44.1 and ranged from 40.2 – 54.4, which was a wider range than the previously mentioned studies. Although a notable trend in PCVs of *F. psychrophilum*-infected fish was not observed, there was significant differences in PCVs of uninfected fish among both years. In 2018, fish collected from Whitefish Bay had median PCVs that were significantly different from all other locations except for Menominee. In 2019, sites within Lake Michigan (Baileys Harbor and Menominee) had significantly different median PCVs from all other locations. However, the factors behind these differences remain to be determined.

In conclusion, this study represents the first report of systemic *F. psychrophilum* infections in wild adult Great Lakes whitefish. This, combined with the observed clinical signs in infected fish, suggest *F. psychrophilum* is capable of causing systemic disease in lake whitefish, a matter of importance given continued declines in body condition and growth of adult lake whitefish and juvenile recruitment in the Great Lakes basin. In this context, the bacterium's ability to not only be transgenerationally transmitted in other salmonids, but also cause substantial early life stage mortality, warrants further investigation. Importantly, results from the molecular analyses performed herein indicate that *F. psychrophilum* variants infecting Great Lakes lake whitefish are distinct from *F. psychrophilum* variants that are widespread in other Great Lakes salmonids and may represent strains with a preference for lake whitefish. With these isolates now available, research on the ability of these new and distinct *F. psychrophilum* strains to infect eggs of Great Lakes lake whitefish may further elucidate the role of *F. psychrophilum* in declining recruitment.

APPENDIX

Figure 1. Adult and juvenile Great Lakes lake whitefish collection sites. LS=Lake Superior; LM=Lake Michigan; LH=Lake Huron. [G]Good recruitment site. [P]Poor recruitment site. Adult collection site =● ; juvenile collection site= ■

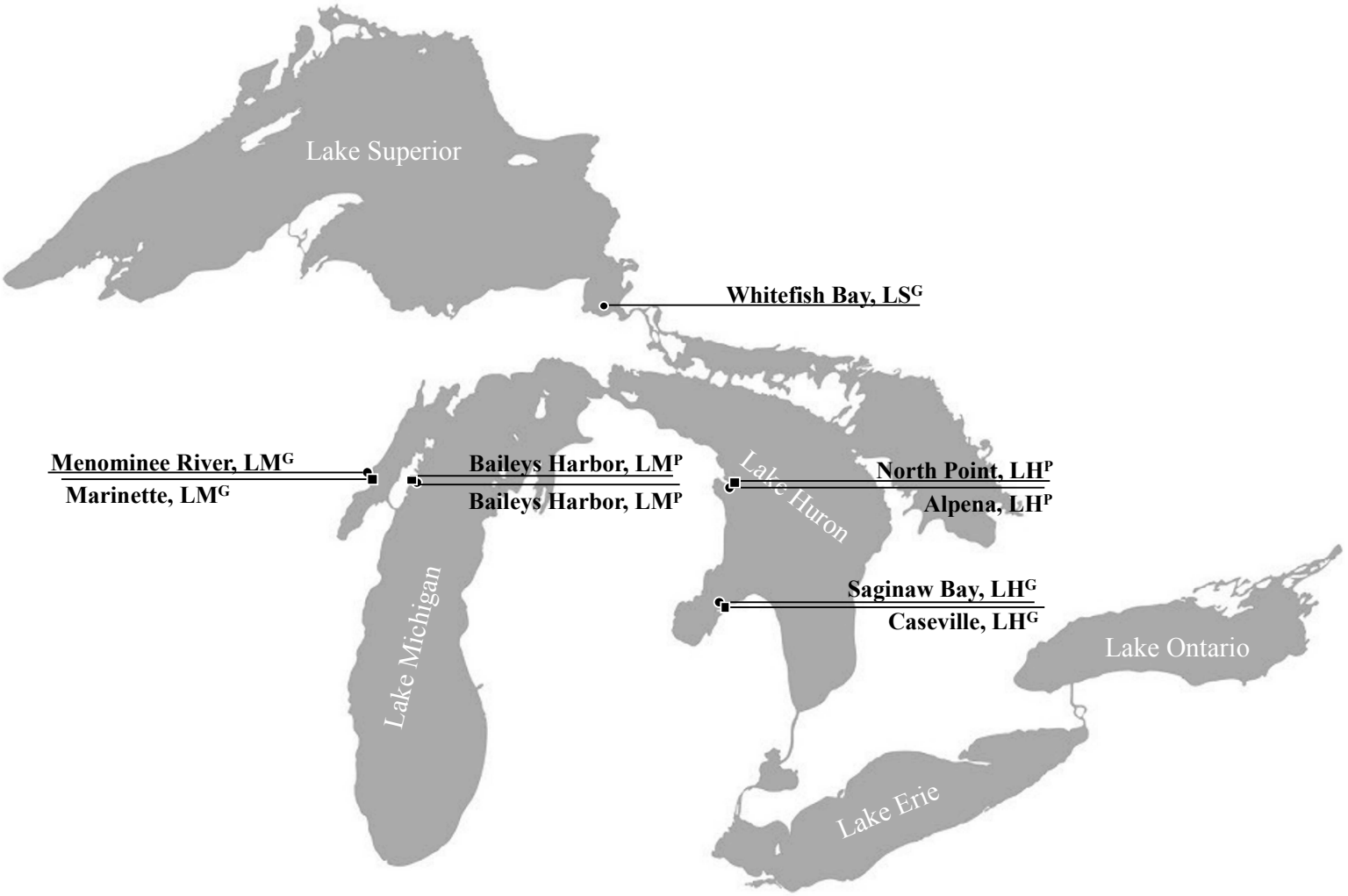

Figure 2. goeBURST diagram depicting the genetic relatedness (as determined via multilocus sequence typing) of the 3 *F. psychrophilum* isolates recovered from adult Great Lakes lake whitefish (shown in red) when compared with all 1,545 previously typed *F. psychrophilum* isolates. Clonal complexes (CC), defined as groups of isolates connected by single-locus variant (SLV) links, are depicted by circles. A CC is named after the most likely founding ST identified as the ST with the highest number of SLVs. Founding STs are named after the ST that has the highest number of SLVs. If STs have the same number of isolates, the CC is named after the earliest ST. The founding ST of a CC depicted in a box.

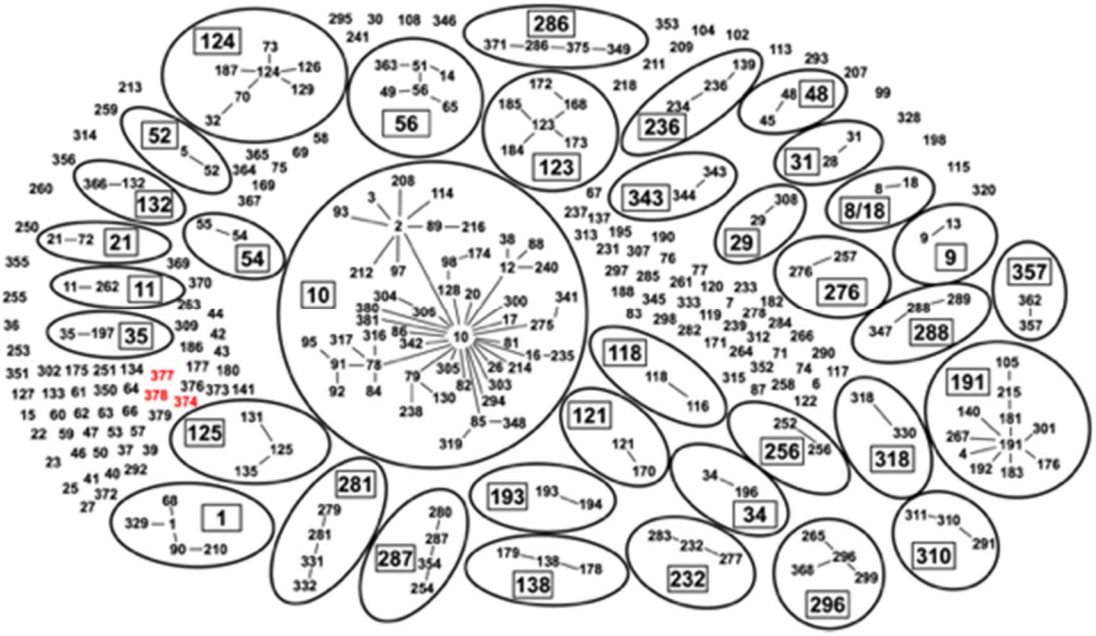

Figure 3. *F. psychrophilum*-infected adult Great Lakes lake whitefish (*C. clupeaformis*) with diffuse petechial hemorrhage throughout the swim bladder and concurrent congestion of the ovaries. Note also the presence of the swim bladder nematode, *Cystidicola* sp., within the swim bladder lumen.

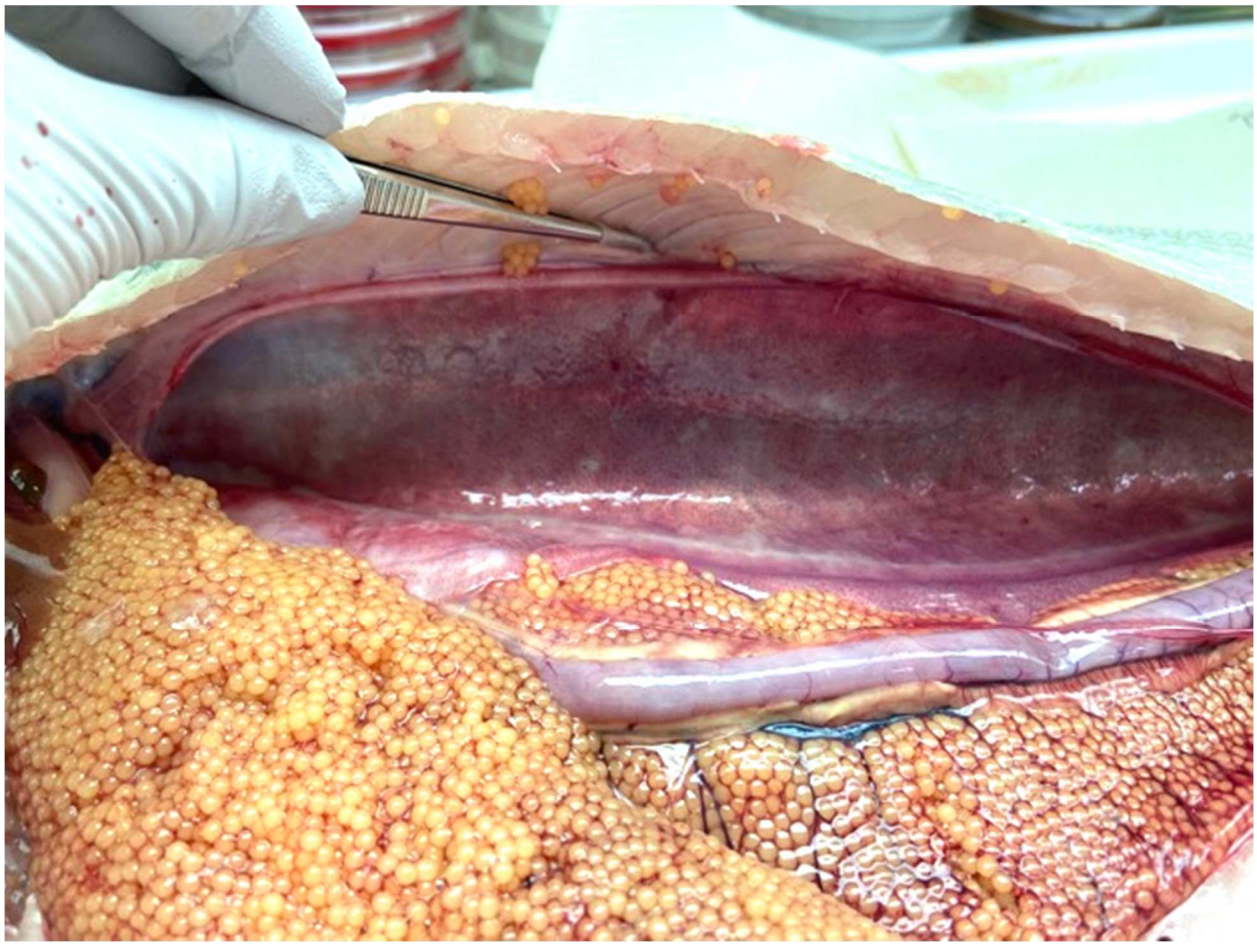

Table 1. Mean length (cm), weight (g), visceral fat index (VFI), packed cell volume (PCV), and *F. psychrophilum* infection prevalence in the kidneys of adult Great Lakes lake whitefish from 2018 – 2019. Mean PCVs exclude those of fish that were found to be infected with *F. psychrophilum*. LS=Lake Superior; LM=Lake Michigan; LH=Lake Huron. Standard deviation of the data is reported in parentheses. [G]Good recruitment site. [P]Poor recruitment site. *F. psychrophilum* was not isolated from adult gonad tissue during this study.

	Mean Length (cm)		**Mean Weight (g)**		**Mean Visceral Fat Index**			**Mean Packed Cell Volume**			***F. psychrophilum* Prevalence (%)**	
Site	2018	2019	2018	2019	2018	2019	Overall	2018	2019	Overall	2018	2019
Whitefish Bay, LS[G]	50.7 (5.1)	52.3 (4.9)	1163.8 (380.7)	1414.7 (549.3)	1.7 (0.8)	1.9 (1.0)	1.8 (0.9)	38.5 (9.6)	41.9 (9.8)	40.2 (9.8)	0/0 (0%)	2/60 (3.3%)
Baileys Harbor, LM[P]	55.2 (6.3)	56.3 (4.7)	1658.7 (454.8)	1511.4 (462.0)	0.2 (0.4)	1.3 (1.0)	0.9 (0.9)	44.3 (10.6)	39.5 (7.6)	41.9 (9.5)	0/0 (0%)	0/0 (0%)
Menominee, LM[G]	45.8 (3.9)	46.4 (3.3)	756.8 (222.0)	798.6 (178.4)	1.1 (0.8)	1.6 (0.9)	1.4 (0.9)	48.0 (7.8)	54.4 (14.6)	51.2 (12.0)	0/0 (0%)	1/60 (1.7%)
Alpena, LH[P]	57.2 (4.6)	56.8 (4.0)	1779.9 (506.4)	1629.9 (448.6)	1.0 (0.7)	1.5 (1.1)	1.8 (0.9)	42.8 (11.0)	41.5 (11.2)	42.1 (11.1)	0/0 (0%)	0/0 (0%)
Saginaw Bay, LH[G]	56.4 (4.3)	56.7 (3.9)	1710.3 (475.7)	1658.0 (436.7)	1.1 (0.9)	1.5 (1.0)	1.3 (1.0)	46.0 (10.2)	44.4 (7.5)	45.2 (9.0)	0/0 (0%)	0/0 (0%)

Table 2. Mean length (cm), and weight (cm) of wild juvenile Great Lakes lake whitefish collected in 2019 and 2020. LS=Lake Superior; LM=Lake Michigan; LH=Lake Huron. Standard deviation of the data is reported in parentheses. [G]Good recruitment site. [P]Poor recruitment site. Only 1 fish was collected from Caseville, LH.

Site	Total Fish Collected	Mean Length (cm)	Mean Weight (g)
Whitefish Bay, LS[G]	25	2.6 (0.2)	<1.0
Baileys Harbor, LM[P]	150	6.1 (0.3)	1.9 (0.3)
Marinette, LM[G]	110	3.4 (0.6)	0.3 (0.1)
North Point, LH[P]	150	3.4 (0.4)	0.3 (0.1)
Caseville, LH[G]	1	2.6	0.2

Table 3. Median packed cell volume (PCV) of uninfected adult lake whitefish in 2018. Significant differences in median PCV were determined via the Kruskal-Wallis rank sum test and Dunn (1964) Kruskal Wallis multiple comparison p-values adjusted with the Bonferroni method (P<0.001).

Comparison	P Adjusted
Alpena – Baileys Harbor	1.000e+00
Alpena – Menominee	4.065e-01
Baileys Harbor – Menominee	1.001e-01
Alpena – Saginaw Bay	1.000e+00
Baileys Harbor – Saginaw Bay	1.000e+00
Menominee – Saginaw Bay	1.000e+00
Alpena – Whitefish Bay	1.097e-02
Baileys Harbor – Whitefish Bay	6.591e-02
Menominee – Whitefish Bay	9.692e-07
Saginaw Bay – Whitefish Bay	3.718e-04

Table 4. Median packed cell volume (PCV) of uninfected adult lake whitefish in 2019. Differences in median PCV was significant by the Kruskal-Wallis rank sum test and Dunn (1964) Kruskal Wallis multiple comparison p-values adjusted with the Bonferroni method (P<0.001).

Comparison	P Adjusted
Alpena – Baileys Harbor	0.366
Alpena – Menominee	0.160
Baileys Harbor – Menominee	0.000
Alpena – Saginaw Bay	1.000
Baileys Harbor – Saginaw Bay	0.055
Menominee – Saginaw Bay	0.679
Alpena – Whitefish Bay	1.000
Baileys Harbor – Whitefish Bay	1.000
Menominee – Whitefish Bay	0.055
Saginaw Bay – Whitefish Bay	1.000

Table 5. Visceral fat index (VFI) of uninfected adult lake whitefish in 2018. Differences in VFI values were determined to be significant by the one-way ordinal permutation test (P< 0.001).

Comparison	P Adjusted
Whitefish Bay – Menominee	1.000e+00
Whitefish Bay – Baileys Harbor	1.910e-13
Whitefish Bay – Alpena	8.436e-06
Whitefish Bay – Saginaw Bay	9.826e-04
Menominee – Baileys Harbor	1.910e-13
Menominee – Alpena	8.436e-06
Menominee – Saginaw Bay	9.826e-04
Baileys Harbor – Alpena	3.005e-06
Baileys Harbor – Saginaw Bay	2.217e-05
Alpena – Saginaw Bay	1.000e+00

Table 6. Visceral fat index (VFI) of uninfected and infected adult lake whitefish from 2018 – 2019. Differences in VFI were determined to be significant by the one-way ordinal permutation test (P<0.001).

Comparison	P Adjusted
Uninfected 2018 – Uninfected 2019	1.295e-07
Uninfected 2019 – Infected 2019	5.763e-02

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Chapter 3:

Field and Laboratory-Based Studies Provide Evidence that Systemic *Carnobacterium maltaromaticum* Infections Cause Disease and Mortality in Great Lakes Lake Whitefish (*Coregonus clupeaformis*)

1. Abstract

Ongoing declines in wild Great Lakes lake whitefish (*Coregonus clupeaformis*) abundance, growth, and early life stage recruitment have generated much concern, as this native species is an important component of a lake's food web and supports valuable commercial fisheries. Although many studies have investigated what factors might be contributing to some of these declines, the potential role(s) of infectious diseases has not been thoroughly examined. We undertook a two-year study to assess the prevalence of multiple fish pathogens in wild adult (n=600) and juvenile (n=436) lake whitefish that were collected from five sites within Lakes Superior, Michigan, and Huron during 2018 and 2019. Three of the sites were considered to have historically good recruitment, while the other two sites were characterized as having poor recruitment. A bacterium producing white, semitranslucent colonies with morphologies ranging from low convex to raised centers with undulate margins was recovered from the kidneys, gonads, and swim bladders of adult fish that also showed a range of gross signs of systemic disease. Phenotypic and biochemical characterization of the recovered isolates revealed they were Gram-positive, oxidase and catalase negative, utilized dextrose, hydrolyzed esculin, produced gamma hemolysis on blood agar, but did not produce H_2S or indole. Subsequent molecular characterization of multiple genetic loci and phylogenetic analyses definitively identified the recovered bacteria as *Carnobacterium maltaromaticum*, the etiological agent of pseudokidney disease. Within lake comparisons revealed that *C. maltaromaticum* infection prevalence (0 – 6.6%) was almost always higher in adult lake whitefish collected from sites associated with poor recruitment than those with historically good recruitment. To assess the effects *C. maltaromaticum* infections have on juvenile lake whitefish health and survival, artificially fertilized (and disinfected) eggs collected from wild lake whitefish were hatchery-reared,

hatched, and subsequently reared under quarantine conditions beginning at 3 months of age. However, before *in vivo* challenge experiments could begin and at approximately 1.8 years of age, laboratory-reared lake whitefish spontaneously developed severe signs of disease, after which mortality ensued. By the end of the disease outbreak, cumulative mortality ranged from 25.1 – 31.7% in both affected tanks. Clinical examination and diagnostic analyses ruled out parasite and viral etiologies; however, a bacterium was recovered from the brains, eyes, and kidneys of affected fish and in some cases, at high intensities (e.g., colonies too numerous to count). Biochemical and molecular characterization identified the bacterium as *C. maltaromaticum*, marking the first time to our knowledge this bacterium has been associated with mortality in captive-reared lake whitefish. Collectively, findings provide evidence that *C. maltaromaticum* infections can cause disease and mortality in GL lake whitefish, thus warranting further investigation into the bacterium's potential role in declining lake whitefish recruitment.

2. Introduction

Lactobacilli (Family *Lactobacillaceae*) are constituents of the microbiota within gastrointestinal tract of many vertebrates, including fish (Ringø and Gatesoupe 1998; Gonzalez et al. 2000, Balcazar et al. 2007). However, some lactobacilli cause serious disease in fish, including *Vagococcus salmoninarum* (Austin and Austin 2007), *Streptococcus*, *Enterococcus*, *Lactococcus garvieae*, and *Carnobacterium* (Ringø et al. 2018). The genus *Carnobacterium* currently comprises 12 species (NCBI 2021) and of those, *C. divergens* and *C. maltaromaticum* (formerly *Lactobacillus piscicola*, and *C. piscicola*; Mora et al. 2003, Hiu et al. 1984, Collins et al. 1987) have been tied to systemic infections and disease in fish (Herman et al. 1985, Starliper et al. 1992). *Carnobacterium maltaromaticum*, the etiological agent of pseudokidney disease

(Foott 1994), has been linked to infections and/or disease in both wild and captive fishes, particularly during spawning and post-spawning phases (Evelyn and McDermott 1961, Herman et al. 1985, Starliper et al. 1992, Austin and Austin 2007) potentially linked to the bacterium's affinity for the gonadal tissues in some fishes (Starliper et al. 1992, Austin and Austin 2007). Thus, there is potential for the bacterium to be potentially passed from infected parents to offspring via the reproductive fluids. Not only is *C. maltaromaticum* associated with disease, but the bacterium has been recovered from the eyes of apparently healthy wild brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta*; Pastorino et al. 2021).

In the Great Lakes basin of North America, widespread *C. maltaromaticum* infections have been detected in wild and hatchery-reared spawning salmonid populations, including several *Oncorhynchus* spp. Loch et al. (2011) recovered the bacterium from external ulcerations and a range of visceral organs of infected fish. In a similar context, systemic infections caused by a *C. maltaromaticum*-like bacterium were detected in wild adult lake whitefish showing gross and histopathological signs of disease that were collected in Lakes Michigan and Huron (Loch et al. 2008). Of note, Loch et al. (2008) found that the prevalence of *Carnobacterium* infections in lake whitefish varied by season and was highest during winter (i.e., the post-spawning period), but whether these infections were present in the gonadal tissues was not investigated. Likewise, the potential for C. *maltaromaticum* infections in adult Great Lakes lake whitefish to be transmitted to juveniles in corresponding nursey habitats remains unknown, as does the effect *C. maltaromaticum* infections have on juvenile lake whitefish health and survival.

Abundance of Great Lakes lake whitefish populations have fluctuated considerably over the past seven decades with the most recent trend suggesting declining abundance across most of the lakes (Ebener 1997, Ebener et al. 2021). Body condition and growth have also been declining in many regions of the Great Lakes (Schneeberger et al. 2005, Mohr and Ebener 2005, Hoyle 2005, Lenart and Caroffino 2016, 2017). Perhaps more concerning, recruitment levels of lake whitefish populations in many areas of the four lower Great Lakes have been declining, which suggests that abundance declines are likely to continue (Mohr and Nalepa 2005, Ebener et al. 2008, Brenden et al. 2010, Lenart and Caroffino 2016, 2017, Ebener et al. 2021). Although many abiotic and biotic factors have been investigated (reviewed in Ebener et al. 2021), the role infectious diseases may be playing in declining recruitment is unclear.

In this context and given the previous detections of systemic *Carnobacterium* infections in adult Great Lakes lake whitefish (Loch et al. 2008), we set out to further investigate the status of *Carnobacterium* infections in wild adult lake whitefish, including assessing the status of infections within the reproductive tissues of adults. We also examined the infection status of wild juvenile lake whitefish linked to adult spawning sites associated with historically good recruitment and recently poor recruitment. And finally, to assess the effects that *C. maltaromaticum* infections have on juvenile lake whitefish health and survival, *in vivo* challenge experiments were planned under laboratory conditions.

3. Materials and Methods

Field-Based Studies

3.1. Collection of adult lake whitefish

During 2018 and 2019, adult lake whitefish were collected in October – November from locations associated with historically good/stable (i.e., stable biomass and abundance) recruitment: Whitefish Bay (Lake Superior), Menominee River (Lake Michigan) and Saginaw Bay (Lake Huron; Figure 4; Mohr and Nalepa 2005, Rennie 2014, Fera et al 2015, Lenart and Caroffino 2017, Ebener et al. 2021). Likewise, adult lake whitefish were collected from two sites with historically poor/low recruitment (i.e., overall less biomass and abundance, and evidence of few early life stage lake whitefish): Baileys Harbor (Lake Michigan) and Alpena (Lake Huron); Mohr and Nalepa 2005, Rennie 2014, Fera et al. 2015, Lenart and Caroffino 2017, Ebener et al. 2021).

Adult fish were collected via commercial trap nets at each location. Attempts were made to collect 30 males and 30 females from each site across three size ranges: <450 cm, 450-550 cm, and >550 cm, to reduce sampling bias. Fish were immediately placed into aerated live wells onboard fishing vessels, then transferred into live wells supplied with compressed oxygen for transportation back to the Michigan State University – Aquatic Animal Health Laboratory (MSU – AAHL). Upon arrival, live fish were immediately euthanized using 250 ml/L of MS – 222 (Tricaine methanesulfonate; Syndel, Ferndale, WA) buffered with 500 mg/L of sodium bicarbonate (Millipore Sigma, St. Louis, MO). All fish handling was performed in accordance with the Michigan State University – Institutional Animal Care and Use Committee Standards (AUF 202100272).

3.2 Clinical examination of adult lake whitefish

Immediately following euthanasia, blood was collected via venipuncture of the caudal vertebral vessel(s) using sterile needles and 5 ml sterile syringes (Beckton, Dickinson and Company, Franklin Lakes, NJ). Length and weight measurements were made for each individual fish, after which thorough external and internal clinical examinations were performed and tissues aseptically collected for bacteriological and virological analyses (see below). During examinations, the visceral fat index (VFI) of each fish was also estimated.

3.3 Collection of wild juvenile lake whitefish

Wild juvenile lake whitefish were collected from May – July 2019, and in June of 2021. Fish were collected from sandy beaches adjacent to the wild adult spawning locations (Figure 4) using a 45.7 m long x 1.8 m tall seine with a 1.8 m x 1.8 m x 1.8 m bag in the center, constructed with 5.1 cm delta mesh. Specifically, fish were collected from Whitefish Bay (Lake Superior), Baileys Harbor (Lake Michigan), Marinette (Lake Michigan), Alpena (Lake Huron), and Saginaw Bay (Lake Huron). Juvenile fish were not collected from Menominee (Lake Michigan) in 2019. Numerous seine hauls were completed at each location, where juvenile lake whitefish were identified by the presence of two dorsal fins including one adipose fin, subterminal mouth, clear fins, and greenish-brown backs with silver sides (Lake Whitefish, 2021) and transferred to a cooler supplied with dissolved oxygen via air pumps for transport back to the MSU – AAHL. Upon arrival to the laboratory, fish were euthanized as described above.

3.4 Clinical examination of wild juvenile lake whitefish

Following euthanasia of wild juvenile Great Lakes lake whitefish, morphometric data were collected, clinical examination performed, and kidney tissues were collected for bacterial isolation (see below). Due to the small size of collected individuals, blood was not collected.

3.5 Bacteriological and virological analyses of wild adult and juvenile lake whitefish

Following external examination, the external surfaces of lake whitefish were sprayed with 70% ethanol, after which coelomic cavities were carefully opened with sterile scissors (one pair per fish) in a laminar flow hood. Tissue from the gonads (adults) and kidneys (juveniles and adults) of individual fish were collected using either sterile disposable 10 µl (adults) or 1 µl (juvenile) loops and directly inoculated onto trypticase soy agar (TSA) and blood agar (TSA with sheep blood, SBA; Thermo Scientific, Pittsburg, PA). Primary cultures were incubated at 22°C for 72 hours and checked for visible bacterial growth, whereby resultant bacteria were sub-cultured onto fresh TSA and subsequently verified for purity. All isolates were inoculated into trypticase soy broth (Thermo Scientific) and incubated at 22°C and checked intermittently until broth became turbid. Then, isolates were supplemented with glycerol (20% volume to volume) and cryopreserved at -80°C for future use.

For initial characterization, isolates were revived from cryostocks onto TSA and incubated for 48 – 72 hours at 22°C, then sub-cultured onto TSA. Following 24-hour incubation and purity verification, isolates were biochemically and morphologically characterized for oxidase (BD BBL™ DrySlide™, Becton, Dickinson and Company, USA) and catalase (hydrogen peroxide solution, 3%; Millipore Sigma, St. Louis, MO) activity, and Gram-stain reactions (reagents from

Thermo Scientific). Isolates were further characterized using tests for bile esculin hydrolysis (on bile esculin agar, Thermo Scientific); motility, indole and hydrogen sulfide production (on sulfur-indole-motility medium, Thermo Scientific), hemolysis, and ability to utilize dextrose in the presence and absence of oxygen (final concentration of 1% dextrose in phenol red broth base, Thermo Scientific), in which all tests were incubated at 22°C and read at 72 hours.

To simultaneously assay for the presence of viruses, kidney, spleen, and heart tissues were aseptically collected as a single pool of tissue and diluted 1:1 (weight per volume) with Earle's salt-based minimal essential medium (Invitrogen, Thermo Scientific), supplemented with 12 mM Tris buffer (Sigma-Aldrich, St. Louis, MO), penicillin (100 IU/mL; Invitrogen), streptomycin (100 µg; Invitrogen), and amphotericin B (250 µg/mL; Invitrogen). Tissues and diluent were homogenized and pooled (5 samples per pool), centrifuged at 5,000 rpm for 30 minutes at 4°C, supernatant collected, then incubated for either 2 hours at 15°C or overnight at 4°C. Immediately following incubation, samples were once again centrifuged at 5,000 rpm for 15 minutes at 4°C. Supernatant was used to inoculate cell cultures of *Epithelioma papulosum cyprini* (EPC; Fijan et al. 1983), Fathead Minnow (FHM; Gravell and Marlsberger 1965), and Chinook Salmon embryo (CHSE-214; Fryer et al. 1965) cells. Following inoculation, cells were incubated at 15°C and examined for cytopathic effects for up to 28 days, with a blind pass onto fresh cells occurring at 14 days post-infection as per the guidelines of the American Fisheries Society Fish Health Section Blue Book (AFS-FHS 2020).

3.6 Bacterial isolate identification with PCR and gene sequencing

Bacterial isolates collected from wild Great Lakes lake whitefish that were presumptively identified as *Carnobacterium* sp. (n=14) following initial morphological and biochemical characterization were subjected to molecular analysis for confirmatory identification. Isolates were revived from cryostock as described above, after which colonies were inoculated into 1 mL of Invitrogen Nuclease-Free Water (Thermo Scientific), centrifuged for 10 minutes at 7,500 rpm, water decanted, and the pellet used for DNA extraction. DNA extractions were performed using the Qiagen DNeasy Tissue Extraction kit (QIAGEN Sciences, Valencia, CA) following the manufacturer's protocol for Gram-positive bacteria. Extracted DNA was subsequently quantified using a Quant-iT DS DNA Assay Kit and a Qubit fluorometer (Life Technologies, Grand Island, NY).

PCR amplification was performed using the primer sets listed in (Table 9). In brief, amplification of a partial stretch of the 16S rRNA gene was conducted using 27F and 1387R primers (Brosius et al. 1978, Marchesi et al. 1998), as well as positions 1526 – 1542 of the 16S rRNA gene and positions 207 – 189 of the 23S rRNA gene using the 16S-4 and 23S-7 primers, which produces a characteristic 600 bp band for *C. maltaromaticum* (Kabadjova et al. 2002, Pellé et al. 2005, Loch et al. 2008). Amplification via PCR was also performed using the PisA forward and PisA reverse primer set, which targets the precursor gene for the piscicolin 126 protein produced by some *C. maltaromaticum* strains (Pellé et al. 2005, Loch et al. 2008).

For the 27F and 1387R and 16S-4 and 23S-7 PCR assays, DNA template (4 µL; diluted to 5 ng/µL) was combined with 10 µL of 2x GoTaq Green Master Mix (Promega, Madison, WI), 5

µL of nuclease-free water, and 0.50 µM/µL of forward and reverse primers for each 20 µL reaction. The cycling conditions for the 27F and 1387R PCR assay began with a denaturation step at 95°C for 5 minutes, followed by 32 cycles of denaturation (95°C) for 30 seconds, annealing (58°C) for 30 seconds, and elongation (72°C) for 1 minute. And lastly, an extension step at 72°C for 7 minutes. The 20 µL reaction for the PisA forward and PisA reverse PCR assay included 4 µL of DNA template (diluted to 5 ng/ µL), combined with 10 µL of 2x GoTaq Green Master Mix (Promega), 4 µL of nuclease-free water, and 1 µM/µL of forward and reverse primers (Pellé et al. 2005, Loch et al. 2008). The cycling conditions for the 16S-4 and 23S-7 and piscicolin 126 PCR assay began with an initial denaturation at 94°C for 10 minutes, followed by 35 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, and a final step of 72°C for 10 minutes (Pellé et al. 2005, Loch et al. 2008). Negative controls consisted of nuclease-free water, and a previously confirmed *C. maltaromaticum* isolate served as the positive control. Resultant PCR products were electrophoresed in a 1.5% agarose gel for 45 minutes at 100V, then visualized under UV transillumination. A 1-kb plus DNA ladder (Invitrogen) was used as a molecular marker.

Purification of generated amplicons from the 27F and 1387R assay was performed by combining 1 µl of the PCR product with 0.25 µl of ExoSAP-IT ™ (Thermo Scientific, Pittsburg, PA) and 3.0 µl of 1 X PCR buffer with $MgCl_2$ (Millipore Sigma, St. Louis, MO), which were then incubated for 20 minutes at 37°C, followed by enzyme activation for 10 minutes at 95°C. Purified amplicons all adult lake whitefish isolates (n=14) were then submitted for bidirectional gene sequence analysis using the 27F and 1387R primers at the Michigan State University - Research Technology Support Facility.

Generated 16S rRNA gene sequences were quality-trimmed using Chromatogram Explorer Lite (version 5.0.2) and then contigs assembled using BioEdit (version 7.2.5). Reference sequences for the type strains of the 12 described *Carnobacterium* spp. were downloaded from the National Center for Biotechnology Information (NCBI, Bethesda, MD) using the BLASTN software. Next, all generated 16S rRNA gene sequences were aligned to all *Carnobacterium* type strains using the CLUSTAL W program from Molecular Evolutionary Genetics Analysis (MEGA; version 10.2.4; Tamura et al. 2013). Model selection for phylogenetic reconstruction was carried out in MEGA, whereby the model with the lowest Bayesian Information Criterion (e.g., Kimura 2-parameter model) was selected. For phylogenetic analyses, neighbor-joining analysis was performed in MEGA and topology robustness were assessed via bootstrap analysis (n=1,000 resamplings).

In Vivo Laboratory-Based Studies

3.7 Rearing of artificially spawned lake whitefish for *in vivo* challenge experiments

Spawning lake whitefish were collected from Elk Rapids in Grand Traverse Bay, MI, by Little Traverse Bay Bands of Odawa Indians personnel in November 2018. Gametes from collected individuals were expressed and eggs were artificially fertilized (1:1 spawning). Eggs were then treated with iodophor and erythromycin (USFWS 2012) and held at the Little Traverse Bay Bands of Odawa Indians' fish hatchery until March of 2019 (i.e., until approximately 3 months post-hatch). Juvenile fish were then transferred to the MSU – AAHL University Research Containment Facility (URCF) and maintained in flow-through PVC tanks, (e.g., tank 6 and tank 7; 485 L) at 12 ± 1 °C in UV-treated well-water supplemented with dissolved oxygen.

Fish were continuously fed a commercial fry diet (Ken's Premium Growth Meal #00) via an automatic feeder. Throughout rearing, tanks were checked, cleaned, and siphoned daily to remove any detritus and uneaten food.

3.8 "Natural" disease outbreak in laboratory-reared juvenile Great Lakes lake whitefish

Juvenile lake whitefish were being reared in preparation for experimental challenges; however, with the onset of the COVID-19 pandemic, the initiation of new *in vivo* experiments was halted in March of 2020. Following these experimental delays and beginning in October of 2020, juvenile lake whitefish in both rearing aquaria began displaying clinical signs of disease and elevated mortality. Because these fish were destined for bacterial challenges experiments, no chemical or antibiotic treatments were pursued; however, salt bath treatments (4.5% sodium chloride immersion bath for 45 – 60 minutes twice daily for 3 days) were implemented, but the disease outbreak continued. Over the course of 7 months, chronic mortality continued (Figure 9) and euthanasia of terminally moribund (e.g., dorsal recumbency and/or apparently distressed) was performed on a daily basis (as needed), whereby all resultant mortalities were immediately processed for bacteriology and virology (see below).

In April of 2021 and approximately 2 weeks after mortalities had subsided, an additional 60 fish from each tank (6 and 7) were randomly selected, euthanized via MS-222 overdose, and subjected to clinical examination and microbiological analyses (see below).

Following euthanasia, morphometric data was recorded, clinical examinations performed, and tissue aseptically collected as described above (Section 3.6). For primary bacterial isolation,

10 µl sterile disposable loops were used to collect kidney tissues, which were directly inoculated onto TSA, as well as Hsu-Shotts medium (HSU; Bullock et al. 1986) and tryptone year extract salts agar (TYES; Holt 1987) that were both supplemented with 4 mg liter^{-1} of neomycin sulfate. Bacterial cultures were also collected from the eyes and brains of representative fish. Furthermore, because fish had grown and the gonads of these juvenile fish had increased in size, these tissues were also collected and inoculated onto TSA, HSU, and TYES using a 10 µl sterile disposable loop.

For virus screening, kidney, spleen and heart tissues were aseptically collected, processed in pools (n=5 fish/pool), and inoculated onto the same 3 cell lines as described above (Section 3.6).

3.9 Bacteriological analyses

Primary cultures were incubated at 22°C (TSA and HSU) and 15°C (TYES) for ≤7 days and checked intermittently for visible bacterial growth. Any resultant bacterial growth was sub-cultured and cryopreserved (Section 3.6) . Initial characterization of isolates collected from laboratory-reared juveniles were performed using the same biochemical characterization tests as previously described (Section 3.6). All presumptively identified *Carnobacterium* sp. isolates were subjected to molecular analysis for confirmatory identification.

3.10 Confirmation isolate identification with PCR and gene sequencing

Representative and presumptively identified *Carnobacterium* isolates were revived from cryopreservation, incubated for 24 hours at 22°C, then DNA extracted as previously described (Section 3.7). PCR amplification using the same three primer sets (Section 3.7), followed by

sequencing of the 16S rRNA gene (Section 3.7) and subsequent phylogenetic analyses (Section 3.7) was conducted.

4. Results

Field-Based Studies

Six hundred wild adult lake whitefish were collected from five sites in Lakes Superior, Michigan, and Huron (Figure 4; Table 7), as were 436 wild juvenile Great Lakes lake whitefish (Figure 4; Table 8). The overall mean length and weight of collected adults was 53.4 cm and 1408.2 g, with variation by site (Table 7). The overall mean length and weight of wild juvenile lake whitefish was 3.52 cm and 0.72 g (Table 8). Virological analyses via tissue culture and virus isolation were performed on EPC, FHM, and CPE cell lines at 15°C for all adult and juvenile lake whitefish (n=1036 total). No cytopathic effect was observed in any samples; therefore, all fish were considered negative for viruses capable of replicating on these cell lines.

Tissues for bacterial isolation were collected from all adult (n=600) and wild juvenile (n=436) Great Lakes lake whitefish. Following approximately 3 – 5 days of incubation on both TSA and SBA at 22°C, white, semitranslucent bacterial colonies with morphologies that ranged from low convex and punctate to raised centers with undulate margins (Fig. 3.7) were recovered from 8 kidney- (n=3 from Whitefish Bay, n=4 from Alpena, n= 1 from Saginaw Bay), 4 gonad- (n=1 from Whitefish Bay, n= 2 from Baileys Harbor, n= 1 from Saginaw Bay), and 2 swim bladder fluid (n=1 from Alpena, n= 1 from Saginaw Bay) cultures. Bacterial infection intensities ranged from 1 – >300 colony forming unit (CFU) per 10 µl of either kidney, gamete, or swim bladder fluid inoculum. Of note, the 2 swim bladder fluid cultures yielded the most prolific

bacterial growth (>300 CFU, i.e., too numerous to count). No bacteria with this morphology were collected from any juvenile Great Lakes over the course of this study.

Phenotypic and biochemical characterization of the fourteen isolates revealed all were Gram-positive, short bacilli that were negative for cytochrome oxidase and catalase activities. All isolates utilized dextrose, hydrolyzed esculin, and were gamma hemolytic on blood agar, but were nonmotile and did not produce H_2S or indole on sulfur-indole-motility medium (Section 3.6). Based on these initial results, the bacteria were suspected as belonging to the genus *Carnobacterium*.

Using the 16S-4 and 23S-7 PCR assay, all 14 adult lake whitefish isolates produced amplicons of approximately 600 base pairs (bp), which is characteristic of *C. maltaromaticum* (Pellé et al. 2005, Loch et al. 2008). When the PisA PCR assay was employed, a *C. maltaromaticum*-characteristic 300 bp band (Pellé et al. 2005, Loch et al. 2008) was generated for 4/14 adult lake whitefish *Carnobacterium* isolates (isolates 34JG collected from Whitefish Bay, 46MSBAG from Baileys Harbor, 10I from Alpena, and 17K collected from Saginaw Bay; Figure 5).

Sequencing of a partial stretch of the 16 rRNA gene, which was PCR-amplified from all 14 isolates using the 27F – 1387R primer set, and subsequent phylogenetic analyses placed them within a single, robustly-supported clade that also contained the *C. maltaromaticum* type strain reference sequence (Figure 5), thereby confirming the identity of the Great Lakes lake whitefish

isolates as *C. maltaromaticum*. However, some minor sequence variations amongst lake whitefish isolates were detected (Figure 5).

The prevalence of *C. maltaromaticum* infections in adult Great Lakes lake whitefish ranged from 0 – 6.6% in 2018 and 0 – 5.0% 2019 across Lakes Superior, Michigan, and Huron (Table 7). Within lake comparisons revealed that prevalence of *C. maltaromaticum* infections were typically higher in lakes associated with poor recruitment than in sites associated with good recruitment. For example, from 2018 – 2019 in Lake Michigan, prevalence of *C. maltaromaticum* was always higher in Baileys Harbor (1.6%), than in Menominee (0%; Table 7). Likewise, in 2018 in Lake Huron, *C. maltaromaticum* prevalence was higher in Alpena (6.6%) compared to Saginaw Bay (3.3%; Table 7). However, in 2019, *C. maltaromaticum* infection prevalence was the same in Alpena (1.6%) and Saginaw Bay (1.6%; Table 7).

External signs of disease that were observed in adult *C. maltaromaticum*-infected lake whitefish included ocular hemorrhage (Figure 7A), generalized erythema, and varying degrees of congestion within the fins. Internally, splenic congestion and swelling, hepatic pallor and friability, renal congestion and swelling, heart pallor, and gonadal congestion and hemorrhage were noted, as was opacity, erythema, and hemorrhage within the swimbladder walls and hyperemia of the swim bladder vasculature (Figure 7B). In two infected fish, a mucoid turbid fluid was present within the swim bladder lumen (Figure 7C), which yielded heavy *C. maltaromaticum* growth (Figure 10). *Carnobacterium maltaromaticum*-infected fish also harbored encysted metacercariae (presumptive *Tetracotyle* sp.) within the ventricle of the heart

(n=8/14), *Cystidicola* sp. within the swim bladder lumen (n=2/14), and acanthocephalans within the gastrointestinal tract (n=1/14).

Laboratory-Based Studies

Beginning in October of 2020 and as the fish continued to be cared for, juvenile lake whitefish (e.g., mean length 23.8 cm and mean weight 118.0 g in Tank 6; mean length 24.3 cm and mean weight 116.9 g in Tank 7) in the two flow-through aquaria began to show gross signs of disease, including ocular hemorrhage (Figure 8A-B) and unilateral to bilateral exophthalmia (Figure 8C). In some instances, the presence of disease signs was followed by mortality and in other cases, terminally moribund fish were euthanized. Eventually, cumulative mortality reached 31.7% in Tank 6 and 25.1% in Tank 7 (Figure 9). As the disease event continued, affected fish began to show more chronic signs of disease concomitant with exophthalmia, including corneal opacity (Figure 8D) and perforation (Figure 8E). Internally, visceral edema, splenic swelling, and erythema within the ovaries were noted.

To determine the cause of this "naturally occurring" disease event that occurred under quarantine conditions, tissues for parasitological, bacteriological, and virological analyses were collected from dead and euthanized fish over the course of the mortality event. Although no viruses or parasites were detected, primary bacterial cultures (TSA, 22°C) taken from the eyes, kidneys, and brains yielded white, semitranslucent bacterial colonies with morphologies that ranged from low convex and punctate to raised centers with undulate margins (Fig 3.8) at intensities ranging from 1 – >300 CFUs per 1 µl or 10 µl of tissue inoculum.

Upon subculture, phenotypic characterization revealed that the bacteria recovered from the eyes, brains, and kidneys of the laboratory-reared juvenile lake whitefish were Gram-positive bacilli that were negative for oxidase and catalase activities. Subsequent molecular characterization of these isolates via the 16S-4 23S-7 PCR assay yielded the *C. maltaromaticum*- characteristic ~600 bp amplicon for all representative isolates (n=11). When these same isolates were tested using the PisA forward and reverse primers, once again all isolates (n=11) yielded 300 bp amplicon characteristic of *C. maltaromaticum*.

Using methods that were similarly performed for the *C. maltaromaticum* isolates recovered from adult lake whitefish, amplification of a partial stretch of the 16s rRNA gene via PCR and subsequent gene sequencing and phylogenetic analyses showed that the isolates recovered from juvenile lake whitefish mortality event fell into the same robustly supported clade that contains the *Carnobacterium maltaromaticum* type strain, confirming their identity as *C. maltaromaticum* (Figure 6).

In an effort to determine *C. maltaromaticum* infection prevalence in lake whitefish surviving the disease outbreak (i.e., approximately two weeks following mortality), 60 fish were randomly collected from Tanks 6 and 7 (n=120 total), euthanized, and clinically and microbiologically examined as described above. The prevalence of *C. maltaromaticum* infections ranged from 3.3% (Tank 6) to 6.6% (Tank 7), whereby the bacterium was recovered from the gonads and eyes (Tank 6) and the kidneys, eyes, gonads, and ascites (Tank 7). Employing the 27F and 1387R PCR assay described above on bacterial isolates presumptively identified as

Carnobacterium sp., and following gene sequencing of produced amplicons, their identity was confirmed as *C. maltaromaticum*.

5. Discussion

Ongoing declines in the condition of adult lake whitefish concurrent with notable declines in the survival and recruitment of juvenile lake whitefish within the Great Lakes continues to alarm fishery managers and scientists alike. A range of abiotic and biotic factors have been proposed as contributing to these declines (reviewed in Ebener et al. 2021) and likely represent important sources of host and environmental stressors on lake whitefish populations within the Great Lakes. In this context, it has long been recognized that infectious diseases result from complex interactions between the affected hosts, their environment, and the infectious disease-causing agent(s) (Snieszko 1974). In the current study, systemic *C. maltaromaticum* infections, as confirmed by isolation and molecular and phylogenetic analyses, were detected in adult spawning phase lake whitefish that concurrently showed gross signs of disease (Figure 7) and in some cases, were of a substantial intensity (Figure 10). Likewise and for the first time, *C. maltaromaticum* infections were detected in the gonads of *C. clupeaformis* collected from Lakes Superior, Michigan, and Huron, and that had already spawned or were nearing spawning. Whether the presence of this bacterium within the gonads and reproductive fluids of infected spawning lake whitefish leads to transgenerational transmission is unknown. Despite this, *C. maltaromaticum* infections were not detected in the 436 lake whitefish that were collected from beaches linked to adult spawning sites with historically good and poor records of lake whitefish recruitment.

In the current study, *C. maltaromaticum* infection prevalence was almost always higher in sites associated with poor recruitment when compared to sites with historically higher recruitment within the same lake (Table 7). Indeed, *C. maltaromaticum* infections were never detected in adult lake whitefish collected from Menominee (good and stable recruitment) in either sampling year but were detected during 2018 and 2019 in fish collected from a site with historically poor recruitment (Baileys Harbor) in the same lake (Lake Michigan). Similarly, *C. maltaromaticum* infection prevalence was twice as high in Alpena (poor recruitment) versus Saginaw Bay (good recruitment) in Lake Huron in 2018. Throughout the study, in fact, only once did this trend not hold true (e.g., within Lake Huron in 2019), when *C. maltaromaticum* infection prevalence was identical (e.g., 1.6%) in both sites. Although a notable trend, it remains unclear whether this pathogen is more prevalent at these sites, or whether other host and/or environmental factors may be predisposing Great Lakes lake whitefish to infections at sites with historically poor recruitment. For example, when comparing the visceral fat index (VFI) of fish from sites within Lake Michigan from 2018 -2019, the mean overall VFI of fish from Baileys Harbor (poor recruitment site) was 0.8, whereas in Menominee (good recruitment site where *C. maltaromaticum* infections were not detected), the mean VFI was 1.4 (see Chapter 2). A higher VFI can be indicative of good nutritional status (Adams 1999), meaning fish from Menominee may be generally healthier and potentially less prone to disease and/or mortality.

A previous study by Loch et al. (2008) also found systemic infections caused by a *C. maltaromaticum*-like bacterium in adult, grossly diseased Great Lakes lake whitefish collected from four sites within Lake Michigan and Lake Huron. Interestingly, lake whitefish were sampled during the fall, winter, spring, and summer seasons over a three-year period in that

study, whereby *C. maltaromaticum* infections were most prevalent in the winter and spring samplings, but were never detected during the fall, which corresponds to the time during which fish were collected and analyzed during the current study. Although seasonal sampling was not undertaken in the current study, the trends that were observed by Loch et al. (2008) suggest that fall sampling represents the "low end" of seasonal prevalence, possibly suggesting even higher *C. maltaromaticum* infection prevalence would have been observed had sampling occurred in the in winter and spring.

Given the continued poor recruitment of juvenile lake whitefish and the previous detections of *C. maltaromaticum* in adult Great Lakes lake whitefish (Loch et al. 2008), another goal of the current study was to determine the effects that *C. maltaromaticum* has on the health and survival of juvenile lake whitefish via *in vivo* laboratory challenge experiments. Thus, the "naturally occurring" disease outbreak that occurred in both tanks (i.e., replicates) of laboratory-reared juvenile lake whitefish despite iodophor and erythromycin egg treatments and quarantine conditions was unfortunate and obviously unplanned. However and given that no studies have yet to examine the virulence of *C. maltaromaticum* to *C. clupeaformis*, we sought to gain a better understanding of the capacity *C. maltaromaticum* has to cause disease and mortality in juvenile lake whitefish despite this unforeseen development. Following clinical examinations and diagnostic testing, no viruses, parasites, or other significant bacteria were detected in laboratory-reared juvenile lake whitefish. Serious signs of disease were observed in laboratory-reared *C. maltaromaticum*- infected fish, including some that match those previously observed in other salmonid species suffering from pseudokidney disease outbreaks (Foott 1994), as well as those observed in wild *C. maltaromaticum* infected adult lake whitefish in the present and a past study

(Loch et al. 2008). The bacterium was isolated from diseased fish throughout the course of the disease outbreak and isolates were definitively identified using biochemical characterization, molecular, and phylogenetic analyses. Infections of *C. maltaromaticum* were also associated with mortality in juvenile fish, and although infections were chronic in nature, mortality was extensive (Figure 9). *Carnobacterium maltaromaticum* infections were detected in multiple tissues, where in some instances infection intensities were similar to those detected in wild fish (Figures 3.7 and 3.8). However, whether stressors such as rearing density or conditions associated with a captive environment are also needed for mortality to occur remain unclear. Following cessation of mortalities, *C. maltaromaticum* was still present within multiple organ systems. Whether this suggests an extended shedding/transmission period remains to be determined.

The piscicolin-126 protein is a class II bacteriocin that produces bactericidal activity against some Gram-positive bacteria (Jack et al. 1996). Loch et al. (2008) found the protein in representative lake whitefish isolates, whereas the current study also found it in *C. maltaromaticum* isolates recovered from wild adult lake whitefish (n=4/14). Notably, all analyzed isolates from the laboratory disease outbreak (n=11/11) were positive for the presence of the piscicolin-126 protein. Whether this discovery means *C. maltaromaticum* isolates recovered from the disease outbreak have a competitive edge over other Gram-positive bacteria within the host or if it provides beneficial effects to the host in other situations remains undetermined.

In conclusion, herein I showed that systemic *C. maltaromaticum* infections were often more prevalent in spawning phase Great Lakes lake whitefish collected from sites with historically poor recruitment when compared to associated with historically good recruitment. Moreover and for the first time, *C. maltaromaticum* infections were detected in the gonads of infected fish, highlighting the possibility for shedding the bacterium along with the eggs and reproductive fluids of infected fish. In this context, *C. maltaromaticum* infections were found to cause a disease outbreak and mortalities in laboratory-reared juvenile lake whitefish, where it was associated with severe disease signs, some of which were previously observed in infected wild fish. Collectively, data presented herein provide evidence that systemic *Carnobacterium maltaromaticum* infections can be associated with disease and mortality in Great Lakes lake whitefish.

APPENDIX

Figure 4. Adult and juvenile Great Lakes lake whitefish collection sites. LS=Lake Superior; LM=Lake Michigan; LH=Lake Huron. [G]Good recruitment site. [P]Poor recruitment site. Adult collection site =●; juvenile collection site= ■

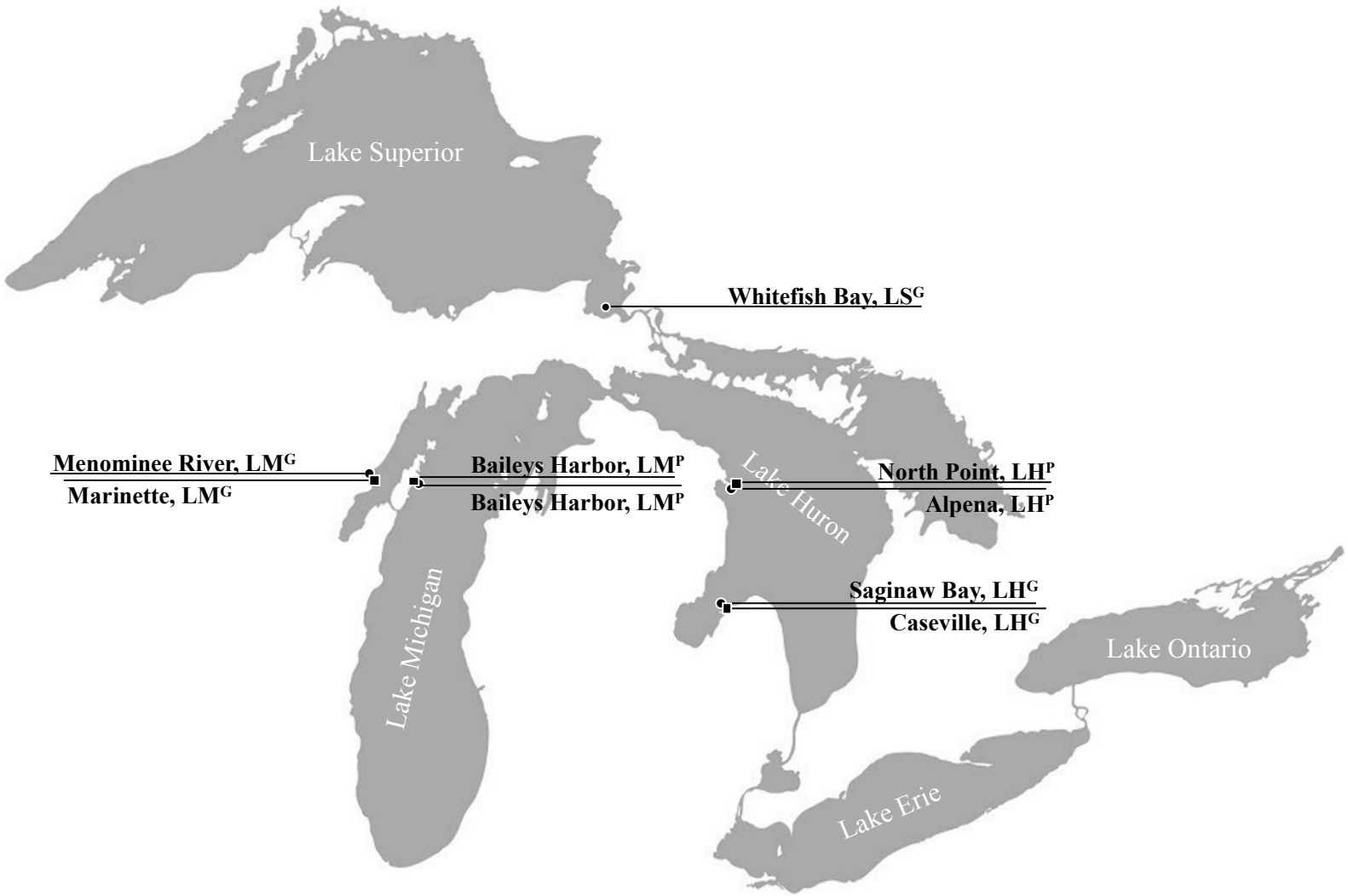

Figure 5. A dendrogram depicting the relationships of bacterial isolates recovered from adult Great Lakes lake whitefish when compared to 16S rRNA gene sequences derived from the type strains of all currently described *Carnobacterium* spp. *Ruminococcus palustris* served as the outgroup and accession numbers from NCBI follow scientific names. Whitefish Bay, Lake Superior[G]= ◆ ; Baileys Harbor, Lake Michigan[P]= ▲ ; Alpena, Lake Huron[P]= ● ; Saginaw Bay, Lake Huron[G]= ■ . [G]Good recruitment site. [P]Poor recruitment site.

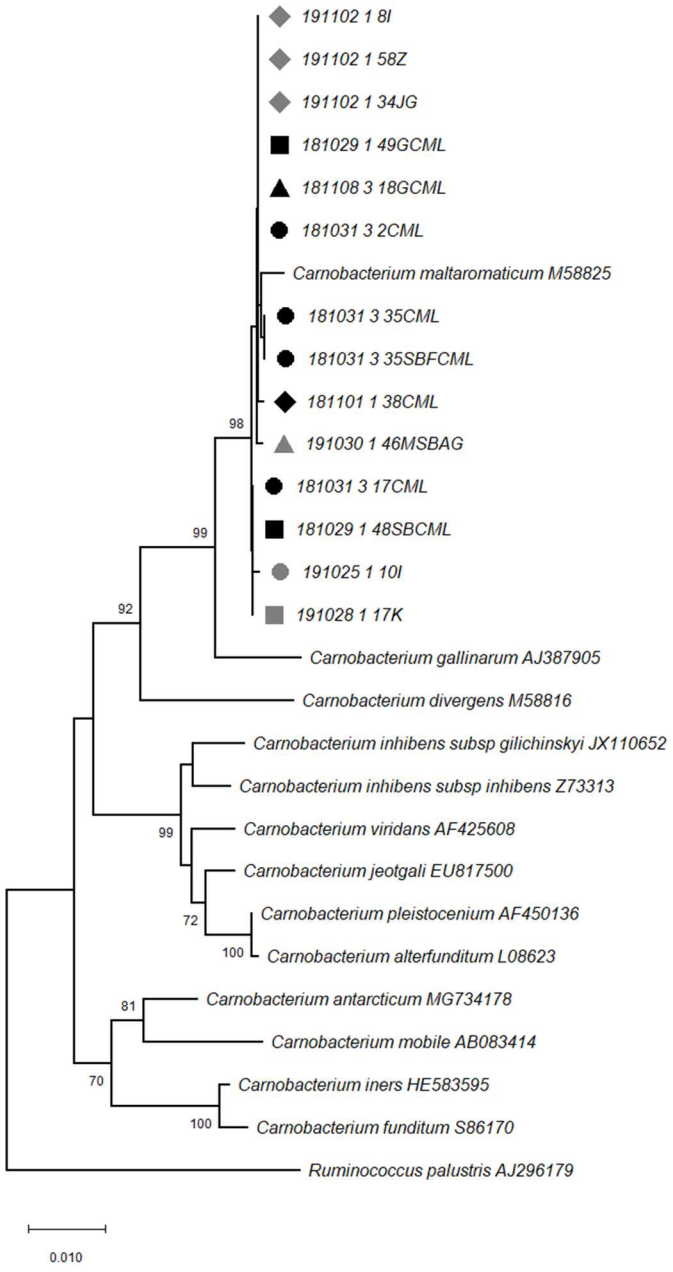

Figure 6. A dendrogram depicting the relationships of bacterial isolates recovered from adult (denoted by filled shapes) and laboratory-reared juvenile Great Lakes lake whitefish (outlined circles) when compared to 16S rRNA gene sequences derived from the type strains of all currently described *Carnobacterium* spp. *Ruminococcus palustris* served as the outgroup and accession numbers from NCBI follow scientific names. Whitefish Bay, Lake Superior[G]=◆ ; Baileys Harbor, Lake Michigan[P]= ▲ ; Alpena, Lake Huron[P]= ●; Saginaw Bay, Lake Huron[G]= ■ ; laboratory-reared juvenile lake whitefish= ○. [G]Good recruitment site. [P]Poor recruitment site.

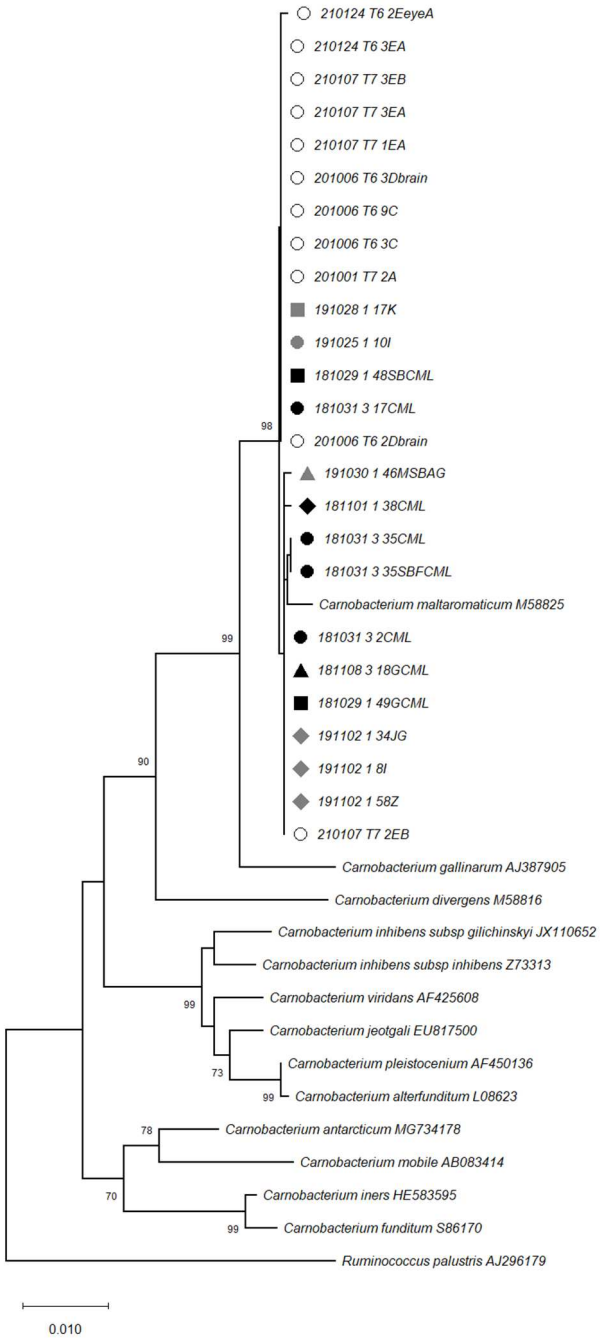

Figure 7. Gross signs of disease that were noted in adult Great Lakes lake whitefish infected with *Carnobacterium maltaromaticum*. (A) Multifocal ocular ecchymoses. (B) Severe hyperemia of the swim bladder vessels and thickening and opacity of the swim bladder wall. (C) Opened swim bladder lumen filled with a turbid, mucoid exudate from which *C. maltaromaticum* was recovered in pure culture.

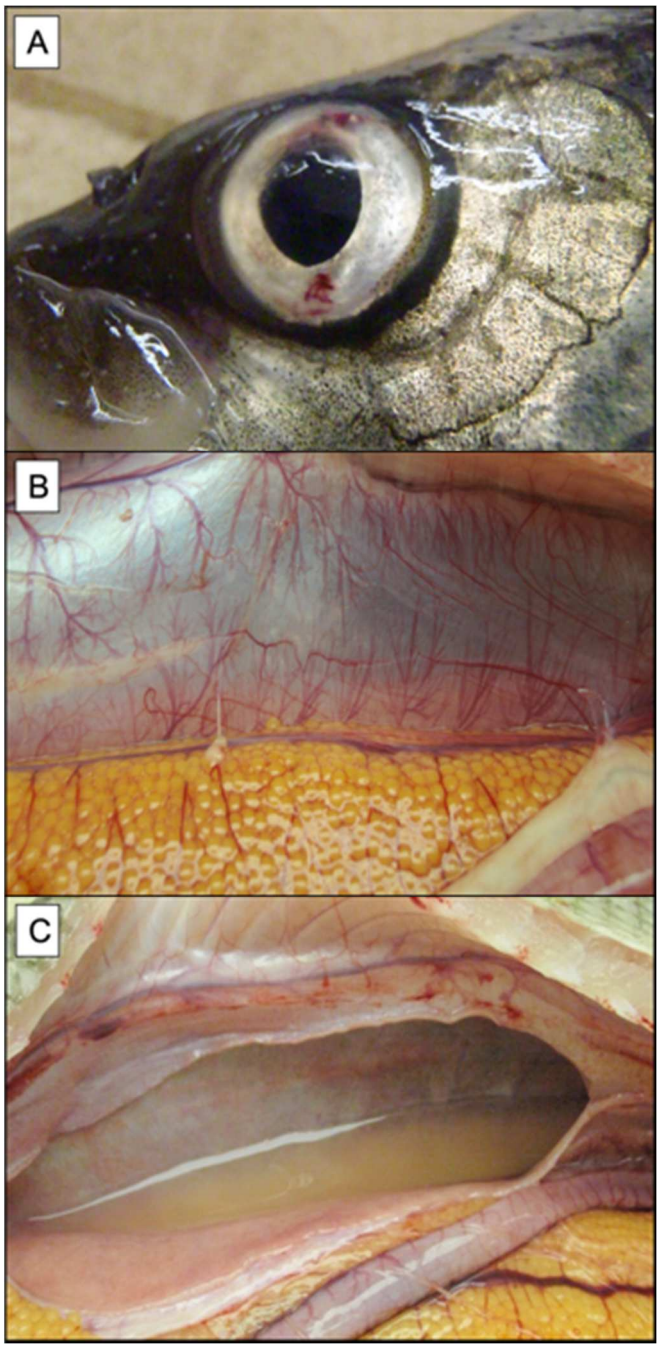

Figure 8. Gross signs of disease that were noted in laboratory-reared juvenile Great Lakes lake whitefish suffering from a *Carnobacterium maltaromaticum* disease outbreak. (A) Ocular linear hemorrhage. (B) Severe intraocular hemorrhage and exophthalmia. (C) Severe unilateral exophthalmia. (D) Severe exophthalmia with corneal opacity and linear hemorrhage. (E) Lenticular perforation of the cornea with concurrent hemorrhage along the base of the eye.

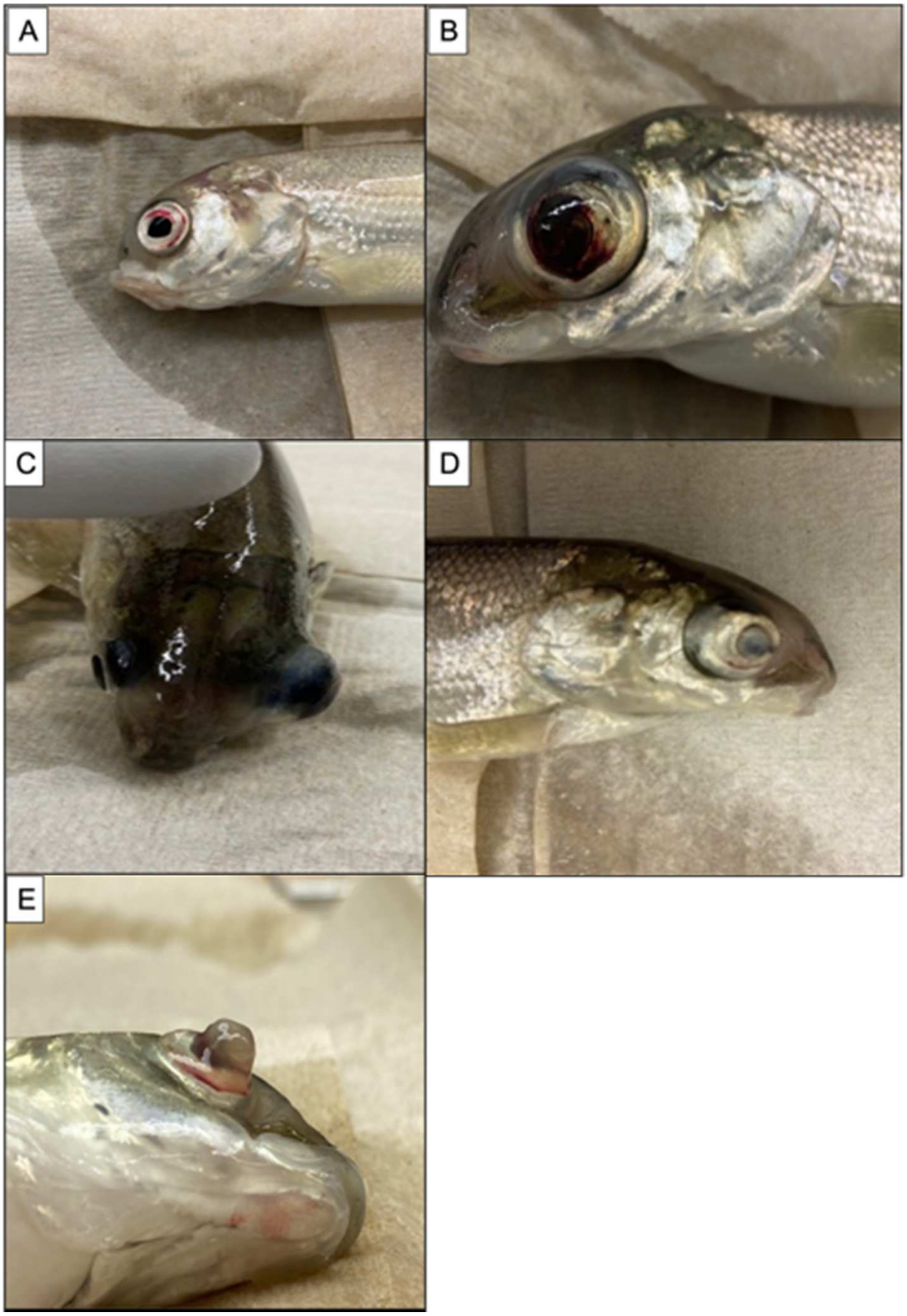

Figure 9. Cumulative mortality (including terminally moribund individuals that were euthanized) in laboratory-reared juvenile Great Lakes lake whitefish during a "naturally occurring" disease outbreak.

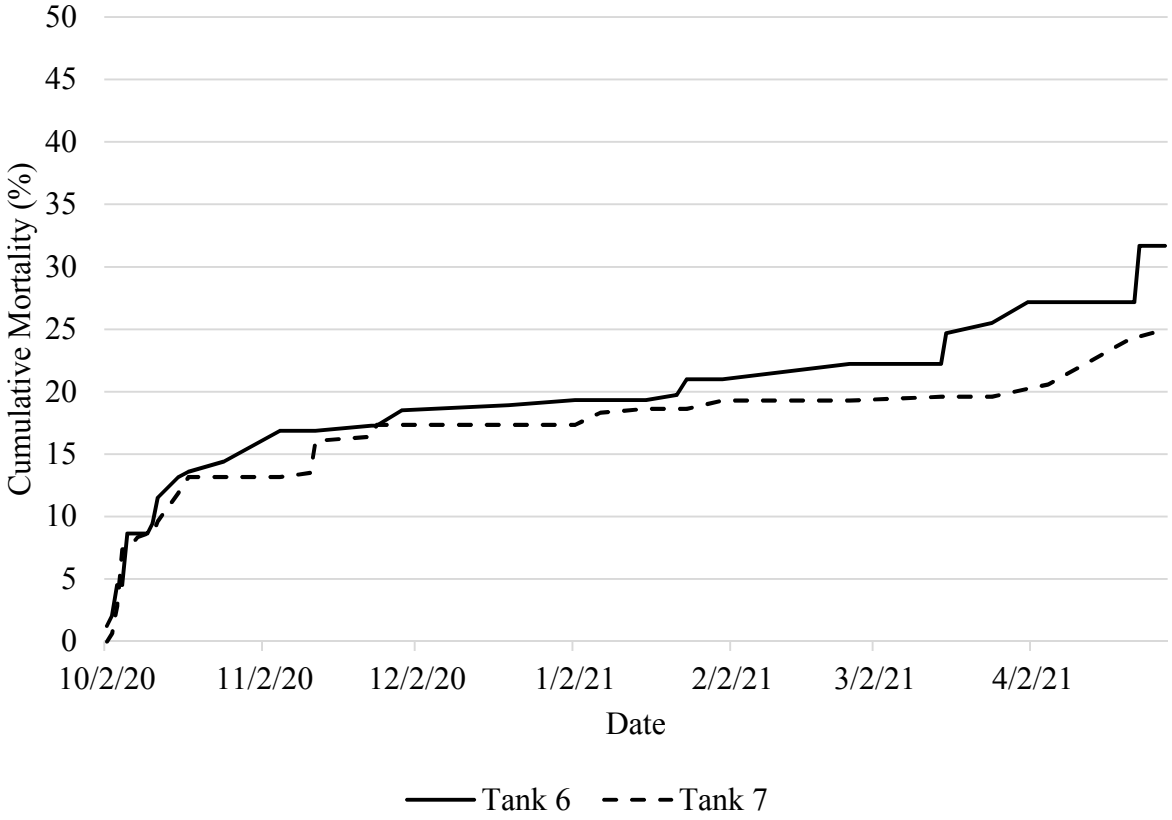

Figure 10. Primary culture (on sheep's blood agar) of *Carnobacterium maltaromaticum* isolated from a mucoid turbid fluid present within the swim bladder lumen of an infected adult Great Lakes lake whitefish. Note the white, semitranslucent bacterial colonies with morphologies that ranged from low convex and punctate to raised centers with undulate margins following incubation at 22°C for 72 hours.

Figure 11. Primary culture of *Carnobacterium maltaromaticum* isolated from within the eye of an infected laboratory-reared juvenile Great Lakes lake whitefish. Tissue yielded white, semitranslucent bacterial colonies with morphologies that ranged from low convex and punctate to raised centers with undulate margins following incubation at 22°C for 72 hours. Picture was taken under dissection microscope.

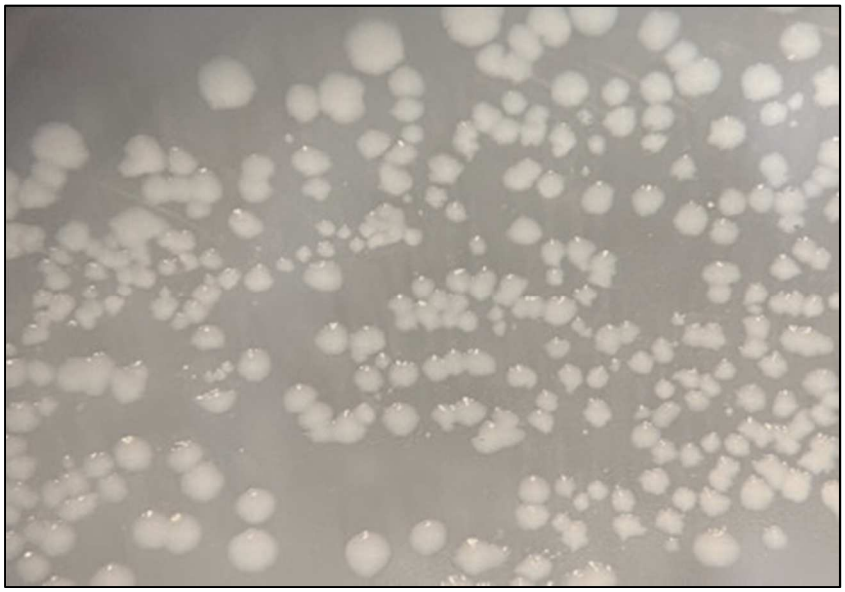

Table 7. Mean length (cm), weight (g), and *C. maltaromaticum* infection prevalence in the kidneys and gonads of adult Great Lakes lake whitefish from 2018 – 2019. LS=Lake Superior; LM=Lake Michigan; LH=Lake Huron. Standard deviation of the data is reported in parentheses. [G]Good recruitment site. [P]Poor recruitment site.

	Mean Length (cm)		**Mean Weight (g)**		***C. maltaromaticum* Prevalence (%)**	
Site	2018	2019	2018	2019	2018	2019
Whitefish Bay, LS[G]	50.7 (5.1)	52.3 (4.9)	1163.8 (380.7)	1414.7 (549.3)	1/60 (1.6%)	3/60 (5.0%)
Baileys Harbor, LM[P]	55.2 (6.3)	56.3 (4.7)	1658.7 (454.8)	1511.4 (462.0)	1/60 (1.6%)	1/60 (1.6%)
Menominee, LM[G]	45.8 (3.9)	46.4 (3.3)	756.8 (222.0)	798.6 (178.4)	0/60 (0%)	0/60 (0.0%)
Alpena, LH[P]	57.2 (4.6)	56.8 (4.0)	1779.9 (506.4)	1629.9 (448.6)	4/60 (6.6%)	1/60 (1.6%)
Saginaw Bay, LH[G]	56.4 (4.3)	56.7 (3.9)	1710.3 (475.7)	1658.0 (436.7)	2/60 (3.3%)	1/60 (1.6%)

Table 8. Mean length (cm), and weight (g) of wild juvenile Great Lakes lake whitefish collected in 2019 and 2020. LS=Lake Superior; LM=Lake Michigan; LH=Lake Huron. Standard deviation of the data is reported in parentheses. [G]Good recruitment site. [P]Poor recruitment site. Only 1 fish was collected from Caseville, LH.

Site	Mean Length (cm)	Mean Weight (g)	Total Fish Collected	Total Fish Cultured	*C. maltaromaticum* Prevalence
Whitefish Bay, LS[G]	2.1 (0.6)	<1.0	25	1	0.0%
Baileys Harbor, LM[P]	6.1 (0.3)	1.9 (0.3)	150	150	0.0%
Marinette, LM[G]	3.4 (0.6)	0.3 (0.1)	110	110	0.0%
North Point, LH[P]	3.4 (0.4)	0.3 (0.1)	150	150	0.0%
Caseville, LH[G]	2.6	0.2	1	1	0.0%

Table 9. Primers that were used for the molecular characterization of bacterial isolates that were analyzed in this study.

Primer Set	Sequences	Sources
27F and 1387R	5'-AGA GTT TGA TCM TGG CTC AG-3' and 5'-GGG CGG WGT GTA CAA GGC-3'	Brosius et al. 1978 Marchesi et al. 1998 Loch et al. 2013
16S-4 and 23S-7	5'-GCT GGA TCA CCT CCT TTC T-3' and 5'-GGT ACT TAG ATG TTT CAG TTC C-3'	Kabadjova et al. 2002 Pellé et al. 2005 Loch et al. 2008.
PisA forward and PisA reverse	5'-GTC ACA GCA TTG ATG CGT ATC-3'and 5'-GAT GTG ATA CAG TCA GCA TGT-3'	Pellé et al. 2005 Loch et al. 2008

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Chapter 4:

In vivo Studies Provide Evidence that Juvenile Great Lakes Lake Whitefish (*Coregonus clupeaformis*) are Susceptible to *Aeromonas salmonicida* subsp. *salmonicida* and Viral Hemorrhagic Septicemia Virus

1. Abstract

Infections caused by fish-pathogenic microbes cause disease in wild Great Lakes fish populations and can be associated with substantial mortality. In the face of ongoing declines in the recruitment and survival of juvenile lake whitefish (*Coregonus clupeaformis*) in the four lower Great Lakes of North America, this study was undertaken to elucidate the virulence of *Aeromonas salmonicida* subsp. *salmonicida* (causative agent of furunculosis; Family *Aeromonadaceae*) and Viral Hemorrhagic Septicemia Virus genotype IVb (etiological agent of viral hemorrhagic septicemia; Family *Rhabdoviridae*), both of which have been recovered from adult Great Lakes lake whitefish, to < 1 year old lake whitefish under laboratory conditions. Eight- and nine-month-old lake whitefish were immersion-exposed to one of five doses of either an *A. salmonicida* subsp. *salmonicida* strain ($6.1x10^1$ – $6.1x10^5$ CFU/ml) recovered from an adult Great Lakes lake whitefish or the Great Lakes VHSV-IVb Index strain (MI03; 1.4 – $1.4x10^4$ PFU/ml), after which gross disease progression, cumulative percent mortality, and median lethal dose (LD_{50}) were assessed. *In vivo* experiments revealed that Great Lakes lake whitefish were highly susceptible to *A. salmonicida* subsp. *salmonicida* and Viral Hemorrhagic Septicemia Virus, whereby severe gross signs that are characteristic of furunculosis and viral hemorrhagic septicemia developed and cumulative percent mortality ranged from 6.6 – 53.3% and 6.6 – 93.3%, respectively. The estimated LD_{50} was $3.6x10^5$ CFU/ml for *A. salmonicida* subsp. *salmonicida* and $5.3x10^1$ PFU/ml for Viral Hemorrhagic Septicemia Virus. Based upon these findings, juvenile *C. clupeaformis* appears highly susceptible to *A. salmonicida* subsp. *salmonicida* and be amongst the most VHSV-IVb susceptible Great Lakes fish species studied to date. Whether these microbial pathogens that are currently widespread within the Great Lakes

basin are contributing to ongoing recruitment declines in lake whitefish warrants further investigation.

2. Introduction

Infections caused by a multitude fish-pathogenic bacteria and viruses cause disease in wild Great Lakes fish populations, and in some instances, have caused substantial mortality in affected wild (Holey et al. 1998, Faisal and Hnath 2005, Elsayed et al. 2006, Faisal et al. 2012) and hatchery-reared populations (Van Vliet et al. 2015, Faisal et al. 2019). Of note, infectious disease outbreaks can also cause serious negative health effects and substantial mortality in fish in their early life stages (reviewed in Woo and Cipriano 2017); however, much of what is known in this regard comes from natural mortality events in hatchery/aquaculture-reared fishes (Cipriano and Holt 2005, Van Vliet et al. 2015, Faisal et al. 2019) and experiments under controlled laboratory conditions (Weeks et al. 2011, Knupp et al. 2021). Indeed, *in vivo* laboratory challenges are not only a primary means of fulfilling Koch's and River's postulates (Rivers 1937) but represent a critical initial step in understanding the ability of microbial pathogens of fishes to cause negative effects on health and survival.

Two well-known microbial fish pathogens, *Aeromonas salmonicida* subsp. *salmonicida* (Family *Aeromonadaceae*), the etiological agent of furunculosis (Shotts 1994), and Viral Hemorrhagic Septicemia Virus (VHSV; Family *Rhabdoviridae*), the causative agent of viral hemorrhagic septicemia (VHS; Batts et al. 2020), have been detected in adult Great Lakes lake whitefish (*Coregonus clupeaformis*) showing gross signs of disease (Loch and Faisal 2010, Loch and Faisal 2011). For example, adult lake whitefish systemically infected with *A. salmonicida*

subsp. *salmonicida* showed severe hemorrhage, exophthalmia, splenomegaly, as well as congestion and fibrous adhesions of the visceral organs (Loch and Faisal 2010). Likewise, VHSV genotype IVb (VHSV-IVb) was detected in wild adult lake whitefish showing disease signs consistent with VHS, including widespread hemorrhage and visceral damage (Thompson et al. 2011, Loch and Faisal 2011, reviewed in Faisal et al. 2012). Of note, both of these microbial pathogens can be present within the reproductive fluids of infected fish (Cipriano et al. 2001, OIE 2021), thus potentially posing a risk of infection to resultant offspring.

Although the ability of both *A. salmonicida* subsp. *salmonicida* and VHS-IVb to cause disease and mortality in a range of fish species is undisputable (reviewed in Woo and Cipriano 2017) and despite the fact that both pathogens have been recovered from wild lake whitefish also showing disease signs, their virulence to Great Lakes lake whitefish has yet to be investigated. Given the continued poor recruitment of juvenile lake whitefish throughout many parts of the four lower Great Lakes (Ebener et al. 2021), the lack of knowledge on the effects that *A. salmonicida* subsp. *salmonicida* and VHSV may have on early life stage and juvenile Great Lakes lake whitefish health and survival is concerning. To this end, I sought to evaluate the *in vivo* virulence of an *A. salmonicida* subsp. *salmonicida* strain recovered from adult Great Lakes lake whitefish (Loch and Faisal 2010) and a VHSV-IVb strain recovered from a massive fish kill within the Great Lakes basin (Elsayed et al. 2006, Loch and Faisal 2011) in < 1 year old Great Lakes lake whitefish under controlled laboratory conditions.

3. Materials and Methods

3.1. Origin of fish for challenge experiments

As a source for laboratory-reared lake whitefish, spawning adults were collected from Elk Rapids in Grand Traverse Bay, MI (Lake Michigan watershed) by Little Traverse Bay Bands of Odawa Indians fish hatchery personnel in November 2020. Gametes were collected and eggs artificially fertilized (1:1 spawning), disinfected with iodophor and erythromycin (USFWS), and held at the Little Traverse Bay Bands of Odawa Indians' Fish Hatchery until April of 2021 (approximately 4 months post-hatch). Juvenile fish were then transferred to the Michigan State University – Aquatic Animal Health Laboratory (MSU – AAHL) University Research Containment Facility (URCF) and maintained in flow-through PVC tanks (485 L; 12 ± 1 °C) in UV-treated well water supplemented with dissolved oxygen. Fish were continuously fed a commercial fry diet (Bio-Oregon BioVita Fry 1.5mm) via automatic feeder. Throughout rearing, tanks were cleaned and siphoned daily to remove any detritus and uneaten food. Prior to challenge experiments (Section 3.4), a subset of fish were screened for the presence of a multitude of fish pathogens, including *A. salmonicida* subsp. *salmonicida* and VHSV, following guidelines of the American Fisheries Society Fish Health Section Blue Book (AFS-FHS 2020). Following verification of a disease-free status, fish were transferred to aerated experimental challenge flow-through glass tanks (37.85 L) supplied with UV-treated water (12 ± 1 °C) for acclimation. All fish handling was performed in accordance with the Michigan State University – Institutional Animal Care and Use Committee Standards (AUF 202100272).

Aeromonas salmonicida **subsp.** ***salmonicida***

3.2 *Aeromonas salmonicida* subsp. *salmonicida* growth kinetics

An *Aeromonas salmonicida* subsp. *salmonicida* isolate (060628-1-Asal-1) originally recovered from a systemically-infected and grossly diseased adult lake whitefish (Loch and Faisal 2010) was revived from cryostock on trypticase soy agar (TSA; Thermo Scientific, Pittsburg, PA), and incubated at 22°C for all *A. salmonicida* subsp. *salmonicida* experimental challenge studies. To determine when strain 060628-1-Asal-1 reached a logarithmic phase of growth, 1 µl of the isolate was inoculated into 40 mls of trypticase soy broth (TSB; Thermo Scientific) and incubated at 22°C for up to 96 hours. Optical density (OD) was measured using a Biowave CO8000 Cell Density Meter (WPA Inc.), and 10-fold serial dilutions were made of the bacterial inoculum at 1, 2, 4, 6, 8, 12, 24, 48, 72, and 96 hours post-inoculation. Dilutions were plated on TSA in duplicates, plates incubated at 22°C for 48 hours, and colonies counted thereafter. From this, it was determined that logarithmic growth was achieved at approximately 18 hours.

3.3 *Aeromonas salmonicida* subsp. *salmonicida* inoculum preparation for experimental challenges

To generate the experimental inocula, strain 060628-1-Asal-1 was inoculated into TSB, vortexed, and incubated for 18 hours at 22°C. Bacterial cells were then harvested via centrifugation (3,700 rpm, 10 min; Thermo Scientific™ Sorvall™ Legend™ X1R centrifuge), after which bacterial pellets were collected and resuspended in sterile 0.65% saline adjusted to an OD of approximately 5.0-5.5, corresponding to ~10^9 CFU/mL (as determined during growth kinetic experiments). The bacterial suspension was then serially diluted 10-fold to create five *A.*

salmonicida subsp. *salmonicida* suspensions corresponding to ~10^8 – 10^5 CFU/ml. To quantify the bacterial concentrations, serial dilutions in 10-fold increments were plated onto TSA in duplicate and incubated at 22°C for 72 hours, after which colony counts were performed.

3.4. *Aeromonas salmonicida* subsp. *salmonicida* median lethal dose experiments

Just prior to *in vivo* challenge experiments, the five *A. salmonicida* subsp. *salmonicida* suspensions were further diluted in UV-treated well (i.e., tank-) water, corresponding to final concentrations of $6.1x10^1$ – $6.1x10^5$ CFU/ml. Next, seventy-five juvenile (mean length 4.4 cm; mean weight 8.4 g) fish (n=5 fish per dose, in triplicate) were immersed in one of the five aerated bacterial suspensions for 1 hour, whereas 15 negative control fish (n=5 fish in triplicate) were immersed in an identical volume of tank water combined with 0.65% sterile saline (i.e., mock challenge). Immediately following immersion exposure to *A. salmonicida* subsp. *salmonicida*, fish were returned to respective aquaria (n=5 fish per tank).

Challenged and mock-challenged juvenile lake whitefish were monitored, fed, and cared for as described above at least twice daily, and the experiment was continued until no mortality occurred for 8 days (i.e., 33 days). Dead fish were subjected to thorough clinical examinations. Length and weight were measured for each individual fish. Following external examination, external surfaces of fish were disinfected with 70% ethanol and the coelomic cavity was opened with sterile scissors in a laminar flow hood and individual organs were examined. For bacteriological analyses, and using a sterile disposable 1 µl loop, individual kidney tissue, as well as tissues from any external skin lesion(s), were inoculated directly onto TSA and incubated at 22°C for 72 hours. Any terminally moribund (e.g., fish in dorsal or lateral recumbency and/or

apparent distress) and fish surviving to 33 days post-challenge were euthanized via 250 mg/L of MS – 222 (Tricaine methanesulfonate; Syndel, Ferndale, WA) buffered with 500 mg/L of sodium bicarbonate (Millipore Sigma, St. Louis, MO), clinically examined, and bacteriologically analyzed as above.

3.5. Identity confirmation of recovered bacterial isolates

Representative bacterial isolates that were recovered from juvenile lake whitefish at each of the challenge doses were sub-cultured, processed for nucleic acid extraction (Qiagen DNeasy Tissue Extraction kit; QIAGEN Sciences, Valencia, CA), and analyzed via the *A. salmonicida* subsp. *salmonicida*-specific endpoint PCR assay of Miyata et al. (1996) as described previously (Loch and Faisal 2010). The presence of a 512 base pair (bp) amplicon was considered confirmatory for *A. salmonicida* subsp. *salmonicida* (Miyata et al. 1996).

Viral Hemorrhagic Septicemia Virus

3.6. Cell culture and virus preparation for experimental challenges

Epithelioma papulosum cyprini (EPC; Fijan et al. 1983) cells were seeded into 35 tissue culture flasks with growth media comprised of minimum essential media (MEM; Invitrogen) supplemented with 29.2 mg/ml^{-1} l-glutamine (Invitrogen), penicillin (100 IU ml^{-1}) and streptomycin (0.1 mg/ml^{-1}; Invitrogen), 10% fetal bovine serum (Thermo Scientific), and sodium bicarbonate (7.5% weight per volume; Sigma-Aldrich). Flasks were incubated at 25°C for 48 hours until EPC cells were confluent. The Great Lakes VHSV-IVb index strain (MI03), originally isolated from wild muskellunge (*Esox masquinongy*; Elsayed et al. 2006), was revived from cryostock as follows. Cellular growth media was removed from all EPC flasks after which

virus (1 ml) and viral maintenance media (2 mls; as described above, but with 2% fetal bovine serum), were inoculated onto 35 flasks and left for 20 minutes at room temperature before adding the additional 7 mls of maintenance media, after which the infected flasks were incubated at 15°C. Once the VHSV-IVb isolate generated ~90% cell lysis of the monolayer (at 4 days post-inoculation), the virus stock was harvested via centrifugation at 5,000 rpm for 15 minutes (4°C) to remove the cells from the virus stock. A total of 350 mls of supernatant was collected into a sterile 1,500 ml glass bottle – this became the virus stock used for experimental challenges.

3.7. Viral quantification

The concentration of the VHSV-IVb stock for experimental challenges was determined via plaque assay on the EPC cell line as described previously (Batts and Winton 1989, Batts et al. 1991, Kim and Faisal 2010). Briefly, EPC cells were grown and seeded into 24-well plates and incubated at 25°C for 48 hours. Next, 10-fold serial dilutions were made by combining 0.1 ml of virus stock with 0.9 ml of maintenance media liquid, vortexing, and repeating this process. Once the serial dilutions were prepared, the growth media was removed from the 24-well plates and the cells were treated with 0.2 ml of polyethylene glycol (PEG) per well and incubated for 10 minutes at room temperature, then 0.1 ml of each virus dilution was inoculated onto PEG-treated cells in duplicate and allowed to absorb onto cells for 20 minutes. Next, each well was overlaid with 1 ml of methylcellulose. Plates were incubated at 15°C for 6 days to allow for plaque formation. Following incubation, the plates were stained using a crystal violet/formaldehyde solution and allowed to air dry for 1 hour before being read. The plaques were counted and the virus concentration was determined to be 1.4×10^6 plaque forming units (PFU/ml virus concentration in the stock).

3.8. Viral Hemorrhagic Septicemia Virus median lethal dose experiments

Five experimental challenges doses, corresponding to 1.4 – $1.4x10^4$ PFU/ml immersion water, were prepared by diluting the VHSV-IVb stock suspension (30 mls; Section 3.7) with 270 mls of maintenance media and tank 2,700 tank water. Seventy-five juvenile fish (n=5 fish per dose in triplicate) were immersed in one of the five aerated VHSV-IVb suspensions (e.g., 1.4, $1.4x10^1$, $1.4x10^2$, $1.4x10^3$, or $1.4x10^4$ PFU/ml immersion water) for 1 hour, whereas 15 negative control fish (n=5 fish in triplicate) were immersed in a volume of 300 ml of maintenance media added to 2,700 mls of water. Immediately following immersion exposure to VHSV-IVb, fish were returned to respective aquaria (n=5 fish per tank).

Challenged and mock-challenged juvenile lake whitefish were monitored, fed, and cared for as described in Section 3.1, and the experiment was conducted for 28 days. Dead fish were subjected to thorough clinical examinations (Section 3.4). Morphometric data (e.g. length and weight) of individual fish was recorded. For virological analyses, kidney, spleen, and heart tissue were aseptically collected from individual fish, homogenized together, and processed for virus isolation (Section 3.9). Terminally moribund and fish surviving the duration of the experiment (i.e., 28 days) were euthanized via MS-222 overdose, after which a necropsy was performed as detailed above.

3.9. Virus re-isolation and molecular confirmation

Kidney/spleen/heart tissues were diluted 1:4 (weight per volume) with Earle's salt-based minimal essential medium (Invitrogen, Thermo Scientific), supplemented with 12 mM Tris buffer (Sigma-Aldrich, St. Louis, MO), penicillin (100 IU/mL; Invitrogen), streptomycin (100

µg; Invitrogen), and amphotericin B (250 µg/mL; Invitrogen). Tissues and diluent were homogenized, vortexed, and centrifuged at 5,000 rpm for 30 minutes at 4°C, after which 300 µl of supernatant was collected, transferred to a new sterile tube, and incubated at either 15°C for 2 hours or 4°C overnight. Following incubation, the supernatant was centrifuged at 5,000 rpm for 15 minutes at 4°C and was immediately inoculated onto EPC cells. Inoculated cells were incubated at 15°C and examined for cytopathic effect (CPE) for up to 28 days, with a blind pass onto fresh cells occurring at 14 days post-infection for samples showing no evidence of CPE. Samples displaying CPE had cells and supernatant harvested and cryopreserved at -80°C for virus confirmation via molecular analyses. If no CPE was observed after 28 days post-inoculation onto EPC cells, samples were deemed negative for VHSV-IVb.

The identity of representative virus isolates recovered from experimentally challenged LWF within each challenge dose was confirmed via the VHSV-specific quantitative real time polymerase chain reaction (qRT-PCR) of Jonstrup et al. (2013). In brief, kidney, spleen, and heart supernatant (see Section 3.9) were processed for nucleic acid extraction (MagMax™ Total Nucleic Acid Isolation Kit; Thermo Scientific) following manufacturer's protocols and assayed as previously described (Standish and Faisal 2017). Samples yielding a C_t value of < 35 were considered positive for VHSV IVb (Jonstrup et al. 2013).

3.10. Median lethal dose

The median lethal dose (LD_{50}) of *A. salmonicida* subsp. *salmonicida* and VHSV-IVb were estimated using the method described by Reed and Muench (1938).

4. Results

Aeromonas salmonicida subsp. *salmonicida*

Mortality in *A. salmonicida* subsp. *salmonicida* challenged juvenile lake whitefish (mean length of 8.4 cm, mean weight of 4.4 g) began on day five post infection (pi) and continued until day 25 (Figure 13). By dose, lake whitefish immersed in the 10^5 CFU/ml (i.e., HD*) A. salmonicida* subsp. *salmonicida* suspension experienced the highest cumulative percent mortality (e.g., 53.3%), whereby mortality occurred between days 5 -14 pi. In the 10^4 and 10^3 challenge doses, mortality reached 13.3% and 6.6%, respectively, between days 17 and 25 pi (Figure 13). No mortality occurred in the 10^2 or 10^1 doses, nor in any mock-challenged lake whitefish throughout the experiment. Under the tested laboratory conditions, the LD_{50} of *A. salmonicida* subsp. *salmonicida* in juvenile lake whitefish was estimated to be $3.6x10^5$ CFU/ml.

A range of external gross disease signs were observed in terminally-moribund and dead *A. salmonicida* subsp. *salmonicida*-challenged fish, including focal to multi-focal petechial hemorrhage in the lateral line, severe gill pallor, bilateral exophthalmia (Figure 12A), and hemorrhagic ulcerations on various areas of the trunk that ranged from shallow and multifocal (current Figure 12B) to deep and focal (current Figure 12C) to focally extensive and cavitating (current Figure 12D). Internally, multifocal petechial hemorrhage within the swim bladder and severe pallor within heart, liver, kidney, and spleen were also noted. No disease signs were observed in mock-challenged fish.

Bacterial cultures taken from the kidneys and external ulcerations of all terminally moribund and dead *A. salmonicida* subsp. *salmonicida* challenged lake whitefish (n=11) yielded pure

profuse bacterial growth consisting of convex, smooth, opaque colonies with entire margins and that produced a brown-diffusible pigment after incubation at 22°C for 48 hours on TSA (Figure 14). Bacterial infection intensities were always high (>300 CFU, i.e., too numerous to count; Figure 14). In the remaining lower doses (10^1 – 10^2 CFU/ml) and the mock-challenged fish, no bacteria were recovered on kidney cultures that were collected at the end (i.e., 33 days pi) of the experiment.

Using the *A. salmonicida* subsp. *salmonicida*-specific endpoint PCR assay of Miyata (1996), all representative bacterial isolates that were recovered from lake whitefish within each of the challenge doses yielded amplicons of 512 bp, confirming their identity as *A. salmonicida* subsp. *salmonicida.*

Viral Hemorrhagic Septicemia Virus

Lake whitefish (mean length of 8.5 cm, mean weight of 4.6 g) mortality occurred in all treatment groups that were immersed in VHSV-IVb, with the first mortalities occurring from day 3 to day 5 pi and the last occurring on day 12 pi (Figure 16). By dose, lake whitefish immersed in the 10^4 PFU/ml (i.e., HD) VHSV-IVb suspension experienced the highest cumulative percent mortality (e.g., 93.3%), whereby mortality occurred between days 2 -10 pi. In the 10^2 and 10^3 challenge doses, mortality reached 60.0% and 73.3%, respectively, by days 11 and 12 pi (Figure 16). A cumulative percent mortality of 6.6% occurred in the two lowest immersion concentrations; however, no mortality occurred in any mock-challenged lake whitefish throughout the experiment. Under the tested laboratory conditions, the LD_{50} of VHSV-IVb in juvenile lake whitefish was estimated to be $5.3x10^1$ PFU/ml.

Terminally moribund and dead fish resulting from exposure to the highest concentration of VHSV-IVb MI03 showed severe signs of disease that in most to all cases included focally extensive ecchymotic hemorrhage within the dorsal musculature (Figure 15A), severe gill pallor (Figure 15B), visceral pallor, and/or severe hemorrhage within and surrounding the kidneys (Figure 15C). In just under half these fish, marked bilateral exophthalmia was observed and in one fish, severe ecchymotic hemorrhage of the swimbladder was noted (Figure 15D). Terminally moribund and dead fish in the 10^3 PFU/ml water challenge dose displayed many of the same disease signs noted above, with the exception that almost half of the fish had severe swim bladder hemorrhage. Fish in the lower dose tanks (1.4 – 10^2 PFU/ml of water) also displayed similar gross disease signs. Disease signs were not observed in surviving fish or any of the mock-challenged fish.

For virus re-isolation, kidney/spleen/heart homogenates from all juvenile lake whitefish (e.g., dead/terminally moribund and survivors) were inoculated onto EPC cells at 15°C. Within 6 – 14 days of incubation, all samples inoculated with tissue homogenates that originated from dead and terminally moribund lake whitefish developed CPE in the form of significant cell lysis (Figure 17). However, no CPE was noted in any samples originating from fish that were euthanized at 28 days pi, including all negative control fish. All representative samples showing CPE from the five VHSV treatment doses were tested using the VHSV-specific qRT-PCR assay of Jonstrup et al. (2013), which yielded Ct values of < 35, thus confirming the presence of VHSV in each sample.

5. Discussion

Despite a range of infectious microbial fish pathogens, including *A. salmonicida* subsp. *salmonicida* and VHSV-IVb, having been detected in adult lake whitefish that concurrently showed gross and/or microscopic disease signs (reviewed in Loch and Faisal 2011, Thompson et al. 2011, Faisal et al. 2012), the effects such infections have on the health and survival of this economically and ecologically invaluable fish species, especially during their early life stages, was heretofore unknown. Herein and for the first time, I show that < 1 year old Great Lakes lake whitefish are not only highly susceptible to VHSV-IVb and *A. salmonicida* subsp. *salmonicida* infection upon immersion exposure, but also suffer from severe disease and in some cases, rapid and substantial subsequent mortality. In fact, the estimated median lethal dose of VHSV-IVb in juvenile lake whitefish of $5.3x10^1$ PFU/ml places this species among the most, if not the most susceptible Great Lakes fish species for which LD_{50} data is currently available. For example, within the Family Salmonidae, Weeks et al. (2011) estimated the LD_{50} of VHSV-IVb MI03 to juvenile lake herring (*Coregonus artedi*), a congener of lake whitefish that is considered highly susceptible to Viral Hemorrhagic Septicemia Virus, to be > 7-fold higher (e.g., $3.85x10^2$ PFU/ml) than that which was estimated for juvenile lake whitefish in the current study. Likewise, the estimated LD_{50} for other salmonids currently present in the Great Lakes basin tested to date exceeds $1.4 x10^6$ PFU/ml (Kim and Faisal 2010), suggesting that lake whitefish may be amongst the most VHSV IVb-susceptible salmonid species within the Great Lakes. Indeed, VHSV-IVb-infected juvenile lake whitefish showed severe signs of disease that are comparable to those found previously in other *Coregonus* sp. under controlled laboratory conditions (Meier et al. 1986, Weeks et al. 2011), such as subdermal hemorrhage on the dorsum and bilateral muscle, and hemorrhage within the swimbladder and muscle surrounding the

kidney. Likewise, similar signs of disease have been noted from wild adult lake whitefish infected with VHSV-IVb (Loch and Faisal 2011).

In a similar context but to a lesser extent, juvenile lake whitefish were determined to be highly susceptible to infection, as well as subsequent disease and mortality, following exposure to *Aeromonas salmonicida* subsp. *salmonicida*. Compared to other salmonids, lake whitefish appear slightly more susceptible to furunculosis than sockeye salmon (*Oncorhynchus nerka*; LD_{50} of $5x10^5$ CFU/ml; McCarthy 1983), but less susceptible than rainbow trout (*O. mykiss*; $9.5x10^4$ CFU/ml) and Atlantic salmon (*S. salar*; $1.8x10^3$ CFU/ml; Adams et al. 1987), both of which are recognized as being the most susceptible to this disease (Cipriano and Bullock 2001). Of note, the disease signs of juvenile lake whitefish infected with *A. salmonicida* subsp. *salmonicida* were comparable to observed signs of disease in adult Great Lakes lake whitefish, including hemorrhage and ulceration (Loch and Faisal 2010), possibly suggesting that adult lake whitefish could also suffer mortality as a result of furunculosis.

Given that the experiments in the current study were conducted under controlled laboratory conditions, the current study does not conclusively prove that such substantial disease and mortality would result from *A. salmonicida* subsp. *salmonicida* and/or Viral Hemorrhagic Septicemia infections in wild populations of juvenile lake whitefish. Nevertheless, the relatively low LD_{50} for both pathogens is below the concentrations that are known to be shed by infected fish (Rose et al. 1989, Kim and Faisal 2012). Likewise, both microbial pathogens can be shed with the reproductive fluids in other fish species (Cipriano et al. 2001, OIE 2021). As evidence shows these microbial pathogens have been detected in wild adult Great Lakes lake whitefish

populations previously (Loch and Faisal 2010, Thompson et al. 2011, Loch and Faisal 2011), does this mean that these infections could potentially be transmitted to juvenile lake whitefish from infected parents? This remains unknown, but now that there is evidence that juvenile lake whitefish are highly susceptible to variants of *A. salmonicida* subsp. *salmonicida* and VHSV-IVb that were originally recovered from within the Great Lakes basin (Elsayed et al. 2006, Loch and Faisal 2010) concurrent with ongoing depressed early life stage recruitment (reviewed in Ebener et al. 2021) including this matter warrants further investigations. In this vein, whether the lack of *A. salmonicida* subsp. *salmonicida* and Viral Hemorrhagic Septicemia infections in the wild juvenile lake whitefish that were analyzed during my field studies (see chapters 2 and 3) is due to these pathogens not being present in these juvenile populations or whether these pathogens cause infected fish to die and thus be "unavailable" for collection is intriguing. Clearly, the role that these and possibly other microbial fish pathogens may be playing in the continued recruitment declines of lake whitefish in the four lower Great Lakes requires additional study.

APPENDIX

Figure 12. Gross signs of disease that were immersion-challenged with *A. salmonicida* subsp. *salmonicida* and confirmed to be systemically infected with the bacterium. (A) Marked bilateral exophthalmia. (B) Shallow multifocal hemorrhagic ulceration on the left lateral aspect. (C) Focal hemorrhagic ulceration penetrating into the underlying muscle on the dorsal aspect of an *A. salmonicida* subsp. *salmonicida* infected fish from which the bacterium was recovered. (D) Deep cavitating and focally extensive ulceration on the left lateral aspect of an *A. salmonicida* subsp. *salmonicida* infected fish from which the bacterium was recovered.

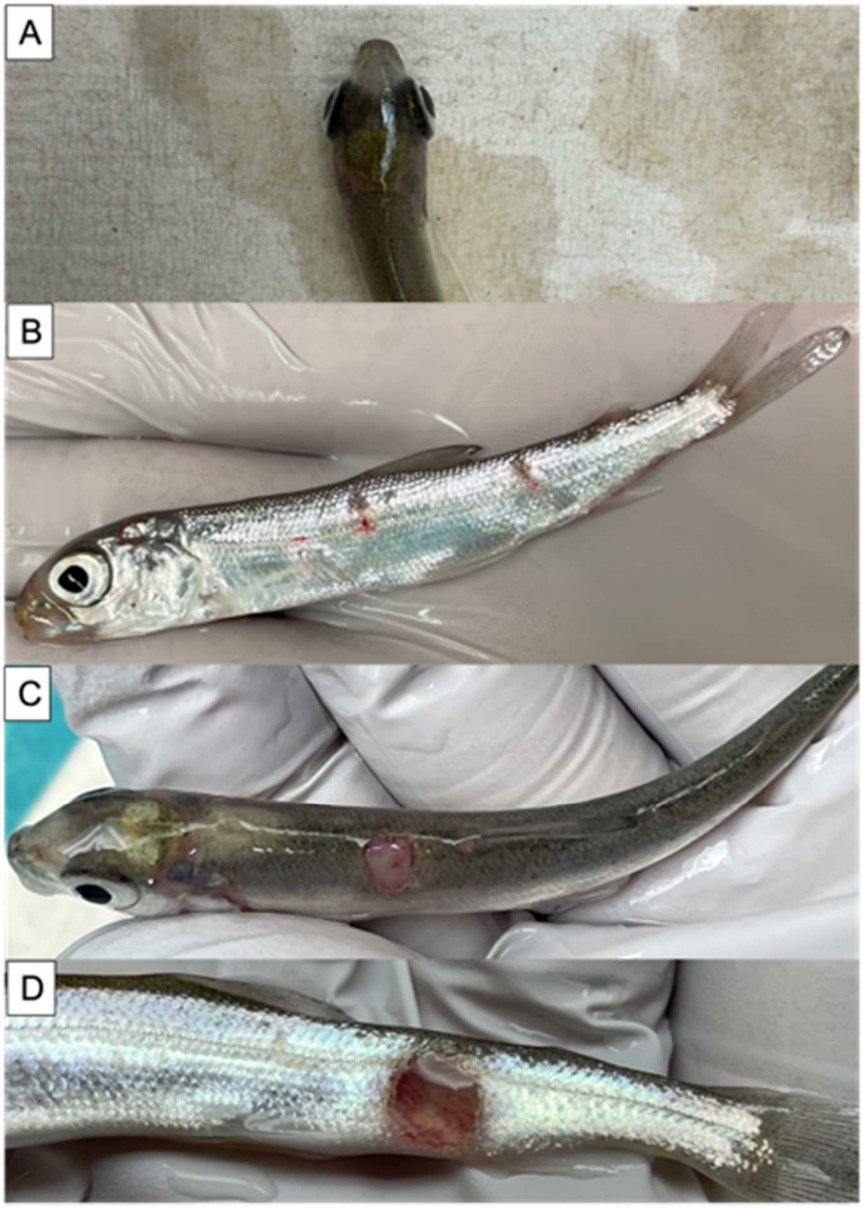


Figure 13. Mean cumulative percent mortality of juvenile lake whitefish following immersion challenge with *Aeromonas salmonicida* subsp. *salmonicida*. Standard error bars are shown.

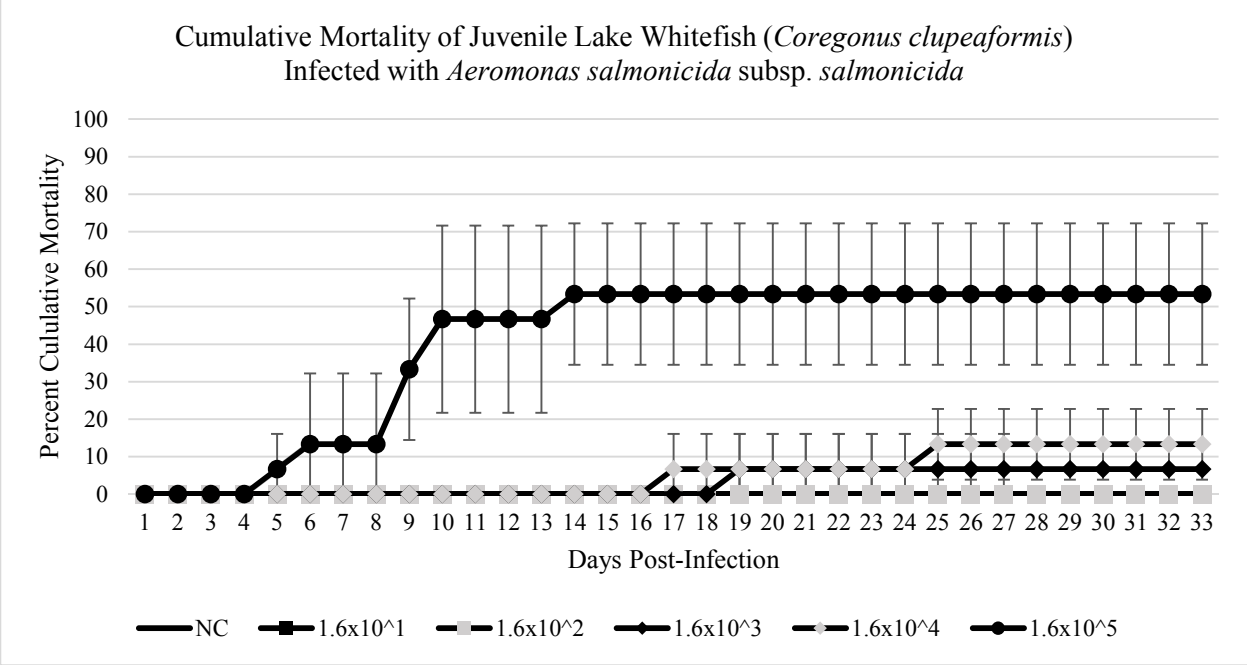


Figure 14. Primary culture on TSA of *Aeromonas salmonicida* subsp. *salmonicida* isolated from kidney culture (1 µl) of juvenile lake whitefish. Note the convex, smooth with entire margins, opaque morphologies that produced a brown pigment after incubation at 22°C for 48 hours.

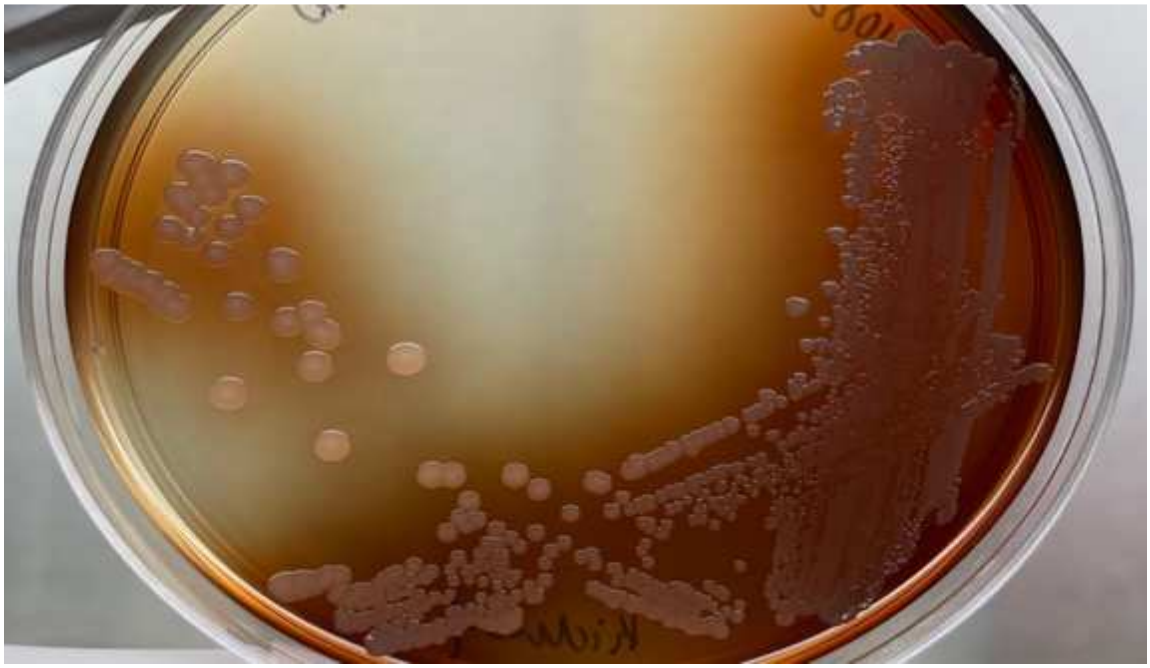


Figure 15. Gross signs of disease in juvenile lake whitefish that were immersion-challenged with VHSV-IVb MI03 and confirmed to be systemically infected with the virus. (A) Marked bilateral exophthalmia and severe focally extensive ecchymotic hemorrhage within the dorsal musculature. (B) Severe gill pallor. (C) Severe renal and peri-renal hemorrhage. (D) Severe ecchymotic hemorrhage of the swimbladder.

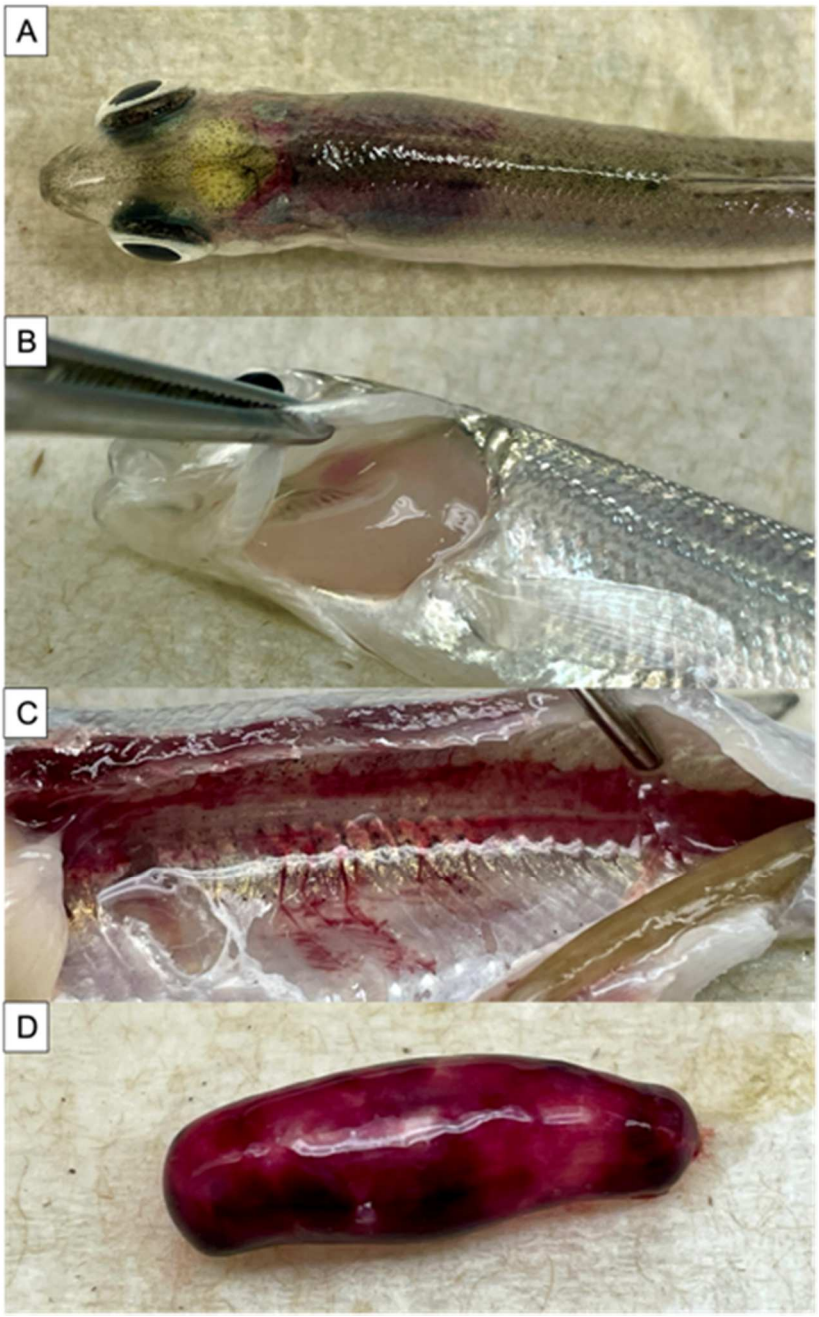


Figure 16. Mean cumulative percent mortality of juvenile Great Lakes lake whitefish over a period of 28 days following immersion challenge with Viral Hemorrhagic Septicemia Virus. Standard error bars are shown.

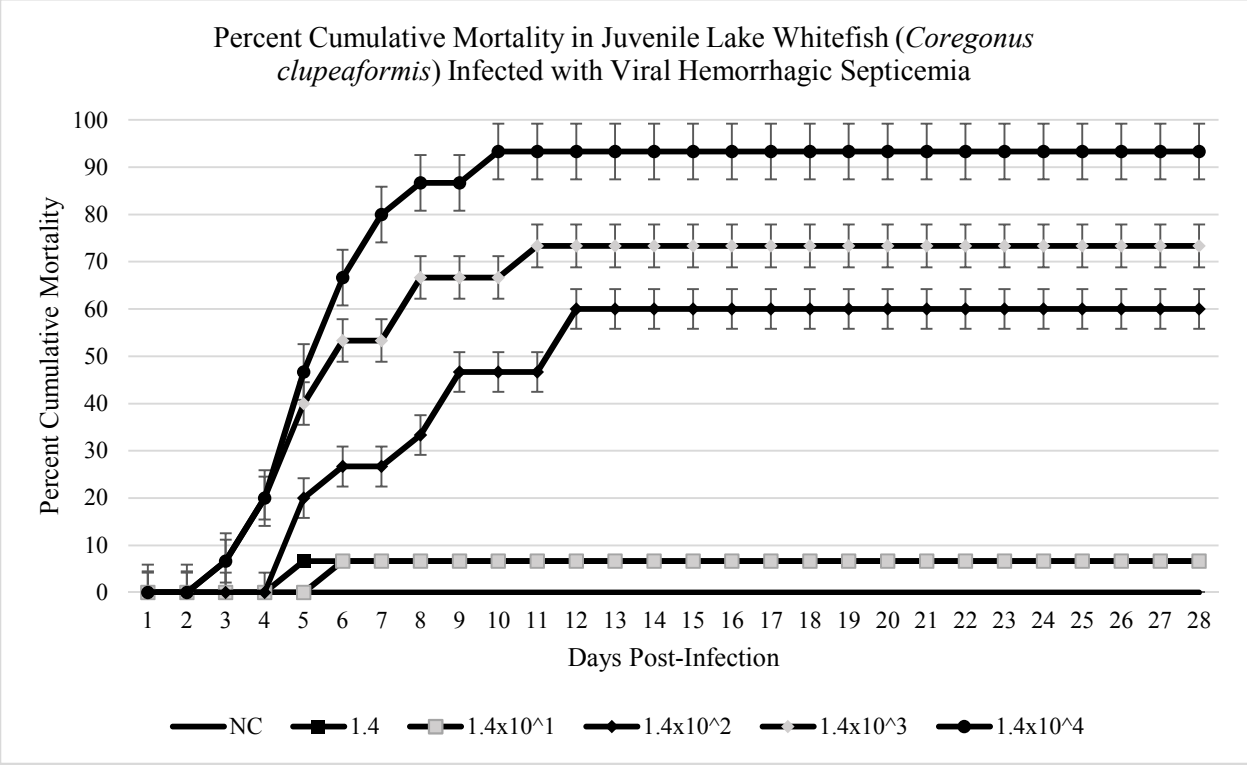
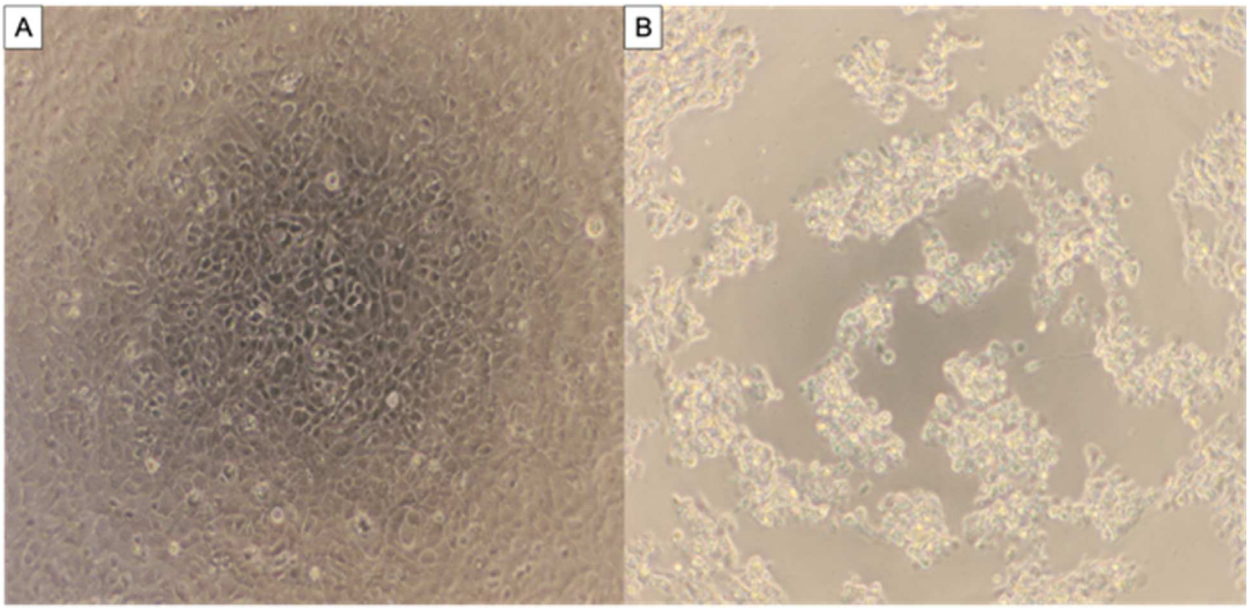


Figure 17. Cytopathic effect on Epithelioma papulosum cyprini (EPC; Fijan et al. 1983) cell line. (A) Negative control cells. (B) Cell lysis following incubation at 15°C for 36 hours.



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Chapter 5:

Conclusions and Future Research

1. Conclusions

Prior to these studies, the potential risks and negative health effects that infectious diseases pose to juvenile Great Lakes lake whitefish were all but unknown, a matter of concern given continued recruitment declines. Several infectious diseases have been detected in adult Great Lakes lake whitefish previously, though whether those same infections were present within the reproductive tissues of adults was completely unknown. This is matter of importance given that several pathogens detected in adult lake whitefish can either be vertically transmitted with the reproductive fluids and/or within the eggs. Moreover, the effects these infectious diseases have on the health and survival of juvenile lake whitefish was completely unknown.

In chapter 2, I describe the isolation of *Flavobacterium psychrophilum,* causative agent of bacterial coldwater disease (BCWD), from wild adult Great Lakes lake whitefish collected from Lakes Superior and Michigan, marking the first time this serious bacterial pathogen has been recovered from *Coregonus clupeaformis* within the Great Lakes basin. Moreover, fish infected with *F. psychrophilum* showed signs of systemic disease, highlighting the possibility that this bacterium could be associated with disease and/or mortality in adults. Despite the detection in adults, *Flavobacterium psychrophilum* was not detected in any wild juvenile lake whitefish examined in this study. Whether this is an indicator of the bacterium's absence in wild juvenile Great Lakes lake whitefish or its presence and ability to induce mortality such that juvenile fish may not be around to be collected, remains unknown. Of note, in depth molecular characterization of *F. psychrophilum* recovered from Great Lakes lake whitefish revealed they comprise novel MLST genotypes, thus further elucidating the diversity of *F. psychrophilum*

within the Great Lakes basin comprise of variants that may have a predilection for lake whitefish.

In chapter 3, and again for the first time, *Carnobacterium maltaromaticum* was recovered from the reproductive tissues of grossly diseased adult Great Lakes lake whitefish collected in Lakes Superior, Michigan, and Huron, which is significant because the bacterium has an affinity to reproductive tissues in other salmonids. Interestingly, when the prevalence of these infections was compared in Lakes Michigan and Huron, *C. maltaromaticum* infections were almost always higher in sites associated with poor recruitment than in sites with historically good recruitment, suggesting that *C. maltaromaticum* could potentially be a factor in declining recruitment in those sites. *Carnobacterium maltaromaticum* was not recovered from any of the 436 wild juvenile lake whitefish examined in this study. However, juvenile lake whitefish being reared for *in vivo* challenge experiments suddenly developed clinical signs of disease and following virological and bacteriological analyses to uncover the cause of this outbreak, I discovered the presence of *C. maltaromaticum* infections within the kidney, eye, brain, gonads, and ascites of affected fish. Collectively, findings revealed systemic infections of *C. maltaromaticum* in juvenile fish, and the ability of *C. maltaromaticum* to induce disease and mortality in *C. clupeaformis*. Furthermore, following molecular analyses, adult and juvenile *C. maltaromaticum* isolates were positive for the presence of the gene encoding for the piscicolin-126 protein. Whether this means isolates recovered from the outbreak of *C. maltaromaticum* have a competitive advantage over other Gram-positive bacteria within the host or if it provides beneficial effects to the host in other situations remains undetermined.

In chapter 4, *Aeromonas salmonicida* subsp. *salmonicida* and Viral Hemorrhagic Septicemia Virus proved to be highly virulent to eight- and nine-month-old juvenile lake whitefish originating from the Great Lakes basin, inducing severe signs of disease and mortality. In fact, the LD_{50} of VHSV-IVb in juvenile lake whitefish of $5.3x10^1$ PFU/ml marks lake whitefish as the most susceptible salmonid species in the Great Lakes basin for which data exists to date. The LD_{50} of $3.6x10^5$ CFU/ml of *A. salmonicida* subsp. *salmonicida* in juvenile lake whitefish also makes this species highly susceptible to infections of the bacterium, meaning the risk of potential vertical transmission from infected adults is highly concerning. Many of the disease signs observed in juvenile lake whitefish throughout these studies were comparable to those observed in other salmonids, including adult lake whitefish, suffering from furunculosis and viral hemorrhagic septicemia, suggesting that lake whitefish in wild habitats may be suffering from these same disease signs observed in laboratory experiments.

Collectively, the findings of my thesis research highlight the potential negative impacts that infectious diseases may be having on the health and survival of juvenile Great Lakes lake whitefish, particularly *Carnobacterium maltaromaticum* and Viral Hemorrhagic Septicemia Virus. Mortality resulting from the natural outbreak of *C. maltaromaticum* in juvenile lake whitefish reared in the laboratory and *in vivo* challenges with Viral Hemorrhagic Septicemia Virus provide evidence that juvenile fish are extremely susceptible to infection and/or disease, thereby highlighting the potential to devastate juvenile lake whitefish populations. In this context, the combination of field and laboratory studies helped elucidate potential infection pathway from infected parent to offspring. The data generated from *in vivo* challenges may also serve as a resource for fishery managers by potentially addressing uncertainties in natural

mortality that can be used in future recruitment models that forecast lake whitefish recruitment to the Great Lakes.

2. Future research

My studies have provided a strong foundation upon which future research can be developed. In chapter 2, I collected isolates of *F. psychrophilum* from infected adult lake whitefish that can now be used to further investigate the potential host preference and virulence of this serious pathogen to juvenile lake whitefish. These isolates could also serve as a resource for BCWD prevention and control strategies, including vaccine development, should lake whitefish hatchery propagation become an emphasis. Additionally, isolates of *F. psychrophilum* recovered in this study can be used to determine the effects these isolates have on the health and survival of juvenile lake whitefish under controlled laboratory conditions, and its potential to be transmitted via the eggs of lake whitefish.

Findings from chapter 3 suggest that prevalence of *C. maltaromaticum* infections in adult lake whitefish populations may represent the "low end" of seasonal prevalence; thus, future field studies investigating microbial pathogen prevalence in adult lake whitefish should consider sampling over multiple seasons over multiple years. *Carnobacterium maltaromaticum* isolates recovered from lake whitefish may be used for future epidemiological investigations by comparing *C. maltaromaticum* strains throughout the Great Lakes, and how lake whitefish isolates relate to strains recovered from other fish species. Although controlled *in vivo* experiments of *C. maltaromaticum* were not conducted during this study, the unplanned outbreak in laboratory-reared juveniles provided an understanding of the effects this bacterium has on the

health of juvenile lake whitefish, such that future controlled *in vivo* experiments of *C. maltaromaticum* may further elucidate its median lethal dose along with shedding and transmission potential.

In chapter 4, *in vivo* studies of *A. salmonicida* subsp. *salmonicida* and Viral Hemorrhagic Septicemia Virus in juvenile lake whitefish provide a framework for future studies to assess the effects of other factors, such as water temperature and host density, may have on the pathogenicity of these infectious microbial pathogens. Additionally, *in vivo* shedding and transmission potential of both pathogens should be investigated, as should the histopathological changes that occur during Viral Hemorrhagic Septicemia Virus and *A. salmonicida* subsp. *salmonicida* infections

During field studies and collection of wild juvenile lake whitefish, I was not able to collect fish from every site nor at the target numbers. Moreover, seining for juvenile lake whitefish occurred during a short time frame (e.g. mid-June – early July), and seining happened between a brief time of day (e.g., between 10 am – 1 pm). In this context, future surveillance of juvenile lake whitefish should extend over a broad time frame, perhaps starting in May and lasting till the end of July. Additionally, future seining should be conducted either later in the evening or even during nighttime when water temperatures are cooler. Moreover, future surveillance should record differences in abundance of juvenile lake whitefish collected and note any differences between the time of day and differences between each month. Likewise, disease investigations and pathogen testing should be paired with ongoing annual surveillance to better understand the

infectious diseases that may be circulating within juvenile Great Lakes lake whitefish populations.