AN EXPLORATION OF VIRAL DISEASES AFFECTING GREAT LAKES LAKE STURGEON (*ACIPENSER FULVESCENS*)

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ABSTRACT

AN EXPLORATION OF VIRAL DISEASES AFFECTING GREAT LAKES LAKE STURGEON (*ACIPENSER FULVESCENS*)

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The lake sturgeon (*Acipenser fulvescens*; LST) is the only sturgeon species in the Great Lakes (GL), where populations are estimated to be <1% of historical abundances. Resultantly, substantial resources are being devoted to the hatchery propagation of LST. Concurrently, more is being uncovered regarding viral diseases that affect sturgeons (e.g. Frog virus 3 [FV3], Nucleocytoplasmic Large DNA viruses [NCLDVs], Acipenserid herpesviruses). To investigate the presence of these viruses, I collected wild adult and hatchery reared juvenile GL-LST (Lake Erie, Huron, Michigan, and Superior watersheds) and utilized specialized/specific molecular and *in vitro* diagnostics. Although FV3 and NCLDVs were not detected in sampled GL-LST, I successfully detected and, for the first time, isolated a herpesvirus (Family *Alloherpesviridae*) from adult GL-LST that, based on genomic analyses, appears distinct from known alloherpesviruses. To assess virulence of this virus (proposed name of lake sturgeon herpesvirus 2, LSHV-2), juvenile GL-LST were immersed in a viral suspension. Within 10 days, disease signs developed in virus-exposed LST, soon progressing to mortality (33% cumulative mortality), where LSHV-2 was re-isolated from representative skin lesions. Next, the efficacy of three disinfectants (Perox-Aid®, Virkon™-Aquatic, and Ovadine®) against LSHV-2 was assessed *in vitro*, where all three substantially decreased viral titers to varying degrees, marking promising means of prevention/control. Whether LSHV-2 has or is contributing to reduced LST abundance in the GL remains to be determined, but my findings provide a foundation to further study and combat this newly uncovered sturgeon-pathogenic virus.

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INTRODUCTION

The lake sturgeon (*Acipenser fulvescens*) is the only native sturgeon species in the Great Lakes, representing the largest and longest-lived fish therein (Scott et al. 1973). Unfortunately, lake sturgeon populations have been estimated to be less than 1% of historical abundances (Hayes et al. 2012). A multitude of factors are believed to have contributed to these declines (Pollock et al. 2015). Although overfishing in the 19th century contributed to lake sturgeon population crashes, other anthropogenic factors, including habitat degradation and dam construction also posed significant threats to lake sturgeon populations (Bajkov et al. 1930, Auer 1999).

Lake sturgeon exhibit several unique life history traits that have led to a mounted resilience to impediments such as poor spawning years and conditions (Crouse 1999, Peterson et al. 2007). Unfortunately, those same traits are now believed to have placed lake sturgeon at an increased risk when faced with a variety of anthropogenic challenges (Crouse 1999). Substantial efforts to restore and conserve lake sturgeon populations and habitats in the Great Lakes have been undertaken, including the captive propagation of lake sturgeon for conservation purposes in hatcheries and streamside rearing facilities. Unfortunately, relatively little is known about the infectious diseases that affect lake sturgeon in wild and hatchery environments. This dearth of knowledge is even more concerning in the face of sizable mortality events that can occur in hatchery-reared LST stocks (up to 92% cumulative percent mortality; GLRI Progress Report and Dr. Ed. Baker, Research Station Manager, MI Dept. of Natural Resources, Pers. Comm.).

While this is happening in the Great Lakes, sturgeon farming has globally expanded, and with it, a growing understanding of the negative consequences of sturgeon pathogens. Some pathogens, specifically viruses, affecting other sturgeon species also appear to be increasing in host and geographic range. For example, Frog Virus 3 (FV3) was recently documented for the

first time in another sturgeon species (*Scaphirhynchus albus*; Waltzek et al. 2014). Additionally, Nucleocytoplasmic Large DNA Viruses (Order Megavirales) have been documented in sturgeons in watersheds surrounding the basin, including lake sturgeon in Manitoba, Canada (USA, Clouthier et al. 2015). Furthermore, viruses in the family *Alloherpesviridae* have been linked to disease and mortality in other sturgeons for decades, including wild and cultured white sturgeon (*A. transmontanus*) in North America (Hedrick et al. 1991, Watson et al. 1995).

Given detection of the pathogens mentioned above often requires specialized and specific diagnostic tests, little is known about their possible presence in the Great Lakes. Herein, I address some critical unknowns surrounding these pathogens: Are they present in the Great Lakes? And if they are, what role might they play in long-standing lake sturgeon population declines or mortality events in hatcheries? Lastly, how can these pathogens be effectively controlled and managed in adult populations? By beginning to address these critical unknowns, we will not only get the first glimpse into the presence of sturgeon pathogenic viruses in the Great Lakes, but also are paving the way for future research on the infectious disease affecting Great Lakes lake sturgeon populations.

1. Study Objectives

This study focuses on viral infections affecting lake sturgeon in the Great Lakes basin of North America. Despite being an iconic fish species of special management concern, little is known regarding the viruses that may be negatively affecting Great Lakes lake sturgeon populations. Furthermore, given the role wild adult lake sturgeon play in the populating of streamside rearing facilities across the basin, understanding the potential pathogens affecting them provides critical information that could be used to mitigate hatchery losses.

One of the initial objectives of this study was to conduct a large-scale lake sturgeon disease surveillance study. Specialized molecular, virological, hematological, and histopathological techniques were used to uncover the presence of sturgeon viruses of potential concern. Although the primary focus of this research is viral diseases of Great Lakes lake sturgeon, a thorough literature review of the parasites and microbial pathogens of sturgeon was conducted. Chapter One of this thesis first details the history of lake sturgeon and provides context as to why infectious disease surveillance is needed before delving into viral, bacterial, fungal, and parasites found in sturgeon populations globally.

Chapter Two of this thesis describes the discovery of a novel alloherpesvirus (Family *Alloherpesviridae*) from Great Lakes lake sturgeon. Therein, I describe the first isolation of an alloherpesvirus from Great Lakes lake sturgeon and characterize the gross and microscopic disease signs in affected adult lake sturgeon. I then describe *in vivo* pathogen exposure experiments revealing that the newly isolated lake sturgeon herpesvirus is capable of inducing mortality in juvenile lake sturgeon (33.3%), a finding that could have implications for lake sturgeon conservation. Research conducted in Chapter Two paves the way for the research focus

of Chapter Four, which aims to arm hatchery personnel with efficacious prevention and control techniques in lake sturgeon rearing facilities.

Chapter Three details another objective of this thesis research, which was to uncover the presence of other sturgeon pathogenic viruses in adult and juvenile Great Lakes lake sturgeon. By employing specialized and specific diagnostic techniques, hundreds of tissue and fluid samples were screened from nearly 300 adult lake sturgeon and over 500 juvenile lake sturgeon were clinically examined and screened similarly.

Following the discovery and isolation of a novel lake sturgeon herpesvirus (Chapter Two), a priority was to determine virus sensitivity to routinely used hatchery disinfectants. In Chapter Four, I describe the susceptibility of the novel lake sturgeon herpesvirus to Nolvasan®, Virkon®-Aquatic, and Ovadine® and give recommendations founded in *in vitro* experiment results.

Lastly, Chapter Five details my overall conclusions and highlights possible avenues of future research.

CHAPTER 1:

LITERATURE REVIEW

1. Lake sturgeon in the Great Lakes basin of North America

Fishes within the Order Acipenseriformes (Phylum Chordata) evolved approximately 200-175 million years ago according to fossil evidence (Bemis, et al. 1997a), and are considered prehistoric fish giants by many. The two families of cartilaginous fishes that comprise this order include *Polyodontidae* (paddlefishes) and *Acipenseridae* (sturgeons). Within the Family *Acipenseridae*, there are four genera: *Huso*, *Pseudoscaphirhynchus*, *Scaphirhynchus*, and *Acipenser*. The lake sturgeon (*Acipenser fulvescens*) is native to North America and was once one of the most abundant North American fishes, where it's historical geographic range spans multiple river and lake basins, including the Hudson Bay, Mississippi River, Coosa River, and St. Lawrence-Great Lakes systems (Fuller, 2022). Native to the Great Lakes basin, the lake sturgeon is the only sturgeon species therein, where it is the largest (>250 lb.) and longest living (up to 150 years) fish (Scott et al. 1973; Hayes et al. 2012). Similar to other sturgeon globally, the lake sturgeon is a treasured fish in the Great Lakes basin, where it remains an iconic historically and culturally important species. Given their history in the Great Lakes and unique life history traits (see below), some view lake sturgeon as a barometer of environmental and ecological health of the basin (Boucher 2022). Furthermore, declines in their populations could be indicative of more substantial alterations in Great Lakes ecology and/or abiotic factors influencing native lake sturgeon populations (i.e., overfishing, dam construction, habitat degradation; Hayes et al. 2012, Hay-Chmielewski et al. 1997).

2. Lake sturgeon biology and life history

2.1. Lake sturgeon biology

The lake sturgeon occupies a unique niche within the Great Lakes basin. Far surpassing all other Great Lakes fish species, the lake sturgeon can reach weights >250 pounds (Scott et al. 1973). The lake sturgeon also possess a series of characteristics found in ancestral fishes including a heterocercal tail and modified ganoid scales, or scutes, in five rows along the dorsal, lateral, and ventral surfaces (Grande et al. 1991). Lake sturgeon also have a protrusible mouth, or oral siphon, used to feed (Harkness et al. 1961). Though dietary habits vary depending on the season, habitat, and food availability, lake sturgeon commonly feed on benthic macroinvertebrates, but will also feed upon fish larvae, fish eggs, small crustaceans, insects, and rarely, other fish (Harkness 1923, Harkness et al. 1961, Stelzer et al. 2008).

2.2. Life history

When compared to other Great Lakes fishes, lake sturgeon have an exceptionally long lifespan, and while there are some disparate conclusions regarding exact age ranges, it is unrefuted that lake sturgeon are very slow to sexually mature (Sulak et al. 2002, Pollock et al. 2015). Evidence suggests female lake sturgeon reach reproductive maturity between 24-26 years old, while males typically mature at 14-16 years (Priegel et al. 1974). The slow maturation rate exhibited by the lake sturgeon, in conjunction with increased energy being allocated to somatic growth throughout the early life stages, results in overall faster growth, thereby quickly lowering predation risk of young (Sunde 1961, Wilson 1987). Lake sturgeon also exhibit protracted spawning habits, with males typically spawning on an annual or biannual basis, and females

habitually spawning once every 3-6 years (Peterson et al. 2007). Notably, lake sturgeon fecundity is exceptionally high compared to many other North American fishes, where during any given spawning year, a female lake sturgeon can release 49,000-667,000 eggs (Harkness et al. 1961, Priegel et al. 1971). Lake sturgeon are broadcast spawners, and although predation remains a threat to their early life stage survival, the sheer volume of offspring often circumvents substantial effects from predators (Wilson et al. 1987). Collectively, these unique life history traits (e.g., delayed maturation, high fecundity, protracted spawning) are believed to convey mounted resilience to consecutive years of poor reproductive output when spawning conditions may be unfavorable (Crouse 1999, Peterson et al. 2007). However and more recently, some of these same characteristics have made the road to population recovery a challenge, as traits such as delayed maturation, and protracted spawning periodicity lead to very slow population growth (Crouse 1999).

2.3. Distinct populations and migratory habits

There are multiple distinct spawning lake sturgeon populations, where genetic diversity is high between them (DeHaan et al. 2006, Welsh et al. 2010). In the context of the Great Lakes basin, there is little spatial and genetic overlap between lake sturgeon populations between all and within individual Great Lakes (McQuown et al. 2003, DeHaan et al. 2006, Welsh et al. 2008). Although different genetically distinct populations throughout the Great Lakes basin exhibit similar spawning behaviors, they have high spawning-ground fidelity (Lyons et al. 1992, DeHaan et al. 2006). In addition to returning to streams, migratory behaviors such as date and breeding locations were found to remain highly consistent on an individual basis, independent of sex, size, and age (Forsythe et al. 2012). Although consistent within separate spawning

populations, some factors, such as migratory habits, vary greatly between separate spawning populations, whereby some migrate much longer distances (Threader et al. 1986, Auer et al. 2007) than others (Basset 1982).

3. Declines in Great Lakes lake sturgeon abundance

3.1. Historic range and abundance

Great Lakes lake sturgeon abundance was once high, with populations estimated to be in millions (Baldwin et al. 1979). In the mid-late 1800s, the lake sturgeon was a top commercial fish species by catch, ranking amongst the five most abundant species (Baldwin et al. 1979). Lake sturgeon catch numbers were highest in lake Erie throughout this period, followed by lake Michigan, Huron, Ontario, and Superior (Baldwin et al. 1979). Since then and due to multiple factors, lake sturgeon population have suffered substantial declines, where estimates suggest populations are <1% historical numbers (Hayes et al. 2012).

3.2. Overharvest of lake sturgeon

Lake sturgeon began to face serious anthropogenic threats in the mid 1800's, when other Great Lakes commercial fisheries (e.g., lake whitefish, *Coregonus clupeaformis* and lake trout *Salvelinus namaycush*) were proving bountiful (Ebener 1997, Wilberg et al. 2004) but lake sturgeon were deemed a nuisance by commercial fishermen due to fishing gear destruction attributed to their size and sharp, bony structure (Tody 1974). As a result, by-caught lake sturgeon were typically destroyed. Not long after being deemed a nuisance to commercial

fishermen, the demand for sturgeon eggs and meat surged and as a result, lake sturgeon harvest increased to unsustainable levels (Tody 1974).

3.3. Habitat degradation

Habitat destruction also poses significant threats to lake sturgeon populations and is believed by many to be the most relevant contemporary threat to Great Lakes lake sturgeon (Beamish et al. 1998, Pollock et al. 2015). The popularity of industries such as logging have subsequently led to an increase of sand and sediment as well as a decrease in large woody debris, concurrently with excessive harvest (Harkness et al. 1961). While sediment-heavy bottoms are generally found to be undesirable spawning habitats for sturgeons, the loss of woody debris also provides lake sturgeon with less cover, leaving them more susceptible to anthropogenic harassment or direct predation (Harkness et al. 1961).

3.4. Dam construction

The construction of physical barriers (e.g., dams, culverts, and lamprey barriers) have directly eliminated lake sturgeon spawning habitats, both by physical destruction and decreased connectivity. Physical barriers are widespread throughout the Great Lakes basin, with the majority of Michigan's large rivers being impacted (Hay-Chmielewski et al. 1997). Not only have physical barriers eliminated both habitat and access to habitat, they have also drastically impacted river dynamics, which may not directly affect lake sturgeon spawning habitat, but explicitly impacts the suitability of a river for lake sturgeon spawning (Hay-Chmielewski 1987).

4. Conservation efforts

In response to declining lake sturgeon abundance, their harvest in the Great Lakes became heavily regulated in the late 20th century (Hay-Chmielewski at al. 1997); however decades prior, it was proposed that fishing regulations were not enough to support restoration of lake sturgeon alone, rather a multifaceted habitat and population focused approach would be required (Bajkov et al. 1930). Although the effectiveness of conservation efforts have been met with varying successes throughout the lake sturgeon's native range, factors such as dam removal, habitat restoration, and implementation of stocking programs have led to clear population improvements (Caroffino et al. 2009, Dieterman et al. 2010, Chalupnicki et al. 2011, Roseman et al. 2011).

4.1. Hatchery-based lake sturgeon conservation in the Great Lakes

Substantial resources are also allocated to the hatchery propagation and rearing of lake sturgeon LST in the Great Lakes with the goal of not only enhancing remnant spawning populations, but also replenishing existing spawning populations (Hayes et al. 2012). To do so, gametes are collected from wild spawning adults and eggs artificially fertilized, or larval lake sturgeon are captured in the drift phase (Holtgren et al. 2007, Bauman et al. 2011, Crossman et al. 2014). Given fidelity of lake sturgeon to their native streams, rearing efforts were further optimized for lake sturgeon by the design of rearing units that can be run on water funneled directly from native streams or rivers, called streamside rearing facilities (SRF; Holtgren et al. 2007). These SRFs are most often populated via capture of larval sturgeon, where captured larvae are raised in rearing units with flow through river or stream water (Holtgren et al. 2007). Larval lake sturgeon are progeny of native adult populations, meaning there is little control over

what larvae are exposed to prior to rearing in an SRF. While there are many streamside rearing units in operation across the basin, there also are a few brick-and-mortar hatcheries such as the Black River Sturgeon Rearing Facility (Cheboygan County, Michigan), which also runs on stream water. While true measurement of success in regard to stocking programs may take decades to measure, stocking efforts have been broadly deployed around the Great Lakes (Hayes et al. 2012). Furthermore, no significant difference in size, movement, or habitat selection has been appreciated in captive reared fish when compared to wild of the same year class (Mann et al. 2011).

Although hatchery conservation of lake sturgeon is promising, many facilities sustain losses that have been linked to diseases caused by fish-pathogenic microbes. Unfortunately, little is known about the diseases of wild adult lake sturgeon. This is of concern, given that gametes and larvae collected from adult lake sturgeon populations in the Great Lakes are utilized to population rearing facilities. The population of rearing facilities using gametes or collecting wild hatched larvae create possible avenues for vertical transmission of potential pathogens from infected parents to progeny. Therefore, understanding pathogens within those populations is critical for our understanding of infectious diseases as an impediment to sturgeon conservation efforts.

5. Infectious disease in sturgeons

Demand for sturgeon products (e.g., caviar, meat) has increased, and given many natural stocks are imperil, sturgeon rearing for consumption has also increased (Bronzi et al. 2011, Bronzi et al. 2014). By the early 2000's, aquaculture-sourced caviar production began overtaking

caviar harvested from wild fisheries, and by the 2010's, aquaculture was the primary source for sturgeon caviar (Williot et al. 2001, Bronzi et al. 2011, Bronzi et al. 2014). Concurrent with the growth of sturgeon aquaculture, more knowledge surrounding infectious diseases affecting sturgeons has also emerged. For example, certain infectious diseases have led to near 95% losses in cultured white sturgeon (Watson et al. 1995, Georgiadis et al. 2001). The following sections of this review will focus on the infectious etiologies of disease in sturgeon in general, beginning with those that have been reported outside of North America, followed by those documented in North America, and finally those that have been identified in Great Lakes lake sturgeon specifically.

6. Infectious agents reported in sturgeon outside of North America

6.1. Parasites

6.1.1. Protozoa

Protozoan parasites have been detected in numerous sturgeon species globally, and make up a sizable portion of blood parasites recovered in sturgeons (Bauer et al. 2002). Some species have been described as specific to sturgeons including, *Trypanosoma anura*, which has been found in the blood of Atlantic sturgeon (*A. schrenckii*) in the Amur river (Levashov et al. 1962). Two species in the genus *Cryptobia* (Family *Kinetoplastida*) have also been detected in Beluga (*Huso huso*), Russian (*A. gueldenstaedtii*), starry (*A. stellatus*), ship (*A. nudiventris*), and sterlet (*A.* ruthenus) sturgeons, where *C. acipenseris* was recovered in all sturgeon species mentioned above. Additionally, *C. pseudoscaphirhynchus* has been detected in the River Amudaria (Aral sea Basin) in the blood of 50% of screened Amur Darya sturgeon (*Pseduoscaphirhynchus*

kaufmanni; Osmanov 1971). Similarly, a *Trichodina* sp. (Family *Trichodinidae*) was recovered from Russian sturgeon co-infected with Nodavirus and showing gross signs of disease (Athanassopoulou et al. 2004). Another *Trichodina* sp. has also been recovered at 100% prevalence in captive reared *A. oxyrinchus* (Atlantic sturgeon) in Poland exhibiting no gross signs of disease (Popielarczyk et al. 2013).

An array of ciliates have also been recovered from sturgeons including additional members of the Family *Trichodinidae* (*Trichodina, Trichodinella, Paratrichodina, Tripartiella, Dipartealla*) and few in the Family *Epistylididae* (*Apiosoma*; Baeur et al. 2002). For example, an *Apiosoma* sp. was recovered from Atlantic sturgeon in Poland but was not linked to gross signs of disease (Popielarczyk et al. 2013). Lastly, Protozoa in the Family *Pleistophoridae* have been found in prevalence as high as 100% across Russia and Europe affecting sterlet, starry, Siberian (*A. baerii*), Beluga, and Russian sturgeons (Skripchenko et al. 1971). Additional details on these parasites are further reviewed by Baeur et al. (2002).

6.1.2. Monogenea

Monogenean parasites have been recovered from multiple sturgeon species (Bauer et al. 2002). *Nitzschia sturionis* has been found in the gills in all sturgeon species in Europe and Russia (Bauer et al. 2002). This parasite is found in brackish and salt water, and has been linked to mortality in starry and ship sturgeon in the mid-20th century (Lutta 1937, 1941). Furthermore, two parasites in the Family *Diclybothriidae* (*Diclybothrium armatum, D. hamulatum*) have been detected ubiquitously across many the sturgeon species primarily inhabiting freshwater in Europe and Russia, though they were not linked to severe gross signs of disease (Bauer 1959).

Lastly and also in the Family *Diclybothriidae*, *Paradyclibothrium pacificum* was recovered from the gills of green sturgeon (*A. medirostris*; Reviewed in Bauer et al. 2002).

6.1.3. Cestoda

Cestoda (Cestodes; Phylum Platyhelminthes) are abundant and have wide host and geographic ranges. Reviewed in Bauer et al (2002), there are at least 20 different species of cestodes that have been reported in sturgeons across Russia and Europe. Although pathogenicity of many species remains to be determined, reports suggest *Amphilina foliacea* is associated with physiological and gonadal changes, likely negatively influencing reproductive success (Davydov et al. 1993).

6.1.4. Trematoda

Reports of Trematodes (Phylum: Platyhelminthes) are widespread throughout Russia and Europe, and ubiquitously affect sturgeon species therein (Reviewed in Bauer et al. 2002, Skrjabina 1974). Most trematodes affecting sturgeons globally are found in the gastrointestinal tract, and despite high prevalence, little is known regarding potential negative health effects (Reviewed in Bauer et al. 2002).

6.1.5. Nematoda

Nematodes are another group of parasites that are widely dispersed geographically and across different species globally (Bauer et al. 2002). At least 30 nematode species in various life stages have been recovered from sturgeons (reviewed in Bauer et al. 2002). While pathogenic effects of many detected nematodes aren't fully understood, studies suggest some nematodes are associated with inflammation of visceral organs (Ivanov et al. 1966).

6.1.6. Leeches

Leeches (Phylum Annelid) are another parasite affecting a diversity of fishes, where there are at least six species found present in Russian and European sturgeons (Bauer et al. 2002). Only one leech reviewed in Bauer et al (2002) appeared unique to sturgeons, *Acipenserobdella volgensis*.

6.1.7. Other parasites

Furthermore, a diversity of Coelenterata (*Polypodium hydriforme*), copepodids (family or some taxonomy), and Ancanthocephala (Thorny-headed worms) have also been recovered from sturgeons outside of North America (Bauer et al. 2002).

6.2. Fungi and water molds

Fungi (Kingdom Fungus) and water molds (Phylum Oomycota) in the Family *Saprolegniaceae* have also been linked to disease in a range of fishes globally, particularly in eggs and other early life stages (Meyers, 1991). In the context of sturgeons, several water molds in the family *Saprolegniaceae* were recovered in a study conducted with fertilized eggs from Persian sturgeon in Iran (Jalipoor et al. 2006). Jalipoor et al. (2006) found that water mold induced egg mortality, determined by gross examination of fungal-infested eggs. Additionally, *Saprolegnia* sp. were recovered from Russian sturgeon co-infected with Nodavirus (Family *Nodaviridae*; see below) in Greece (Athanassopoulou et al. 2004).

6.3. Bacteria

6.3.1. Bacterial pathogens in sturgeons

Bacterial pathogens in several families have been linked to extensive disease and mortality in multiple sturgeon species (Kayis et al. 2017).

6.3.2. *Flavobacteriaceae*

Bacteria in the family *Flavobacteriaceae* are Gram-negative rod-shaped bacteria, some of which are notorious for inducing disease and mortality in cultured fishes (reviewed in Loch et al. 2015). Of those causing disease is *Flavobacterium hydatis,* both of which was recovered from sturgeons showing gross signs of disease, colloquially referred to as “Bacterial hemorrhagic septicemia” (Timur et al. 2010). *F. columnare* has been shown to induce disease in Siberian

sturgeon (Brun et al. 1991), but not in Gulf sturgeon (*A. oxyrinchus*; Altinok et al. 2001). Additionally, an *F. johnsoniae*-like bacteria has been recovered from Russian sturgeon in Turkey (Karatas et al. 2010). Concerningly, the recovery of a *F. johnsoniae*-like bacteria was associated with severe gross signs of disease including lethargy, hemorrhagic ulcerations, and skin lesions associated with increased mucus (Karatas et al. 2010). *Flavobacterium* spp. have also been recovered as possible opportunistic pathogens in Russian sturgeons co-infected with Acipenserid iridovirus-European (AcIV-E; Family *Iridoviridae*) or Nodavirus (Family *Nodaviridae*; Athanassopoulou et al. 2004).

6.3.3. *Aeromonadaceae*

Aeromonas hydrophila has been recovered from sturgeons showing gross signs of disease colloquially referred to as "Bacterial hemorrhagic septicemia," similar to *F. hydatis* (Timur et al. 2010). Similarly, *A. hydrophila* has been recovered as possible secondary infections in AcIV-E or Nodavirus infected Russian sturgeons (Bigarre et al. 2016, Athanassopoulou et al. 2004). Also in the family *Aeromonas*, *A. sobria* has been recovered from Siberian sturgeon and *A. veronii* from Beluga sturgeon (*H. huso*) exhibiting gross signs of disease (Kayis et al. 2017, Ciulli et al. 2020).

6.3.4. *Mycobacteriaceae*

Bacteria in the genus *Mycobacterium* have also shown to be problematic in sturgeon culture. *M. chelonie*, was isolated from farmed Russian sturgeon exhibiting tumor-like skin lesions, *M. marinum* from hybrid sturgeon in Taiwan, and *M. salmoniphilum* from other diseased farmed

Russian sturgeon (Antoufermo et al. 2014; Chang et al. 2014; Righetti et al. 2014). A diversity of *Mycobacterium spp.* were also recovered and identified in Chinese and Atlantic sturgeons (*A. sinensis*; Zhang et al. 2015).

6.3.5. *Enterobacteriaceae*

Bacteria in the family *Enterobacteriaceae* have also been recovered from sturgeons showing gross signs of disease. For example, *Enterobacter* spp., *Serratia liquefaciens*, and *Hafnia alvei* have all been detected as possible secondary infections in sturgeon co-infected with Acipenserid iridovirus-European (AcIV-E; Ciulli et al. 2016, Bigarre et al. 2016). Reviewed in Santi et al. (2017), *Citrobacter* sp. and *Yersinia* spp. have also been found in Siberian and Russian sturgeons; however pathogenicity remains to be determined.

6.3.6. Other bacteria

A diversity of other bacterial pathogens have been recovered from farmed sturgeon globally. For example, *Chryseobacterium indologenes* has also been recovered as possible opportunistic pathogens in Russian sturgeons co-infected with AcIV-E or Nodavirus. Additionally, *Streptococcus dysgalactiae*, another globally damaging fish pathogen (Netto et al. 2011; Nomoto et al. 2006), was recovered from moribund Atlantic sturgeon in central China (Yang et al. 2009). Similarly, *Vibrio anguillarum* has also been linked to disease and mortality in beluga and Russian sturgeons (Austin et al. 2007).

Furthermore, *V. alginolyticus* and a *Pastuerella* sp. were isolated from the liver and kidney of Siberian sturgeons showing gross signs of disease including hemorrhagic lesions (Costinar et al. 2010). *Lactococcus lactis* has been isolated from diseased hybrid sturgeon (*H. huso* x *A. gueldenstaedtii*) in Taiwan, where cumulative mortality of affected lots reached 100% (Chen et al. 2012). Similarly, *Plesiomonas shigelloides*, a known facultative fish pathogen (Faisal et al. 1987), has been isolated from European sea sturgeon (*A. sturio*) in Germany (imported from Russia) wherein the effected lots had ~5% mortality and mild inappetence (Klein et al. 1993). Of note, mortality decreased upon improvement of environmental conditions (Klein et al. 1993). Likewise, *Acinetobacter* spp. have been recovered in sturgeons in Europe of which were co-infected with AcIV-E (Ciulli et al. 2016, Bigarre et al. 2016). Lastly, *Pseudomonas alcaligenes* was recovered from the internal organs of Russian sturgeon (*A. sinensis*) showing gross signs of disease including gill pallor, anemia, pale visceral organs accompanied by petechial hemorrhaging, and occasional small amounts of clear ascites (Xu, et al. 2015). The same study also concluded hybrid sturgeon (*H. dauricus* x *A. schrenckii*) were susceptible to *P. alcaligenes*, wherein doses ranging from $2x10^6$ $2x10^8$ resulted in 33-100% cumulative percent mortality in just over two weeks (Xu, et al. 2015). Other potentially pathogenic bacteria in the families *Pseudomonas*, *Chryseomonas*, *Sphingobacterium*, *Chromobacterium*, *Chryseobacterium*, and *Shewanella* have been detected in sturgeons outside of North America (reviewed in Santi et al. 2017, Ciulli et al. 2020).

6.4. Viruses

Diseases caused by viruses seem to be the most problematic and plentiful based on the numerous reports in sturgeon species. Viruses are often classified based on a combination of

their nucleic acid composition (RNA vs. DNA), strandedness (single or double stranded), Sense (positive of negative), and method of replication (Baltimore 1971). I begin by reviewing DNA then RNA viruses affecting sturgeons outside of North America before focusing on North America, then lake sturgeon specifically.

6.4.1. Nucleocytoplasmic Large DNA Viruses

The term Nucleocytoplasmic large DNA viruses (NCLDVs) refers to a taxonomically diverse group of viruses in the Order Megavirales (Colson et al. 2012). This group of viruses are referred to as NCLDVs due to their genome size compared to other viruses, however it should be noted that the NCLDVs are not a formal taxonomic grouping. Within this group are viruses in the families *Poxviridae, Iridoviridae, Asfarviridae, Phycoviridae, Ascoviridae, Mimiviridae,* and the proposed family *Marseilleviridae* (Colson et al. 2012, Colson et al. 2013). Also within this group are genetically similar NCLDVs documented to affect sturgeon species, sometimes referred to as sturgeon NCLDVs (Clouthier et al. 2015). Although many of the sturgeon NCLDVs are documented only in North America, outside of North America, white sturgeon iridovirus (WSIV, proposed Family *Mimiviridae*; Clouthier et al. 2018) and Acipenser Iridovirus-European (AcIV-E, Family *Mimiviridae*; Bigarre et el. 2017, Clouthier et al. 2018) are two NCLDVs of concern.

White sturgeon iridovirus. WSIV is a double stranded DNA virus that was first documented in North American white sturgeon (*A. transmontanus*, Hedrick et al. 1990). While most work with further understanding the characteristics and pathogenicity with WSIV has been done in North America (detailed below), recent detections have been documented in a range of *Acipenser* species in Poland, Italy, and Germany (Hofsoe-Oppermann et al. 2019). Detections were

associated with grossly healthy fish, suggesting research is needed to assess the risk of WSIV to European sturgeons (Hofsoe-Oppermann et al. 2019).

Acipenserid iridovirus-European. Acipenserid iridovirus-European (AcIV-E) is also a double stranded DNA virus first detected in Russian sturgeon in Northern Europe following years of unexplained mortality events (Adkinson et al. 1998). Further research looking at various degrees of mortality events in other farmed sturgeon species exhibiting gross signs of disease (e.g., *H. huso*, *A. baerii*, and *A. naccarii*) detected AcIV-E via histological and molecular methods (Bigarre et al. 2016). More recent work has also linked AcIV-E to mortality events in starry sturgeon and sterlet (Mugetti et al. 2020). There are now multiple recognized strains of AcIV-E (Clouthier et al. 2018, Pallandre et al. 2019).

6.4.2. Acipenserid herpesviruses

Acipenserid herpesvirus-1. Acipenserid herpesvirus 1 (AciHV-1, formerly white sturgeon herpesvirus-1, WSHV-1; Family *Alloherpesviridae;* Order Herpesvirales) was first detected in California (US; Hedrick et al. 1991a; see below), and has since been detected in Italy, likely a result of the shipment of live white sturgeon from California (USA; Kurobe et al. 2005).

Acipenserid herpesvirus-2. Acipenserid herpesvirus 2 (AciHV-2, former WSHV-2; Family *Alloherpesviridae*, genus *Ictaluriviridae*), first isolated and described in North American white sturgeon, has since been detected in farmed Siberian and hybrid sturgeon (*H. huso* x *A. ruthenus*) in Russia (Watson et al. 1995, Shchelkunov et al. 2009). Upon initial detection, the virus was recognized as Siberian sturgeon herpesvirus (SbSHV); however genomic analyses revealed SbSHV is likely a strain of AciHV-2 (Doszpoly et al. 2010, Doszpoly et al. 2017). Although the

SbSHV strain of AciHV-2 had already been linked to sturgeons exhibiting gross signs of disease and mortality, experimental infections revealed this virus could induce 100% cumulative mortality in juvenile Siberian sturgeon under experimental conditions (Hanson et al. 2016).

6.4.3. Other viral diseases

A diversity of other viral pathogens have been detected in sturgeons (Athanassopoulou et al. 2004, Vicenova et al. 2011, Radosavljevic et al. 2019). Piscine rhabdoviruses pose significant risks to fish species globally (LaPatra et al. 2016) and the only rhabdovirus detected in sturgeons is spring viremia of carp virus (SVCV, Family *Rhabodoviridae*), where it was isolated from farmed Siberian sturgeon in the Czech Republic exhibiting gross signs of disease (Vicenova et al. 2011). Furthermore, Nodavirus (RNA virus, Family *Nodaviridae*; Nishizawa et al. 1997) was detected for the first time in Russian sturgeon in Greece, where it caused clinical signs of disease such as lethargy and ataxia (Athanassopoulou et al. 2004, Xylouri et al. 2007). Lastly, genetic evidence of cyprinid herpesvirus 3 (CyHV-3, Family *Alloherpesviridae*) has been detected in farmed and experimentally exposed Atlantic, Russian, and hybrid sturgeon; however no gross signs of disease were evident (Kempter et al. 2009, Pospichal et al. 2016).

7. Diseases affecting sturgeons in North America

7.1. Parasites

As described above, there are a diversity of parasites that have been recovered from sturgeons globally (Bauer et al. 2002). In addition to those mentioned above, there are also many

parasites affecting sturgeon species in North America including Protozoa, Monogeneans, Cestoda, Trematoda, and others (Margolis et al. 1986, Bauer et al. 2002).

7.1.1. Protozoa

Similar to other parasites described herein, despite reports little is known regarding the pathogenicity of protozoans in sturgeons in North America. Published reports are also sparse, however *Cryptobia salmositica* has been recovered from white sturgeon showing no gross signs of disease (Hoffman et al. 1998).

7.1.2. Monogenea

Reports detail at least two monogenean species in North American sturgeons. One monogenean species (*Nitzschia. superba*) has been recovered from the gills of sturgeons of North America (Hoffman 1998), and is believed by some to be closely related, and possible the same species, as *N. sturionis*, which has been recovered from ship and starry sturgeon in the Aral Sea (see above, reviewed in Bauer et al. 2002). Another Monogenea species recovered from shortnose sturgeon is *Diclybothrium atriatum*, recovered from fish showing no gross signs of disease (Choudury et al. 1996).

7.1.3. Cestoda

At least two cestode (Phylum Platyhelminthes) species have been recovered from white sturgeon (Margolis et al. 1986). While no gross signs of disease were associated, the prevalence

of *Diphyllobothrium* sp. and *Amphilina bipunctata* in white sturgeon ranged from 5.6-36.1% (Margolis et al. 1986).

7.1.4. Trematoda

Trematodes (Phylum Platyhelminthes) have been documented in white sturgeon in British Columbia, where prevalence was roughly 25% (Margolis et al. 1986). Additionally, a trematode, *Pristicola sturionis,* has been recovered from North American sturgeons (species not specified, Skrjabina, 1974). Possible pathogenic effects of the above trematodes were not assessed. Furthermore, a marine trematode (*Tubulovesicula lindbergi*) has also been detected in Columbia River white sturgeon (Becker et al. 1970).

7.1.5. Nematoda

In addition to being found outside of North American, nematodes (Phylum Nematoda) have been detected in white sturgeon in the Columbia River in North America where gross signs of disease were not observed (Chitwood et al. 1950, McCabe et al. 1993). The same nematode detected in the Columbia River (*Cystoopsis acipenseris*, Chitwood et a. 1950) has also been detected in Fraser River white sturgeon (Margolis et al. 1970) also not associated with disease.

7.1.6. Leeches

Leeches (Phylum Annelid) have been recovered from shortnose sturgeon (*A. brevirostrum*; Smith et al. 1980). Infection was not associated with mortality, and a total of six different leech species were recovered during surveillance (Smith et al. 1980).

7.1.7. Other Organisms

Amoebic gill disease (AGD), caused by *Neoparamoeba perurans*, has been linked to mortality in hatchery reared pallid sturgeon (*S. albus*) in the US (Hughes et al. 2000). Therein, cumulative percent mortality reached 100% in some affected tanks, and fish exhibited signs of AGD including gill epithelial hyperplasia and lamellar fusion (Hughes et al. 2000). An Acanthocephala species (*Corynosoma strumosum*, Phylum Acanthocephala) has been recovered from white sturgeon in the Fraser river with no associated gross signs of disease (Margolis et al. 1986).

7.2. Fungi and water molds

Fungal diseases have also been documented in North American sturgeons (Steckler et al. 2014). Amongst those detected include *Veronaea botryosa*, which was found in farmed juvenile and young adult Siberian and white sturgeons with a range of external and internal clinical signs of disease (Steckler et al. 2014). While there are no existing treatments to the systemic phaeohyphomycosis caused by *V. botryosa*, further investigations determined feed supplementation could help mitigate associated hatchery losses (Soto et al. 2021).

7.3. Bacteria

Few bacterial pathogens have been detected and published in existing North American sturgeons (*A. transmontanus*, *A. fulvescens*, *S. albus*, *S. platorhynchus*, *A. brevirostrum*, and *A. oxyrhynchus*).

7.3.1. *Streptococcus* spp.

Currently, most reports are from bacteria recovered from white sturgeon. Amongst those include *Streptococcus innae*, recovered from a mortality event in the US Pacific Northwest (Soto et al. 2017). In this instance, infected fish had severe ulcerative skin lesions and internal gross signs of disease such as bloody ascites, petechial hemorrhaging, and organ pallor, and were also found to be co-infected with AciHV-2 (Soto et al. 2017). *S. innae* is now broadly considered an emerging threat to global aquaculture, including in white sturgeon (Pierzan et al. 2019). Recent studies have linked *S. innae* to additional mortality events and generated data linking experimental exposure to disease and mortality (Pierzan et al. 2019, Nguyen et al. 2020).

7.3.2. Other bacteria

Another report of bacterial disease in white sturgeon occurred in Idaho (US), where fish were found to be infected with *Chlamydiae*-like obligate intracellular parasite, resulting in epitheliocystis (Groff et al. 1996).

7.4. Viruses

In North America, there are a diversity of sturgeon pathogenic viruses that effect most sturgeon species in the US and Canada (Mugetti et al. 2020).

7.4.1. Nucleocytoplasmic Large DNA Viruses

NCLDVs are the largest and most diverse group of viruses affecting North American sturgeons. Members of the unofficial grouping NCLDVs that are known to infect North American sturgeons include Namao virus, Missouri River sturgeon iridovirus, British Columbia white sturgeon iridovirus, Shortnose sturgeon virus, and white sturgeon iridovirus (Clouthier et al. 2015, Clouthier et al. 2018).

White Sturgeon Iridovirus. One of the most problematic NCLDVs in North America is white sturgeon iridovirus (WSIV; Clouthier et al. 2015, Hick et al. 2016, Clouthier et al. 2018). First identified in North America in 1988 and linked to mortality in multiple white sturgeon farms in California, WSIV has induced upwards of 95% cumulative mortality in infected lots of fish (Hedrick et al. 1990). Following detection in 1988, Hedrick et al. conducted pilot WSIV transmission trials that revealed infected fish are capable of transmitting virus to naïve fish, inducing subsequent disease and mortality (Hedrick et al. 1990). Future detections of WSIV and the deployment of newly developed sturgeon specific cell lines (e.g., WSS-2, Hedrick et al. 1991b) also led to successful virus isolation (Hedrick et al. 1995).

Following the first detections, this virus was found to be the causative agent of numerous sturgeon mortality events in North America (LaPatra et al. 1994, Soto et al. 2017, Raverty et al. 2003). Although transmission isn't yet fully understood, it's hypothesized that horizontal

transmission is most common. Furthering this, a study conducted by Drennan et al. compared iodine treatments and lack thereof across a series of eggs fertilized via gametes collected from wild adults in the Koontenai River, where WSIV is endemic (Drennan et al. 2006). There were no significant WSIV-related differences in early life stage mortality across treatment groups (Drennan et al. 2006). Further studies comparing fish reared on Koontenai River water vs. municipal water revealed fish reared on river water tested positive for WSIV infection, whereas those reared on municipal water did not, further supporting horizontal transmission as the primary mode of transmission (Drennan et al. 2006). Additionally, preliminary evidence suggests rearing conditions have impacts on WSIV-associated mortality (Georgiadis et al. 2001, LaPatra et al. 1996).There are now multiple molecular and culture based diagnostic tools available for detection of WSIV, as well as published suggested protocols for targeted tissue collection (Hedrick et al. 1995, Kwak et al. 2005, Clouthier et al. 2015, Hick et al. 2016) Unfortunately, WSIV is considered endemic to many substantial river systems in North America (LaPatra et al. 1994, Raverty et al. 2003, Drennan et al. 2006).

Namao virus. The most recently discovered member of this grouping of viral diseases is Namao virus (NV, proposed Family *Mimiviridae*, Clouthier et al. 2018). NV was recovered while investigating a wild mortality event of lake sturgeon in 2009 and 2010. Moribund fish presented with inappetence, anorexia, erratic behavior, diffuse hemorrhaging, and hyper-production of mucus (Clouthier et al. 2015). To investigate the cause of disease in afflicted fish, a PCR assay targeting the major capsid protein (MCP) gene of other sturgeon NCLDVs was employed, resulting in the amplification of a 219 bp portion of the now described NV MCP gene (Clouthier et al. 2015). Despite molecular detection, attempts made to isolate virus *in vitro* on *Epithelioma papulosum cypri*ni, Chinook salmon embryo-214, white sturgeon skin-1, and white

sturgeon spleen-2 were unsuccessful; however virions were apparent in electron micrographs of epithelial cells (Clouthier et al. 2013). To date, this is the only published report of NV.

Missouri River sturgeon iridovirus. Missouri River sturgeon iridovirus (MRSIV, proposed Family *Mimiviridae*, Clouthier et al. 2018) is believed to be endemic to the Missouri and Mississippi River basins, where it affects pallid and shovelnose sturgeon (Kurobe et al. 2011). First detected in hatchery reared pallid and shovelnose sturgeon populations in South Dakota in 1999, affected fish exhibited gross signs of disease such as lethargy, emaciation, rostral inflammation, and reddening of the mouth and base of fins. Following detection and characterization of MRSIV, subsequent experiments revealed the newly described virus was capable of inducing substantial mortality in pallid and shovelnose sturgeon, could be readily transmitted between species, and could establish carrier states in adult Missouri river sturgeons, with a potential risk of transmission despite absence of clinical disease signs (Kurobe et al. 2011). MRSIV remains only detected in shovelnose and pallid sturgeon in the Missouri and Mississippi river basins. Further research included the development of MRSIV-targeted diagnostic techniques by Kurobe et al. (2011), and MRSIV is also readily detected using assays described in Clouthier et al. (2015; Kurobe et al. 2011).

Other sturgeon NCLDVs. Other NCLDVs detected in Canadian sturgeon populations include British Columbia white sturgeon iridovirus (BCWSV, proposed Family *Mimiviridae*, Clouthier et al. 2018) and shortnose sturgeon virus (SNSV proposed Family *Mimiviridae*, Clouthier et al. 2018). BCWSV, originally thought to be WSIV and later determined to be a distinct virus (Raverty et al. 2003, Clouthier et al. 2015), has only been reported once in white sturgeon. In 2001, infected fish from one aquaculture facility exhibited signs of disease including lethargy and inappetence (Raverty et al. 2003). Similarly, SNSV has only been reported once in shortnose

sturgeon, where infected fish we're also coinfected with Acipenserid herpesvirus 2, and the iridoviral causative agent was initially believed to be WSIV (LaPatra et al. 2013). Later work determined the iridoviral causative agent to be genetically distinct, thus the name shortnose sturgeon virus (Clouthier et al. 2015). SNSV and BCWSV can both be diagnosed via the conventional and quantitative PCRs designed by Clouthier et al. (2015).

7.4.2. Adenoviruses

White sturgeon adenovirus (WSAdV-1) is a double stranded DNA virus recently separated into the new genus *Ichtadenovirus* and re-named *Sturgeon ichtadenovirus A* (Benko et al. 2011, Dozspoly et al. 2019). WSAdV-1 is another pathogen of concern in the context of sturgeons and was first reported in 1984 in juvenile white sturgeon (Hedrick et al. 1984). Suffering chronic mortality, microscopic examination of intestinal and spiral valve tissues via electron microscopy revealed the presence of electron-dense, hexagonal adenovirus-like particles, and histopathological changes (e.g., nuclear hypertrophy of gastro-intestinal epithelial cells) were also appreciated (Hedrick et al. 1984). Following detection of this virus, preliminary susceptibility studies were conducted and revealed *in vitro* susceptibility to WSAdV-1 when exposed to infected tissues via intraperitoneal injection (Hedrick et al. 1984). Later analyses determined the etiology of the mid-80s white sturgeon mortality event was indeed a fish-associated adenovirus (namely white sturgeon adenovirus) and described new diagnostic techniques to detect WSAdV-1 (Benko et al. 2002, Kovacs et al. 2003).

7.4.3. Iridoviruses

Frog virus 3 (Family *Iridoviridae*; genus *Ranavirus*; Chinchar et al. 2017) is a double stranded DNA virus that also poses a threat to North American sturgeons. Overall, viruses in the family Iridoviridae are highly problematic, with infections often resulting in gross signs of disease and/or mortality in most fishes (Plumb et al. 1996, Anders 1989). FV3 is of particular contemporary concern given its recent host range expansion into clinically diseased pallid sturgeon in the Missouri and Mississippi River basins. Furthermore, experimental exposures provided evidence of disease manifestation and mortality following exposure to FV3 (Waltzek et al. 2014, Stilwell et al. 2022).

7.4.4. Alloherpesviruses

Viruses in the Family *Alloherpesviridae* are well-known for their ability to induce mortality in fish across North America (Hanson et al. 2016). Amongst those currently published as present in sturgeon are the previously referenced AciHV-2 and AciHV-1 (Hedrick et al. 1991a, Watson et al. 1995). Both viruses, first detected in the 1980s in California white sturgeon, have been linked to mass mortality in farmed and wild white sturgeons in the Pacific Northwest region of the US and farmed shortnose sturgeon in eastern Canada (Hedrick et al. 1991a, Watson et al. 1995, Georgiadis et al. 1999, 2000, Kelley et al. 2005, LaPatra et al. 2014).

Acipenserid herpesvirus-1. In California, farmed white sturgeon infected with AciHV-1 exhibited no specific gross signs of disease explicitly linked to AciHV-1, however percent mortality in affected lots reached 50% (Hedrick et al. 1991a). AciHV-1 was successfully isolated using specific cell lines developed by Hedrick et al. (1991b), and cytopathic effects were

described as the formation of syncytium (Hedrick et al. 1991a). Electron micrographs revealed the presence of hexagonal herpesvirus-like virions, and histopathologic changes were apparent in the epidermis (Hedrick et al. 1991a).

Acipenserid herpesvirus-2. AciHV-2 (genus *Ictalurivirus)* was incidentally detected as part of a disease surveillance program initiated in white sturgeon (Watson et al. 1995). AciHV-2 was initially isolated on WSS-2 cells from reproductive fluids collected from a grossly normal adult white sturgeon, with CPE described as focal rounding, enhanced refractility, and enlargement of cells (Watson et al. 1995). The same virus was also detected via cell culture in a variety of visceral organs and external tissues (Watson et al. 1995). While fish in which AciHV-2 was first detected did not exhibit gross signs of disease, later detections occurred in shortnose sturgeon exhibiting gross signs of disease in the form of semi-translucent, raised foci of apparent cutaneous thickening (LaPatra et al. 2013). Currently, AciHVs are readily detected via cell culture using host-specific cell lines and molecularly by employing generalized alloherpesvirus primers (Hedrick et al. 1991b, Kurobe et al. 2008). The two viruses and associated strains are also hypothesized to fall into two robustly supported separate groupings (Kurobe et al. 2008).

8. Geographic and host range expansions of sturgeon-pathogenic viruses

Of the viruses described above as occurring in North America, many appear to have increased in host and geographic range, seemingly encroaching towards the Great Lakes, and/or have demonstrated the ability to spread widely and rapidly. For example, white sturgeon iridovirus once detected was subsequently detected in multiple facilities in the Pacific northwest (US) and Canada (LaPatra et al. 1994, Drennan et al. 2006, Raverty et al. 2003). Despite these

range expansions and continuous detections amongst white sturgeon, there are currently no published reports of WSIV in the Great Lakes basin. WSIV is also currently listed as an emergency pathogen in the Great Lakes Fishery Commission - Fish Health Committee Model Program for the Great Lakes, given the potential threat it could pose to Great Lakes lake sturgeon populations (Phillips et al. 2014).

Additionally, FV3, a notorious amphibian pathogen that has a tremendous and diverse host range, was for the first time was documented in the Family *Acipenseridae* (e.g., pallid sturgeon, Waltzek et al. 2014). Alarmingly and as described above, FV3 resulted in approximately 95% cumulative mortality in effected groups of pallid sturgeon. Further raising alarm, is that FV3 has been detected in amphibian populations in both Wisconsin and Minnesota (Granoff et al. 1965).

9. Diseases affecting lake sturgeon

Unfortunately, literature surrounding infectious diseases affecting lake sturgeon is relatively sparse when compared to other commercially valuable sturgeon species. However, this should not be confused with a lack of presence of infectious diseases, because until recent years surveillance of wild lake sturgeon populations was minimal. In fact, many publications regarding diseases of lake sturgeon are from recent decades.

9.1. Parasites

Covered thoroughly above, parasite infections are common in sturgeon species globally and within North America. Parasite infections have also been documented in lake sturgeon, and while few, reports are detailed below. Of note, large scale surveillance was conducted in the 1990s, wherein a diversity of parasitic organisms were recovered (Choudhury et al. 1993). The reports below do not encapsulate the extent of the following parasites in the Great Lakes, rather detail published reports in lake sturgeon.

9.1.1. Monogenea

One Monogenean parasite (*Diclybothrium atriatum*) was detected in lake sturgeon showing no gross signs of disease through the course of parasite surveillance (Choudhury et al. 1996).

9.1.2. Cestoda

At least two species of cestodes were recovered from adult lake sturgeon sampled in the Rainy and Nelson rivers in Canada, as reported in Choudhury et al. (1993). The two Cestodes, *Proteocephalidea* sp. and *Tetraphyllidea* sp., were found in <1% prevalence (Choudhury et al. 1993).

9.1.3. Trematoda

Infections with Trematodes have also been documented in lake sturgeon, where Choudhury et al. (1993) described four species found in multiple rivers throughout Canada. The species

included: *Azygia longa*, *Crepidostomum auriculatum, Diclybothrium armatum*, *Diplostomum* sp. and were not associated with gross signs of disease.

9.1.4. Nematoda

Five species of nematode were recovered in the parasite surveillance study conducted by Choudhury et al. (1993) including: *Cystidicoloides* sp., *Raphidascaris* sp., *Rhabdochona cascadilla*, *Spinitectus acipenseri*, and *Truttaedacnitis clitellarius*. Similar to parasites described above, no associated signs of disease were observed in infected fish.

9.1.5. Other parasites

Other parasite infections with Coelenterata (*Polypodium hydriforme*; Dick et al. 1991), leeches (*Placobdella montifera*), and Acanthocephala (four spp. detailed in Choudhury et al. 1993) were also detected in lake sturgeon surveyed in Canada (Choudhury et al. 1993).

9.2. Bacteria

Little is known regarding bacterial infections in lake sturgeon; however unpublished data from the Michigan State University - Aquatic Animal Health Laboratory suggest bacterial pathogens of concern including *Flavobacterium* spp. and *Aeromonas* spp. have been found in streamside rearing facilities, sometimes in association with mortality events.

9.3. Viruses

9.3.1. Namao virus

Clouthier et al. (2013) recently described previously mentioned Namao virus (see above), which is closely related to the devastating WSIV, and linked to significant hatchery mortality events in Manitoba, Canada.

9.3.2. Frog virus 3 and white sturgeon iridovirus

While not yet found in wild lake sturgeon, studies conducted by Hedrick et al. revealed that lake sturgeon are also susceptible to infection with WSIV, though gross signs of disease and mortality were not appreciated (Hedrick et al. 1992). Also amongst those pathogens documented in lake sturgeon is FV3, where a personal communication cited in Waltzek et al. described lake sturgeon as being susceptible to FV3 in a controlled laboratory pilot experiment (Waltzek et al. 2014).

9.3.3. Lake sturgeon herpesviruses

Through the duration of completing this literature review, a report of an alloherpesvirus in wild lake sturgeon in Wisconsin (USA) was published (Walker at al. 2022). Therein, the authors describe the molecular detection of a virus similar to other acipenserid herpesvirus, but genetically distinct.

10. Conclusions

Lake sturgeon remain a species of special management concern in the Great Lakes. While much is understood about the anthropogenic factors influencing population declines, a critical knowledge gap needs to be addressed: what affect could infectious diseases be having on wild adult and hatchery reared juvenile lake sturgeon? Given the drastic difference in number of publications regarding infectious diseases in other sturgeon species compared to lake sturgeon, it is likely there is much still to learn regarding pathogen presence in this treasured Great Lakes fish. Literature regarding sturgeon pathogens in North America within the last decade has shown a concerning trend: viruses affecting other sturgeons, including NCLDVs, alloherpesviruses, and FV3, are demonstrating host and geographic range expansions. Most recently and alarmingly, molecular evidence of an alloherpesvirus was recovered in Great Lakes (Lake Michigan) and Great Lakes-adjacent waterways.

By conducting surveillance for possible pathogens that pose the greatest threat to Great Lakes lake sturgeon populations, critical unknowns regarding pathogen presence and potential negative effects can be addressed. Furthermore, knowledge acquired through surveillance of adult Great Lakes lake sturgeon can bolster hatchery rearing efforts by identifying pathogens of special concern when rearing juvenile lake sturgeon. Though the lake sturgeon continues to face an uphill battle to population recovery, continued and apparently successful conservation and management strategies could be well-supplemented with an improved understanding of infectious diseases endemic to or emerging in existing populations.

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CHAPTER 2:

FIRST ISOLATION OF A HERPESVIRUS (FAMILY *ALLOHERPESVIRIDAE*) FROM GREAT LAKES LAKE STURGEON (*ACIPENSER FULVESCENS*)

1. Abstract

Lake sturgeon (*Acipenser fulvescens*; LST) is the only native sturgeon in the Great Lakes (GL), but due to multiple factors, their populations are estimated to be <1% of historical abundances. Little remains known about infectious diseases affecting adult and juvenile GL-LST in hatchery and wild settings. Therefore, a two-year disease surveillance study was undertaken, resulting in the detection and first *in vitro* isolation of a herpesvirus from wild adult LST with grossly apparent cutaneous lesions inhabiting two GL watersheds (e.g., Huron and Erie). Histological and ultrastructural examination of lesions revealed proliferative epidermitis associated with herpesvirus-like virions. A virus with identical ultrastructure was recovered from lesions *in vitro*. Partial DNA polymerase gene sequencing placed the virus within the Family *Alloherpesviridae*, with high similarity to a molecularly detected lake sturgeon herpesvirus (LSHV) from Wisconsin, USA. Genomic comparisons revealed ~84% ANI between Wisconsin and Michigan strains, leading to the proposed classification of LSHV-1 and -2 for the two taxa. When naïve juvenile LST were immersion-exposed to LSHV-2, severe disease and ~33% mortality followed, with virus re-isolated from representative skin lesions. Results collectively show LSHV-2 is associated with skin damage in wild adult LST, disease and mortality in juvenile laboratory exposed LST, and highlight this virus as a potential threat to GL LST conservation.

2. Introduction

The lake sturgeon (*Acipenser fulvescens*; Family *Acipenseridae*) is the only indigenous sturgeon species in the Great Lakes of North America, where it is also the largest and longest

living fish (Hay-Chimielewski and Whelan 1997). Unique life history traits, such as delayed maturation, sporadic spawning, and low natural recruitment, have placed lake sturgeon at increased risk for human-induced mortality. Indeed, several anthropogenic factors (e.g., overharvesting, habitat degradation, dam construction) have been linked to declines in both numerical abundance and distribution range of Great Lakes lake sturgeon (Hayes and Caroffino 2012). Despite substantial efforts to rehabilitate lake sturgeon, which include hatchery supplementation, their abundance in the Great Lakes is estimated to be <1% of historical levels (Hay-Chimielewski and Whelan 1997, Hayes and Caroffino 2012).

Despite these substantial population declines, little is known about the infectious diseases of lake sturgeon when compared to other sturgeon species (Order Acipenseriformes). This is a matter of concern given the propensity of disease to impede hatchery-based conservation and aquaculture productivity for sturgeon worldwide (Bigarré et al. 2017, Yu-Ping and Di 2005), including in North America. In particular, viral diseases are important sources of mortality in a range of sturgeon species, including white sturgeon (*A. transmontanus*), pallid sturgeon (*Scaphirhynchus albus*), shovelnose sturgeon (*S. platorhynchus*), and shortnose sturgeon (*A. brevirostrum*) and have been detected in sturgeon populations across North America (Kurobe et al. 2011, Waltzek et al. 2014, Raverty et al. 2003, Hedrick et al. 1990, Clouthier et al. 2013).

Among the viruses associated with disease outbreaks in sturgeon, herpesviruses within the Family *Alloherpesviridae* have been identified as important pathogens of multiple sturgeon species. The first report of herpesvirus infections in sturgeon was made in juvenile white sturgeon in California, USA, in 1990 (Hedrick et al. 1991). The disease-causing virus, now known as Acipenserid herpesvirus-1 (AciHV-1; syn. white sturgeon herpesvirus 1), along with the formally classified *Acipenserid herpesvirus 2* (AciHV-2; genus *Ictalurivirus*; Watson et al.

1995), are now recognized as important sources of mortality in juvenile captive-reared white sturgeon in the western USA (Hedrick et al. 1991, Watson et al. 1995, Georgiadis et al. 2000, Soto et al. 2017). Subsequent studies have uncovered similar herpesviruses in Siberian sturgeon (*A. baerri*) in Russia, wild white sturgeon of the Columbia River in Oregon and the Snake River in Idaho, and in cultured shortnose sturgeon (*A. brevirostrum*) from northeastern Canada (Kelley et al. 2008, Doszpoly and Shchelkunov 2010, Doszpoly et al. 2017, LaPatra et al. 2014). Importantly, AciHVs have been isolated from the reproductive tissues of sexually mature and apparently healthy wild white sturgeon, raising the possibility of virus transmission from infected broodstock to offspring (Watson et al. 1995). Despite this group of viruses having been detected in various North American sturgeon populations, an AciHV has never been isolated from wild lake sturgeon. However, just prior to submission of the current manuscript, Walker et al. (2022) reported the detection of a novel alloherpesvirus in lake sturgeon in Wisconsin, USA, via a degenerate PCR assay and electron microscopy (Walker et al. 2022). Although this virus, termed lake sturgeon herpesvirus, was found in association with skin lesions, it's pathogenic potential was not determined.

Given the lack of reports of AciHV-1 and AciHV-2 in the Great Lakes basin in conjunction with their ability to cause sizeable mortality events in other sturgeon species, the Great Lakes Fishery Commission - Great Lakes Fish Health Committee designated these viruses (e.g., "white sturgeon herpesvirus") as Emergency Fish Pathogens in the 2014 Model Program for Fish Management in the Great Lakes (Phillips et al. 2014). However, during a study investigating the infectious diseases of Great Lakes lake sturgeon, a replicating agent with acipenserid herpesvirus-like characteristics was initially detected and subsequently isolated from grossly appreciable skin lesions in adult lake sturgeon from the Lake Huron and Lake Erie watersheds of

the Great Lakes basin. As such, the primary objectives of this study were to initially characterize and identify the replicating agent and investigate the tissue changes associated with naturally occurring infections in adult lake sturgeon. Following the successful isolation of this alloherpesvirus, an additional goal was to characterize its capacity to cause disease and/or mortality in juvenile lake sturgeon via *in vivo* challenge experiments under controlled laboratory conditions.

3. Materials and methods

3.1. Fish and sampling

During May and June of 2019 (year one), adult lake sturgeon were sampled from two locations within the Great Lakes basin: a) spawning or near-spawning phase adult lake sturgeon were captured via hand netting by Michigan Department of Natural Resources (MDNR) personnel in the Black River (Lake Huron watershed, Presque Isle County, Michigan); and b) adult non-spawning lake sturgeon were sampled from the St. Clair River (Lake Erie watershed, St. Clair County, Michigan) by MDNR personnel via trotline.

Upon capture, fish were gently restrained while partially immersed in water for gross examination (<3 minutes) and non-lethal tissue collections. Biometric data (i.e., length, weight, girth) were recorded, after which, whole blood was collected by caudal venipuncture. A portion of whole blood was immediately transferred into a sterile heparinized tube (Heparin sodium salt, ThermoFisher Scientific Waltham, MA, USA) for subsequent packed cell volume (PCV) measurement, and the remainder was placed into a 1.5 ml tube and diluted 1:4 (W:V) with viral

growth media utilized for virological analyses (250 µL; see below) and placed in an ice-chilled cooler. If fish were ready to spawn, reproductive fluids and/or eggs were gently expressed, collected from the cloaca via syringe, saved in a sterile 5 mL centrifuge tube or Whirl Pak™ (VWR, Radnor, PA, USA), and immediately placed on ice. When skin lesions of concern were present, a small amount of tissue was biopsied (<250 mg) using forceps and either a sterile scalpel or scissors. The biopsied tissue was saved in RNA Later (Sigma-Aldrich, St. Louis MO, USA) on ice for molecular analyses, in a 2 oz whirl-pak containing viral growth media (see below) on ice for virus isolation, and in some cases, the biopsied tissue was saved in 10% buffered formalin for histopathological analyses. During the year one field season, all samples for virus isolation were frozen at -20°C after collection and prior to further analyses.

In May 2020 (year two) and due to the COVID-19 pandemic, adult lake sturgeon were exclusively sampled from the Black River site. All clinical examination and tissue sampling procedures were identical to year one, with the following exceptions: a) within hours of collection, all reproductive fluid samples (750 µL) were diluted in sterile viral growth media consisting of Earle's salt-based minimal essential medium (EMEM; Invitrogen, Carlsbad CA, USA) supplemented with 10% BD Bacto^{TM} tryptose phosphate broth (TPB; ThermoFisher Scientific, Waltham MA, USA), 2% Fetal Bovine Serum (FBS; Gemini Bioproducts, Sacramento CA, USA), penicillin (100 IU mL^{-1}), streptomycin (100 µg mL^{-1}; Invitrogen, Carlsbad CA, USA), gentamicin sulfate (0.1 mg mL^{-1}; Sigma-Aldrich, St. Louis, MO, USA), amphotericin B (2.5 µg mL^{-1}; Sigma-Aldrich, St. Louis MO, USA), and buffered with sodium bicarbonate (pH 7.4-7.6; Sigma-Aldrich, St. Louis MO, USA) at a ratio of 1:4 (V:V) respectively; b) whole blood samples were similarly diluted and collected directly into sterile viral growth media (1:10, V:V); c) skin lesion tissues were collected directly into one mL of

sterile viral growth media, not exceeding a ratio of 1:4 (W:V); and d) all samples for virus isolation were maintained on ice and/or at 4°C (i.e., unfrozen) prior to inoculation onto cells. Furthermore, using Fisher's Exact Test in Microsoft® Excel® (Version 2207 Build 16.0.15427.20182), I checked for significant differences between the presence of any grossly observable virus-suspect skin lesions in males and females collected from the Black River in 2019 and 2020.

3.2. Hematology

Less than eight hours post-collection, heparinized blood from each fish was aliquoted into a micro-hematocrit tube, spun at 16,000 rpm in a CritSpin™ Hematocrit Centrifuge (Iris Sample Processing, Westwood, MA, USA) for 2 minutes, after which the packed cell volume (PCV) was measured (Grant 2015).

3.3. Histopathology

Representative, formalin-fixed skin lesions that appeared grossly as thickened opaque foci were submitted to the Michigan State University Veterinary Diagnostic Laboratory, where they were paraffin embedded, sectioned (5 µm), and stained with hematoxylin and eosin (H&E) according to routine methods (Prophet et al. 1992). Stained preparations were then examined via light microscopy for histopathological changes.

3.4. Virological analysis via cell culture

3.4.1. Cell culture

Five cell lines were utilized for cell culture analysis: *Epithelioma papulosum cyprini* (EPC; Fijan et al. 1983), Chinook salmon embryo (CHSE-214; Lannan et al. 1984), white sturgeon skin (WSSK-1; Hedrick et al. 1991), white sturgeon gonad (WSGO; Watson et al. 1998), and a white sturgeon and lake sturgeon hybrid spleen cell line (WSxLS). All cells were maintained in 75 cm^2 cell culture flasks (Corning, Corning, NY, USA) at 25°C (EPC) or 21°C (CHSE, WSSK-1, WSxLS, WSGO). Growth media for EPC, WSSK-1, WSxLS, and WSGO cell lines were comprised of EMEM supplemented with 10% TPB, 10% FBS, penicillin (100 IU mL^{-1}), streptomycin (100 µg mL^{-1}), amphotericin B (2.5 µg mL^{-1}), 2mM L-Glutamine, and buffered with sodium bicarbonate (pH 7.4-7.6), CHSE cells were grown in Eagle's MEM (with Earle's salts, nonessential amino acids, and sodium pyruvate; ATCC, Manassas, VA, USA), nystatin (20 µg mL^{-1}; Sigma-Aldrich, St. Louis, MO, USA), L-glutamine, FBS, penicillin, and streptomycin as above. For virus isolation, cells were grown in 96-well, flat bottom plates (Corning, Corning, NY, USA) to 80%-90% confluency (<48 hours) in a medium as described above but modified to contain 2% FBS (WSSK, WSxLS; MEM-2-T) or 5% FBS (EPC, CHSE; MEM-5-T) and buffered with UltraPure Tris (Invitrogen, Carlsbad, CA, USA) to pH 7.4-7.6 (AFS-FHS 2020).

3.4.2. Sample processing

For samples collected during year one, whole blood, reproductive fluids, and skin lesion tissues were thawed on ice and diluted (1:4 W:V) with EMEM supplemented with TPB,

penicillin (100 IU mL^{-1}), streptomycin (100 µg mL^{-1}), gentamicin sulfate (0.1 mg mL^{-1}), amphotericin B (2.5 µg mL^{-1}), and buffered with UltraPure Tris (to pH 7.4-7.6; Invitrogen, Carlsbad, CA, USA). Samples were then homogenized and centrifuged (5,000 RPM, 30 minutes, 4°C) (Boonthai et al. 2017). After centrifugation, whole blood sample supernatants were further diluted 1:5 with the sterile viral growth medium described above for inoculation onto cells. The supernatant was incubated for 2 hours at 15°C or for 24 hours at 4°C in preparation for inoculation onto cells (AFS-FHS 2020). Due to a lack of virus isolation in year one, sample processing was modified for year two samples as described above (section 2.1).

3.4.3. Virus isolation

Clarified supernatant was centrifuged just prior to inoculation (5,000 RPM, 15 minutes, 4°C) onto the cells. All samples from year one and two were inoculated onto EPC, CHSE, WSSK, and WSxLS, whereas a subset of samples from year one were inoculated onto WSGO. After inoculation, all samples and cell lines were incubated at 15°C. After a 14-day observation period, a second passage was performed on all samples, with no centrifugation step in between to allow for potential cell-to-cell virus transmission. Inoculated cell cultures were passed two to four more times for a total of 60 days on cells. If cytopathic effects (CPE) were noted, samples were passed into 25cm^2 culture flasks containing fresh cells (<48 hours old). If CPE persisted, supernatant and cells were preserved at -80°C in a solution of 20% FBS and 20% glycerol and/or frozen un-diluted for further analyses.

3.5. Electron microscopy

For transmission electron microscopy, representative formalin-fixed skin lesions that had been fixed in neutral-buffered, 10% formalin solution were trimmed into 2 mm pieces and postfixed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer for 2 hours. Tissues were serially dehydrated in acetone and embedded in Poly/Bed 812 resin (Polysciences Inc., Warrington, PA, USA) in flat molds. Sections were obtained with a Power Tome XL ultramicrotome (Boeckeler Instruments, Tucson, AZ, USA). To identify areas of interest, we stained semithin (0.5-μm) sections with epoxy tissue stain and examined them under a light microscope. Ultrathin (70 nm) sections were then cut, mounted onto 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined under a 100 CXII transmission electron microscope (JEOL, Peabody, MA, USA). Similarly, cells from cell culture samples (see below) exhibiting cytopathic effects (CPE) were harvested, centrifuged at 600 RPM (5 minutes) and supernatant discarded. The cell pellet was then fixed in 10% buffered formalin and processed as above.

3.6. Molecular and gene sequence analyses

3.6.1. Nucleic acid extraction

Representative skin lesion biopsies were processed for molecular analyses by thawing RNALater (Sigma-Aldrich, St. Louis, MO, USA)-preserved tissues at 4°C, followed by centrifugation (5,000 RPM, 10 minutes) to pellet the tissue and allow for removal of the RNALater via micropipette. Following a single rinse with sterile phosphate buffered saline

(PBS), nucleic acid (NA) extraction was performed using the DNeasy Blood and Tissue kit (Qiagen Inc., Germantown, MD, USA) according to the manufacturer's protocol for animal tissue. The concentration of extracted NA was quantified via a Qubit™ fluorometer using the double-stranded DNA broad range assay kit (dsDNA BR Assay Kit; Invitrogen, Carlsbad CA, USA). Likewise, cell culture samples showing CPE, along with uninoculated negative control samples, were harvested for NA extraction and subsequent PCR analyses. In brief, once samples were passed and CPE observed (5-14 days post passage onto fresh cells), NA was extracted using the DNeasy Blood and Tissue kit following the manufacturers protocol for cultured cells.

3.6.2. Endpoint PCR analysis

Because the gross appearance of the observed skin lesions was like those linked to acipenserid herpesviral infections in other sturgeon species (Hedrick et al. 1991, Watson et al. 1995), the degenerate endpoint PCR assay developed by Hanson et al. (2006) was employed (Hanson et al. 2006). The HV (5' CGG AAT TCT AGA YTT YGC NWS NYT NTA YCC-3') and Cons lower (5'-CCC GAA TTC AGA TCT CNG TRT CNC CRT A-3') primers, which amplify a conserved 497 bp region of the DNA polymerase gene of several alloherpesviruses, including AciHV-1 and AciHV-2, and the cycling parameters of Kurobe et al. (2008) were utilized (Kurobe et al. 2008). Each 25 µL reaction was comprised of 12.5 µL 2x GoTaq Green Master Mix (ThermoFisher Scientific, Waltham, MA, USA), 5 µL of DNA template, and 40 pmol of each primer, with the remaining volume comprised of sterile, nuclease free water (Invitrogen, Carlsbad, CA, USA). Nuclease-free water, NA extracted from fin tissues collected from apparently healthy and previously sampled adult lake sturgeon, and NA extracted from

uninoculated WSxLS cells served as template for negative control reactions, whereas NA extracts from previously confirmed AciHV-1 and AciHV-2 isolates were used as template for positive control reactions. Each PCR reaction was conducted in a Mastercycler Pro S 6325 Vapo protect PCR System (Eppendorf, Hamburg, Germany). The PCR products were subsequently separated by gel electrophoresis in a 1.5% agarose gel infused with SYBR Safe DNA Gel Stain (ThermoFisher Scientific, Waltham, MA, USA; 1 mM) at 100V for 30 minutes and then visualized via UV transillumination under a UVP DigiDoc-It Imaging System (UVP, Upland, CA, USA). A TrackIt™ 1 kb plus DNA ladder (Invitrogen, Carlsbad, CA, USA) was utilized for reference of amplicon size.

3.6.3. Gene sequence analysis

Representative cell culture and skin lesion samples yielding amplicons of ~ 500 bp were purified using the QIAquick Spin Kit (Qiagen Inc., Germantown, MD, USA) and submitted to the Genomics Technology Support Facility (Michigan State University, East Lansing, MI) for bidirectional sanger sequencing using the HV and Cons primers of Hanson et al. (2006). Additionally, a subset of amplicons from PCR-positive cell culture and skin lesion samples were purified as above and subsequently cloned using the TOPO-TA cloning kit with TOP-10 *Escherichia coli* chemically competent cells (Qiagen Inc., Germantown MD, USA) following the manufacturer protocols. Up to six resultant clones from each sample were screened for the target amplicon size via PCR analysis using primers M 13 Forward-20 (5’ GTA AAA CGA CGG CCA G 3’) and M 13 Reverse (5’ CAG GAA ACA GCT ATG AC 3’) and the following cycling parameters: 94°C for 10 minutes, 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and

72°C for 45 seconds, and a final extension at 72°C for 10 minutes. PCR products were then visualized via gel electrophoresis as described above. Cloning-derived amplicons (n=3-6 per sample) were then purified, quantified, and submitted for sequencing as described above.

3.6.4. Phylogenetic analyses

All generated chromatograms were analyzed for quality and trimmed using Chromatogram Explorer (version 5.0.2.3). Next, contigs were assembled using the BioEdit sequence alignment editor (version 7.2.5; Hall et al. 1999) via the Contig Assembly Program. Generated sequences of the polymerase gene were initially analyzed using the nucleotide Basic Local Alignment Search Tool (BLASTn) software from the National Center for Biotechnology Information (NCBI) nucleotide database. Reference sequences showing similarity were downloaded from NCBI, as were outgroups, and an alignment between study and reference sequences performed using ClustalW in MEGAX (Version 10.2.4; Kumar et al. 2018). Phylogenetic analyses were conducted in MEGAX using neighbor joining analysis (Tamura et al. 2004), where evolutionary distances were determined using the maximum likelihood method (Saitou et al. 1987).

3.6.5. Nanopore sequencing and genomic analyses

Genomic DNA was prepared using the Puregene DNA kit (Qiagen Inc., Germantown, MD, USA) following the manufacturers protocol for cultured cells. Preparations from lake sturgeon herpesvirus isolate 200413-11TC were sequenced using a rapid barcoding kit (RBK-004, Oxford Nanopore Technologies, Oxford, UK) on a R9.4.1 flow cell. Raw fastq sequences were filtered

to a minimum read quality of 15 and a minimum length of 2000 bp using NanoFilt (De Coster et al. 2018). The filtered data, 220,725,293 bases in 57,034 reads, was assembled using Canu v2.0 (Koren et al. 2017). The nanopore reads were re-mapped to the assemblies at a median 69-fold depth of coverage and consensus base calls were produced by Medaka v1.2.3 (https://github.com/nanoporetech/medaka) which produced two contigs (119, 069 and 87,627 bp). The assembled contigs were annotated in Geneious Prime® 2022.0.1 (Biomatters, Ltd., Auckland, New Zealand) based on GenBank Accession number (Acc. No.) OK485036 (Lake sturgeon herpesvirus strain Wolf River; Walker et al. 2022) and regions corresponding to the DNA packaging terminase subunit 1 (Acc. No.: OK485036: ORF19) and DNA polymerase catalytic subunit (Acc. No.: OK485036: ORF36) were extracted for analyses. Comparable sequences were identified by Blastn searches for somewhat similar sequences of the NCBI non-redundant nucleotide (nr/nt) database for the 2,100 bp CDS of the DNA packaging terminase subunit 1 and the 4,638 bp CDS of the DNA polymerase catalytic subunit. In addition to Blastn searches, MAFFT alignments were performed on nucleotide and translated amino acid sequences using the complete CDS of DNA terminase and DNA polymerase sequences from the Wolf River lake sturgeon herpesvirus strain (Acc. No.: OK485036) and the Acipenserid herpesvirus 1 (AciHV-1) strain UC Davis (Acc. No.: OK275734; OK485036; Walker et al. 2022). The complete CDS of the DNA packaging terminase subunit 1 and DNA polymerase catalytic subunit will be deposited in GenBank (Johnston et al. 2022, *under review*)

Average nucleotide identity (ANI) estimations were made for the lake sturgeon herpesvirus Wolf River strain (Acc. No.: OK485036) and lake sturgeon herpesvirus isolate 200413-11TC recovered in the present study using both best hits (one-way ANI) and reciprocal best hits (two-way ANI) as calculated by Goris et al. (2007). To account for the smaller viral genomes,

combinations of minimum alignment lengths (125, 250, 500, 750 and 1000 bp) and minimum alignment identities (70%, 75%, 80%, 85%) were tested. Fragment options included a window size of 1000 bp and step size of 200 bp. To provide a frame of reference, similar comparisons were performed on two strains of Ictalurid herpesvirus 1 (Channel catfish virus strain Auburn 1 [Acc. No.: NC_001493] and Blue catfish alloherpesvirus strain S98-675 [Acc. No.: MK392382]) as well as comparisons between Cyprinid herpesvirus 1 strain NG-J1 (Acc. No.: JQ815363) and Cyprinid herpesvirus 3 strain GY-01 (Acc. No.: MK260013).

3.7. *In vivo* assessment of isolated herpesvirus virulence to lake sturgeon

3.7.1. Origin of fish for virus exposure experiments

During May of 2020 and 2021, fish were spawned at the Black River Streamside Rearing Facility following previously described protocols and rearing practices (Crossman et al. 2011, Crossman et al. 2014, Bauman et al. 2015, Bauman 2015, Bauman et al. 2016). Upon reaching eight weeks of age, fish were transported to the Michigan State University-Research Containment Facility (MSU-URCF), where they were reared under quarantine conditions in a 680L flow through tank supplied with ultraviolet-treated, oxygenated well water (10°C $\pm$ 1°C; ~9 L/min). Fish were fed frozen blood worms (Brine Shrimp Direct, Ogden UT, USA) 1-2 times per day (~2% body weight), and the tank was cleaned and siphoned 1-2x daily to remove detritus/uneaten food. Prior to challenge, a subset of fish were screened via tissue culture for the presence of viruses, including AciHVs, and verified to be free from infection. Briefly, fish were euthanized in Tricaine-S (MS-222; Syndel, Ferndale WA, USA; 250 mg/L) buffered with

sodium bicarbonate (500 mg/L), tissues collected, processed, and inoculated onto cells as detailed in section 2.5.

3.7.2. Preparation of virus inoculum for exposure experiments

Lake sturgeon herpesvirus isolate 200413-11TC**,** originally recovered from the skin lesion of an adult lake sturgeon sampled from the Black River in year 2 (see results), was rapidly thawed from -80°C and inoculated (25 µL) onto a 96 well plate of WSxLS cells, prepared as described above. Upon development of CPE, cells and supernatant were passed into 25cm^2 culture flasks until CPE again developed. Virus infected cells/supernatant were then harvested and a Tissue Culture Infectious Dose$_{50}$ ($TCID_{50}$) performed by serially diluting virus 10-fold in sterile MEM-2-T (10^{-1}-10^{-7}) and inoculating all dilutions onto 96 well plates, which were observed for the development of CPE for 21 days, at which point wells with CPE were counted and the $TCID_{50}$ calculated (Reed and Muench 1938).

3.7.3. Pilot exposure experiments

To guide a replicated virus exposure study, multiple small pilot experiments were undertaken. Juvenile lake sturgeon (n=five/treatment; mean weight 1.74 g, Table 1) were exposed to Great Lakes lake sturgeon herpesvirus isolate 200413-11TC via intracelomic (IC) injection or bath immersion and after exposure, were maintained in flow-through (water temperature of 10°C $\pm$ 1°C; ~1.5L/min) or recirculating (water temperature of 22°C $\pm$ 1°C) 37.8L aerated glass aquaria (Table 1). For IC injection exposure, fish were anesthetized with

sodium bicarbonate-buffered (250 mg/L) MS-222 at a concentration of 125 mg/L and once sedate, injected with 25 or 50 µL ($1.53x10^2$ - $3.20x10^5$ $TCID_{50}$/mL) of virus infected or negative control cell suspensions (Table 1) using sterile 1 mL syringes and 25-gauge needles. Following injection, fish were released back into their experimental tanks and monitored for recovery. For pilot bath immersion exposures, fish were immersed in a 125 mL suspension of virus (treatment group; $6.40x10^5$ $TCID_{50}$/mL; Table 1) or cell growth media (negative control group) for 60 minutes at a concentration of one part virus suspension or negative control media to nine parts water (low dose) or one part virus suspension/media to four parts water (high dose). Following immersion exposure, fish and the virus or media suspensions were gently poured back into their respective 37.8L flow-through glass aquaria.

Fish were monitored and cared for daily as described above. All dead fish were examined and multiple tissues (e.g., eye, fin, gill, kidney, spleen, heart, oral siphon, and/or barbels) aseptically collected and processed as previously described for inoculation onto EPC, CHSE, WSSK, and WSxLS cell lines. For pilot experiments, cell culture samples showing the characteristic CPE observed on primary isolation were considered positive for the Great Lakes lake sturgeon herpesvirus. Terminally moribund fish and fish surviving until completion of the pilot experiments (i.e., 202-295 days post-challenge) were euthanized via MS-222 overdose as above and analyzed similarly. All challenge experiments were conducted in accordance with the MSU-Institutional Animal Care and Use Committee (AUF: 201900057 and 202200033).

3.7.4. Replicated immersion exposure experiment

Guided in part by results from pilot experiments, a larger scale and replicated immersion exposure experiment was undertaken. For experimental inoculum, virus was cultured and quantified as described above. Fish (mean weight of 1.20 g; n=8 fish per replicate tank, n=6 replicates) were immersed in a 120 mL suspension of lake sturgeon herpesvirus isolate 200413-11TC at a concentration of one-part virus suspension to three parts tank water with aeration ($1.28x10^6$ $TCID_{50}$/mL). Another six replicate tanks (n=8 per replicate) were similarly mock challenged with one-part WSxLS cell/media suspension to three parts clean tank water with aeration. Following a 60-minute immersion exposure, fish and the respective suspension were gently poured into their respective 37.8L flow-through glass aquaria maintained at 10°C $\pm$ 1°C (1.5 L/min). As detailed previously, terminally moribund fish and fish surviving until the completion of the immersion challenge (112 days) were euthanized as described above and tissues (internal organs, siphon, barbel, fin, gill, lesions) collected for virus isolation and molecular analyses. In brief, select cell culture supernatant samples were analyzed using the primer set developed by Hanson et. al (2006) as described above, and a subset of samples were cloned and sequenced using methods described in sections 2.5.1-2.5.3 and 2.6.1-2.6.3. Significant differences in cumulative percent mortality between virus-exposed and mock-exposed replicates were assessed via conducting a Fisher's Exact Test in Microsoft® Excel® (Version 2207 Build 16.0.15427.20182).

4. Results

4.1. Gross findings in adult Great Lakes lake sturgeon

During year one, 137 spawning or near-spawning adult lake sturgeon (n=111 males, n=26 females) of varying lengths and weights (Table 2) were examined from the Black River. Among the examined fish, 4 (median length, weight, and girth of 146.2 cm, 26.8 kg, and 63.8 cm, respectively) of 26 females and 5 (median length, weight, and girth of 141.8 cm, 21.36 kg, and 53.4cm, respectively) of 111 males from this location presented with focal (Fig. 1a) to multifocal (Fig. 1d) to coalescing (Fig. 1b-c) semitranslucent to opaque, whitish, slightly raised plaque-like lesions on the fins, body, and/or head (Table 2). Likewise, among the 76 adult lake sturgeon examined from the St. Clair River in year one, one (length of 120.8 cm, weight of 41.4 kg, and girth of 49.2 cm) had grossly similar skin lesions on the body and head (Fig. 1d; Table 2). During year two, 79 adult spawning or near-spawning lake sturgeon (n= 54 males, n= 25 females) of varying lengths and weights (Table 2) were examined from the Black River. Among the examined fish, 6 (mean length, weight, and girth of 157.6 cm, 31.4 kg, and 60.25 cm) of 25 females and 6 (mean length, weight, and girth of 136.2 cm, 18.0 kg, and 50.25 cm) of 54 males presented with similar raised, opaque lesions on the trunk, fins, cranium, and opercula. Across all sites and sampling years, fish with skin lesions had a length, weight, and girth of 146.9 cm, 24.7 kg, and 55.25 cm, compared to 136.3 cm, 22.5 kg, and 52.2 cm in fish without grossly appreciable skin lesions.

When comparing the presence of skin lesions in males and the presence of skin lesions in females in 2019 using Fisher's Exact Test, a one-tailed p-value of 2.42×10^{-23} was observed, indiciating significant differences between sexes. However in 2020, a one-tailed p-value of 0.13

was observed, indicating no significant differences in lesion presence between males and females sampled.

4.2. Hematology

Fish from all sites in year one and two had a mean PCV of 32.2%, with some variation by sampling site and year (Table 2). Overall, fish presenting with the skin lesions had a mean PCV of 33.9%, compared to a mean PCV of 32.6% in fish without skin lesions. No clear trends in PCV were appreciable by site or year (Table 2); however, female lake sturgeon had slightly lower mean PCVs in both year one and two at the Black River site when compared to males (Table 2). PCV measurements were comparable to reference intervals established by DiVincenti et. al and Cassle et. al (2013).

4.3. Histopathology

Sections of fin, gills and the site of gross lesions were examined from five fish sampled from the Black River in year two. Fins and gills were examined from two additional fish, all of seven had skin abnormalities suspect for Acipenserid herpesviruses and were from the same site and year. None of the fish had lesions in the gills. The microscopic changes in the gross lesion site were most severe in fish #8, but overall similar in all five fish. There was marked ballooning degeneration of the deeper portion of the stratum spinosum of the epidermis (Fig. 2a). Affected cells were enlarged with lightly eosinophilic to clear cytoplasm and their nuclei were swollen with marginated chromatin (Fig. 2b). Within the nuclei, there was often lightly eosinophilic, flocculent material forming central inclusions that displaced chromatin. The more superficial

portion of the epidermis had undergone necrosis and there was loss of nuclear detail, marked karyorrhexis and sloughing of epithelial cells. Multifocally there was transmural necrosis also affecting the superficial portion of the underlying lepidotrichia (Fig. 2c). In the subepidermal portion of the connective tissue surrounding the lepidotrichia was loss of cellular detail and multiple foci of karyorrhexis. In the deeper portions of the connective tissue separating the mineralized spines was extensive neovascularization and proliferation of fibroblasts (granulation tissue) with dense infiltrates of small lymphocytes (Fig. 2d). Multiple segments of the mineralized spins were partially lysed, had scalloped edges and were surrounded by proliferating fibroblasts or were completely lost in other areas (Fig. 2e). There was common clustering of pigmented cells in the superficial connective tissue. In all submitted skin samples, there was marked epidermal hyperplasia with severe intercellular edema (spongiosis) in the stratum spinosum. Keratinocytes focally lost orientation, forming small clusters of cells, and mitotic figures were common. There were dense infiltrates of small lymphocytes in the lower portion of the stratum spinosum focally extending into the stratum basale (Fig. 2f). Lymphocytes were commonly surrounded by a clear halo and surrounded individual necrotic keratinocytes. There was often clustering of pigmented cells within the superficial stratum spinosum of the more normal appearing epidermis forming a transition zone to the deeper portions of the epidermis that were infiltrated by lymphocytes.

4.4. Cell culture

In year one, 119 whole blood, 91 reproductive fluid, and 8 skin lesion samples collected from the Black River and 57 whole blood, 3 reproductive fluid, and 1 skin lesion samples collected from the St. Clair River were inoculated onto the EPC, CHSE, WSSK-1, and WSxLS cell lines,

with a subset also being inoculated onto WSGO. After a 60-day observation period, there was no observable CPE in any of the samples on any cell lines. In year two, 67 whole blood, 47 reproductive fluid, and 12 skin lesion samples collected from the Black River were inoculated onto EPC, CHSE, WSSK-1, and WSxLS. No CPE was detected in any whole blood or reproductive fluid samples, nor in any of the skin lesion samples inoculated onto the WSSK, EPC, or CHSE cell lines. In contrast, CPE, in the form of large, multinucleated cells (i.e., syncytia), were observed on the WSxLS cell line (Fig. 3) in 11 of the 12 skin lesions (91.6%), beginning as early as five days post inoculation. As the observable CPE progressed and by two weeks post-inoculation, all cells had either detached, lysed, or formed syncytia. Subsequent passages resulted in identical syncytia formation and individual isolates were cultured and cryopreserved for further analyses.

4.5. Electron microscopy

When transmission electron microscopy (TEM) was utilized on representative skin lesions and WSxLS cells showing CPE, large numbers of herpesvirus-like particles (~85 nm) with electron-dense nuclei were observed within the degenerating nuclei of affected cells (Fig. 4a-d).

4.6. PCR, gene-sequencing, and phylogenetic analyses

4.6.1 Sanger sequencing and phylogenetic analyses

Based on the gross similarity of the observed skin lesions to those associated with alloherpesviruses in other sturgeon species, the degenerate PCR assay of Hanson et al. (2006) targeting the DNA polymerase gene was used to test nine skin lesions from the Black (n=8) and St. Clair (n=1) Rivers in year one, and twelve skin lesions from the Black River in year two. Among them, four year-one skin lesions from the Black River and one from the St. Clair River yielded ~500 bp amplicons, as did six year-two skin lesions from the Black River and six representative suspect virus isolates recovered on the WSxLS cell line. Direct Sanger sequencing yielded sequences ranging from 468-529 bp and sequence comparisons using BLAST revealed highest similarity (ranging from 96.2%-99.8%) to the newly detected lake sturgeon herpesvirus Wolf River strain (GenBank Accension Number: OK485036), followed by 76.7%-82.0% similarity to an AciHV-1 DNA polymerase gene reference sequence (GenBank Accension Number: EF685904.1). When representative amplicons from both sampling years and sites (e.g., 3 from year one and 3 from year two) were cloned and subsequently sequenced (3-6 clones per sample), the generated sequences all were most similar to the same newly detected lake sturgeon herpesvirus Wolf River strain (GenBank Accension Number: OK485036; 97.5%-98.4%), followed by 80.9%-82.0% similarity to an AciHV-1 DNA polymerase gene reference sequence (GenBank Accension Number: EF685904.1). Subsequent phylogenetic analyses using a partial stretch of the DNA polymerase gene via the Neighbor-Joining method (Kimura 2-parameter with gamma distribution) placed all sequences generated in this study into a robustly supported clade also containing the lake sturgeon herpesvirus Wolf River reference sequence that was most closely related to, yet distinct from, the AciHV 1 clade (Fig. 5; Felsentien 1985, Kimura 1980).

4.6.2. Nanopore sequencing and genomic analyses

The draft genome generated for lake sturgeon herpesvirus isolate 200413-11TC consists of two contigs (119,069 and 87,627 bp). Blastn searches produced no direct matches to any alloherpesvirus isolates in GenBank (Table 3, 4), but did reveal a high degree of similarity (99.36%) across a 156-bp fragment of the DNA packaging terminase subunit I to lake sturgeon herpesvirus strains from the Chippewa (OL440177), Wolf (OL440173, OL440175) and Wisconsin (OL440170) Rivers in Wisconsin (Walker et al. 2022). MAFFT alignments of the 4,638 bp DNA polymerase catalytic subunit CDS from lake sturgeon herpesvirus isolate 200413-11TC with lake sturgeon herpesvirus (OK485036) and AciHV-1 from White Sturgeon (OK275729) revealed 92.6% nt (93.4% aa) similarity to lake sturgeon herpesvirus ORF36 and 79.8% nt (86.5% aa) similarity to AciHV-1. Similarly, MAFFT alignments of the 2,100 bp DNA packaging terminase subunit I CDS with the Wolf River lake sturgeon herpesvirus (OK485036) and AciHV-1 (OK275729) revealed 86.8% nt (92.8% aa) similarity to lake sturgeon herpesvirus ORF19 and 85.5% nt (92.3% aa) similarity to AciHV1 (OK275734).

Across a series of alignment lengths and identities, the lake sturgeon herpesvirus Wolf River strain and the genomically characterized herpesvirus isolate recovered in the current study (Lake sturgeon herpesvirus isolate 200413-11TC) averaged 84.02% ANI (SD: 2.8074%; range 81.21-89.08%; Table 5). Comparably, across the same series of parameters, Channel catfish virus and Blue catfish alloherpesvirus averaged 94.10% (SD 0.53; range 93.38-94.82%) (Table 6). Cyprinid Herpesvirus 1 and Cyprinid Herpesvirus 3 were too divergent to make comparable analyses (Table 7).

4.7. *In vivo* challenge experiments

Following small-scale pilot challenges of juvenile lake sturgeon to multiple concentrations of the newly isolated lake sturgeon herpesvirus via multiple exposure routes (Table 1), gross disease signs, in the form of decreased reactivity to feed, lethargy, and peri-oral hemorrhage (Fig. 6a), began to manifest at 3-31 days post-exposure (PE). Other signs of disease observed throughout the pilot studies included enophthalmia and focal dermal ulceration on the head and/or body of the fish (Fig. 6b-c). Eventually, severe lethargy that progressed to fish lying in dorsal recumbency, severe ulceration of the caudal fin into the peduncle with associated hemorrhage (Fig. 6d), and/or mortality began on day 8 PE and continued until day 84 PE (Table 1). Tissues from representative, grossly apparent skin lesions in both immersion and IC injection pilot experiments were inoculated onto WSxLS cells, after which CPE, in the form of syncytia, was observed within seven days. There was no evidence of CPE on CHSE, EPC, or WSSK, nor was virus recovered from other tissues sampled (i.e., oral siphon, barbel, eye, visceral organs).

Based in part on results from pilot experiments and experimental exposure studies with other acipenserid herpesviruses (Hedrick et al. 1991, Watson et al. 1995), an immersion route of exposure was selected for a larger scale, replicated challenge experiments. In the replicated immersion challenge, fish were exposed to lake sturgeon herpesvirus isolate 200413-11TC at a concentration of 1.28×10^6 $TCID_{50}$/mL, after which gross signs of disease were noted in virus-exposed fish beginning 10 days PE. Early signs of disease included moderate to severe lethargy when compared to negative controls across all replicates, and overall decreased reactivity to feed. Additionally, hemorrhaging around the oral siphon began 10 days PE (Fig. 7a). At 15-16 days PE, more severe gross signs of disease began to manifest, including mild corneal opacity and mild-moderate erosion of the caudal fin. Within 48 hours of caudal fin erosion onset, a

subset of affected fish had near complete loss of the caudal fin and ulceration extending deep into the caudal peduncle musculature, often accompanied with moderate-severe hemorrhaging (Fig. 7b, c). Disease signs continued to progress to marked emaciation, more widespread hemorrhaging around the oral siphon and caudal peduncle, and complete loss of the caudal fin and posterior portions of the caudal peduncle (Fig. 7d). No gross signs of disease were noted in negative control, mock-exposed fish. Mortality in virus-exposed fish began 19 days PE and continued through 76 days PE, where all dead or terminally moribund and euthanized fish had grossly similar ulcerations of the caudal peduncle and resultant near complete loss of the caudal fin. Following conclusion of this experiment at 112 days PE, cumulative percent mortality (CPM) across all virus-exposed replicates reached a mean of 33.3% (Fig. 8) compared to a mean CPM of 0.0% across all negative control, mock-exposed replicates. Using a Fisher's Exact Test to compare CPM of virus-exposed fish to mock-exposed fish, a one-tailed p-value of 0.0000034 was observed, indicating significantly more mortality occurred in virus-exposed juvenile lake sturgeon when compared to those that were mock-exposed.

Upon inoculation of collected tissues (e.g., siphon, barbel, visceral organs, lesions) onto the WSxLS cell line, CPE (i.e., formation of syncytia) that was identical to what was observed in the herpesvirus infected adult lake sturgeon developed in representative skin lesion samples (Fig. 9), but not in cells inoculated with other tissues. Subsequent PCR analyses of representative cells showing CPE using the degenerate primer set described above [31] yielded an ~500 bp amplicon; following cloning and Sanger sequencing, recovered virus isolates were confirmed to be the same lake sturgeon herpesvirus. Upon euthanasia of surviving fish at the end of this study and inoculation of tissues onto cells, there was no evidence of a replicating agent.

5. Discussion

Herein, we describe the first *in vitro* isolation of an alloherpesvirus from wild lake sturgeon in general, as well as the first detections of an alloherpesvirus in lake sturgeon inhabiting the Lake Huron and Lake Erie watersheds of the Great Lakes basin, North America. These findings are significant for several reasons. First, Great Lakes lake sturgeon populations are estimated to be <1% of historical abundances, and substantial resources are currently being allocated to the restoration of this iconic Great Lakes fish (Hay-Chimielewski and Whelan 1997). In this context, the present study not only showed that the presence of actively infectious virus was associated with skin lesions in wild adult lake sturgeon that serve as gamete sources for hatchery-based conservation, but also that the newly isolated alloherpesvirus can induce severe disease and subsequent mortality in experimentally exposed juvenile LST. Although this virus was not recovered from reproductive fluids in the current study, other alloherpesviruses infecting other sturgeons (e.g., white sturgeon; *A. transmontanus*) have been recovered from the reproductive fluids of spawning adults showing gross signs of disease (Watson et al. 1995), raising suspicions of possible vertical transmission and subsequent negative effects in offspring. Whether this newly detected virus has been or is leading to disease and/or mortality in wild and hatchery reared juvenile lake sturgeon remains unknown and warrants priority attention. Second, other acipenserid herpesviruses (formerly known as white sturgeon herpesviruses; Hedrick et al. 1991, Watson et al. 1995) are currently listed as emergency pathogens in the Great Lakes Fishery Commission-Great Lakes Fish Health Committee Fish Health Model Program (Phillips et al. 2014). This list, curated based on the potential threats certain pathogens pose to Great Lakes fishes, harbors significant fish pathogens believed to not yet be in the Great Lakes basin; therefore, isolation of this virus is highly relevant to Great Lakes lake sturgeon management and

may necessitate revision of the current list of emergency pathogens. Third, alloherpesviruses have been shown to have immunomodulatory effects in other fishes, leading to immunosuppression and increased potential for opportunistic infections (Adamek et al. 2013, Faisal et al. 2019). For example, previous work found that when common carp (*Cyprinus carpio*) were experimentally infected with Cyprinid herpesvirus 3, infection negatively affected the mucosal barrier of the skin and associated immune system activity (Adamek et al. 2013). Furthermore, infection with Salmonid herpesvirus 3 (EEDV) in lake trout (*Salvelinus namaycush*) induced skin ulcerations which were found to be co-colonized with multiple fish-pathogenic bacteria, including *F. psychrophilum* and *Aeromonas* spp. (Faisal et al. 2019). Similarly, Acipenserid herpesvirus 2 has also been linked to co-infections with *Streptococcus iniae* in white sturgeon (*A. transmontanus*; Soto et al. 2017). Collectively, literature supports the ability of alloherpesviruses to both negatively affect the immune system and subsequently provide opportunities for secondary or co-infections with other fish pathogens (Adamek et al. 2013, Soto et al. 2017, Faisal et al. 2019). Lastly, herpesviruses are known to establish latency and chronic infections in hosts, where stressors, such as spawning or environmental factors could lead to resurgence of active infections (Faisal et al. 2019, Cohen 2020). This was demonstrated during the resurgence of salmonid herpesvirus 3 (EEDV; *Alloherpesviridae*) in hatchery-reared lake trout (*S. namaycush*), where disease and mortality attributed to EEDV was thought to be induced by a combination of environmental stressors (e.g., temperature fluctuations and heavy rainfall) in addition to rearing densities (Faisal et al. 2019). Whether similar stressors and immunomodulatory effects are associated with infection emergence, status, or severity in the context of this newly isolated alloherpesvirus remains to be determined.

Although the present study reports the first isolation of an alloherpesvirus from lake sturgeon, Walker et al. (2022) recently reported the molecular detection of an alloherpesvirus in four rivers in Wisconsin (US), including within the Lake Michigan watershed (Walker et al. 2022). Following initial screening using the PCR assay designed by Hanson et al. (2006) and subsequent genome sequencing and assembly, Walker et al. (2022) determined the virus recovered from WI lake sturgeon was an alloherpesvirus most similar to, yet distinct from AciHV-1, and proposed the name lake sturgeon herpesvirus [21,31]. Interestingly, using the same degenerate primer set of Hanson et al. (2006) and subsequent sequence analysis of partial DNA polymerase gene sequences, the virus detected in the present study was similar, yet not identical, to the lake sturgeon herpesvirus Wolf River strain (Walker et al. 2022, Hanson et al. 2006). Additional comparison of the genomes generated by Walker et al. (2022) and this study revealed high similarity across a small (i.e., 156 bp) portion of the DNA packaging terminase subunit I, and ~93% similarity across the entire DNA polymerase catalytic subunit CDS (Walker et al. 2022). However, ANI comparisons across the available genomic data for the two lake sturgeon viruses revealed only 84% similarity (Table 5). Considering these results and acknowledging that strict criteria to delineate alloherpesvirus taxa are not fully established, we posit that the virus detected in the current study is most similar to, yet distinct from, the lake sturgeon herpesvirus recently described by Walker et al (2022). In fact, the %ANI between the lake sturgeon herpesvirus Wolf River strain detected by Walker et al. (2022) and the current study is ~10% lower than the %ANI for two catfish alloherpesviruses (e.g., Channel catfish virus and blue catfish alloherpesvirus; Table 6) that are believed to represent distinct taxa. Indeed, work done by Venugopalan et al. further supported the separation of channel catfish virus and blue catfish alloherpesvirus into separate taxa through host-specificity and serum neutralization

studies (Venugopalan et al. 2021). Therefore, we propose the virus described in Walker et al. (2022) and the virus described herein be respectively referred to as lake sturgeon herpesviruses 1 and 2 pending additional taxonomic investigations (Walker et al. 2022).

Lake sturgeon herpesvirus 2 was detected in infected adult lake sturgeon residing in two (e.g., Huron and Erie) Great Lakes watersheds, where the virus was associated with grossly apparent skin lesions. Of note, these lesions were not only grossly and microscopically similar to those reported by Walker et al. (2022), but also to white sturgeon infected with AciHV-2 (Hedrick et al. 1991). Thus, it appears these viruses may have a similar disease progress despite representing different viral species and infecting different hosts. In the present study, the prevalence of these herpesvirus-linked skin lesions varied by sex in the Black River during both study years, whereby lesions were 2.2-3.4 times more prevalent in females than males. Infections with other fish pathogens (e.g., *A. salmonicida* and *Carnobacterium maltaromaticum*) in other fish species nearing and/or undergoing spawning have also been documented as more prevalent in females (Loch et al. 2011, Loch et al. 2011). Potential factors behind the observed differences in virus-linked lesion prevalence by sex could involve the complex relationship between stress, sex, and the immune system (Campbell et al. 2021). Not only have female fish been documented to have higher cortisol levels during spawning that may dampen the immune response, but evidence also suggests female spawning fish have a less robust immune response compared to males (Hou et al. 1999, Shepherd et al. 2012, Campbell et al 2021). Similarly, differences in the herpesvirus-linked skin lesion prevalence was apparent by sampling site, where prevalence was lower in the St. Clair River than the Black River in 2019 (1.1% vs. 6.6%; Table 2). Whether this relates to differences in spawning behavior, environmental factors and/or differences between the two populations is unknown currently. However, it is noteworthy that in the Black River, nearly

all fish were at the spawning phase, whereas fish in the St. Clair River were primarily pre-spawning phase, and spawning associated stress has been linked to emergence of active herpesvirus infections from latency and generally decreased immune parameters (Pickering et al. 1987, Hou et al. 1999, Cohen et al. 2020).

Hematologic analyses revealed no significant differences in PCVs between fish with LSHV-associated lesions compared to those without. There is little published information regarding any relationship between hematologic parameters, such as packed cell volume, and infection with alloherpesviruses. Overall, in this study, we found no substantial differences in PCV between male and female fish from any site in any year, and all PCV averages fell within published sturgeon (*A. fulvescens* and *A. gueldenstaiedtii*) reference intervals (17%-38% and 16%-34%; DiVincenti et al. 2013, Cassle et al. 2020).

To clarify the effects that the newly recovered lake sturgeon herpesvirus may have on the health and survival of juvenile lake sturgeon, *in vivo* laboratory challenge experiments were completed. Results definitively showed that the newly isolated LSHV-2 is indeed capable of causing disease and subsequent mortality in juvenile lake sturgeon immersed in a virus-laden suspension under laboratory conditions. Likewise, these experiments revealed that LSHV-2 was capable of replicating in naïve hosts and could be re-isolated from the resultant skin lesions, collectively fulfilling Rivers postulates (Rivers 1937). Notably, the progression of grossly apparent disease signs was remarkably consistent across virus-exposed replicates, whereby fish developing gross disease signs showed lethargy and decreased reactivity to feed, which eventually progressed to complete loss of the caudal fin and deep ulceration into the caudal peduncle in every mortality (n=16). Somewhat surprisingly, the gross disease signs in LSHV-2-exposed fish showed uncanny similarity (e.g., ulcerative lesions) to those often associated with

Flavobacterium columnare, causative agent of columnaris disease (Davis 1922, LaFrentz et al. 2022). However, tissues from caudal ulcerations were collected from representative fish in all replicate tanks and inoculated onto Hsu-Shotts medium using routinely employed methods (AFS-FHS 2020, Bullock et al. 1986) and no yellow pigmented bacteria, including *F. columnare*, were recovered (data not shown). Considering these findings, fishery managers and fish health professionals/clinicians should be aware that LSHV-2 appears to induce disease signs that could be readily confused with columnaris disease, potentially leading to delayed or misdiagnosis, especially without deployment of species-specific *in vitro* and specialized molecular techniques.

Also in the present study and for the first time, a lake sturgeon herpesvirus from lake sturgeon was successfully isolated and propagated *in vitro*. The type of CPE induced by the LSHV (i.e., syncytia formation) was highly similar to that noted in AciHV 1 and 2 (Hedrick et al. 1991, Watson et al. 1995, Doszpoly et al. 2010). Notably, propagation of this alloherpesvirus was only possible using a cell-line that included cells derived from the original host, the lake sturgeon, and is consistent with reports of strong host specificity often exhibited by alloherpesviruses (Hanson et al. 2011). In addition to requiring specialized reagents, results from the current study affirm protocols established by the World Organisation for Animal Health (OIE), in that attempts to isolate an alloherpesvirus *in vitro* should avoid freezing of tissues (WHO 2022). Additionally, the use of a viral transport media formulated based on recommendations from the American Type Culture Collection and American Fisheries Society-Fish Health Section (AFS-FHS 2022, ATCC 2022) also seems to have enhanced successful virus recovery and may relate to the importance of antibiotics and fetal bovine serum for virus stability and replication. Based upon the results herein, when attempting to recover LSHVs *in vitro*, we recommend the following process be followed: a.) collect tissue and fluid samples directly into

viral growth media (<1:4; W:V) detailed in section 2.1; b.) maintain virus at 4°C (i.e., unfrozen) and inoculate onto cells within five days of collection; and c.) utilize cell lines derived, at least in part, from lake sturgeon.

6. Conclusions

This study describes the first isolation of an alloherpesvirus from wild adult lake sturgeon that concurrently exhibited gross cutaneous skin lesions. The recovered virus proved virulent to naïve juvenile Great Lakes lake sturgeon, whereby immersion exposure resulted in the development of disease and subsequent mortality and re-isolation of the virus from the skin lesions of affected hosts. Genetic and genomic characterization of the virus detected in this study revealed it was most similar but distinct from a very recently described LSHV (Walker at al. 2022). Based upon these results and until further taxonomic studies are undertaken, we propose the virus detected in Walker et al. and the virus isolated herein be referred to as lake sturgeon herpesvirus 1 and 2, respectively. Although much remains unknown about the effects LSHV-1 and 2 may have had or be having on LST populations, reports of the closely related Acipenserid herpesviruses being detected in reproductive fluids raises the potential for transgenerational transmission in lake sturgeon with unknown consequences for wild populations and hatchery conservation efforts alike. Research conducted in the present study is paving the way for ongoing and future research efforts seeking to arm those rearing lake sturgeon in the Great Lakes with efficacious management tools reduce the negative health risks this virus poses to Great Lakes lake sturgeon populations and restoration.

APPENDIX

Figure 1. Semi-translucent to opaque, whitish, slightly raised plaque-like observed on adult lake sturgeon in the Black (a-c) and St. Clair (d) Rivers indicated with black arrows. Lesion intensity varied from focal (a) to multifocal (d), to multifocal and coalescing (b-c) on the fins, head, and body.

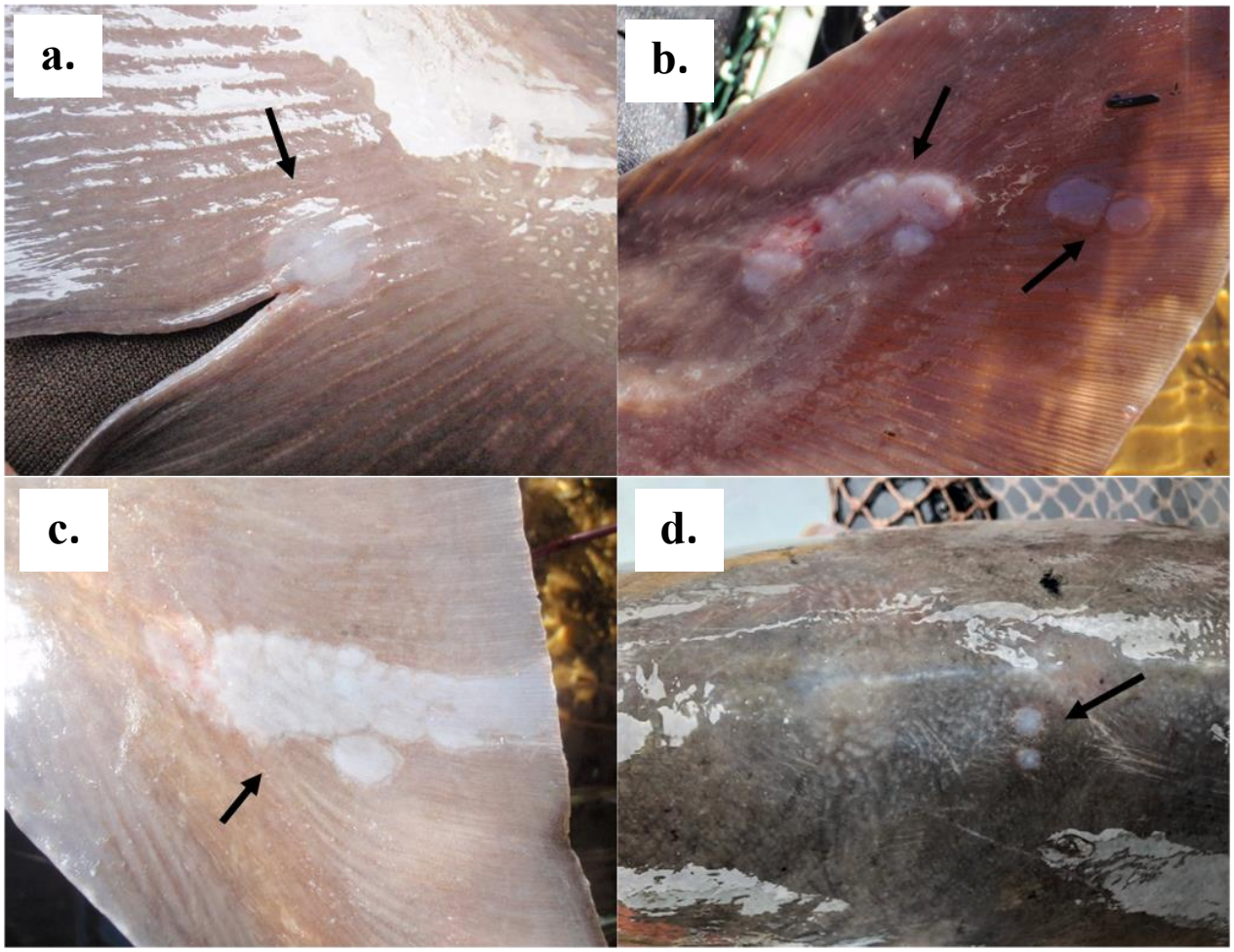

Figure 2. a) Marked ballooning degeneration of the deeper portion of the stratum spinosum of the epidermis. b) Affected cells are enlarged with lightly eosinophilic to clear cytoplasm and their nuclei are swollen with marginated chromatin. c) Transmural necrosis affecting the superficial portion of the lepidotrichia. d) Extensive granulation tissue with dense infiltrates of small lymphocytes separating mineralized spines. e) Segments of mineralized spins were partially lysed, had scalloped edges and were surrounded by proliferating fibroblasts. f) Dense infiltrates of small lymphocytes in the lower portion of the stratum spinosum focally extending into the stratum basale.

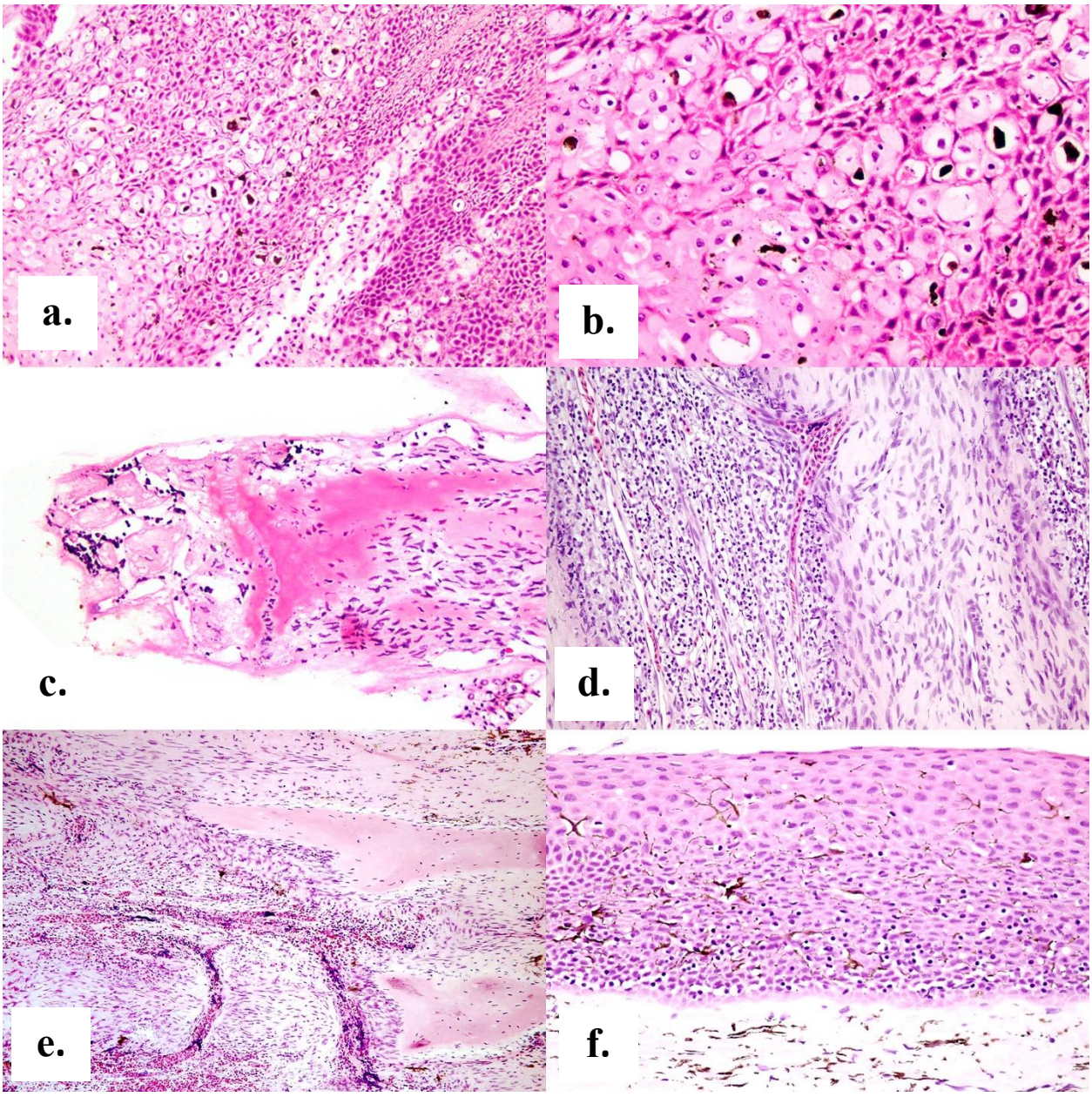

Figure 3. a) A normal mono layer of white sturgeon x lake sturgeon (WSxLS) cells post-infection with tissue infected with virus. Arrows indicate large, multinucleated giant cells, or syncytia.

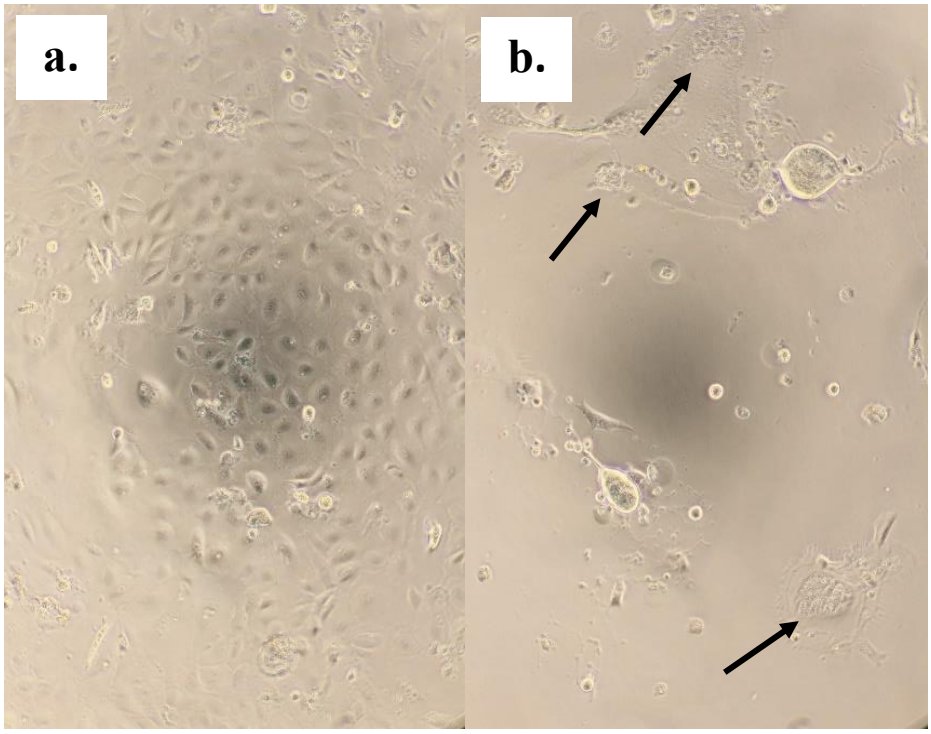

Figure 4. a) Large numbers of herpesvirus particles (arrows) in degenerating nucleus of an epithelial cell in the stratum spinosum of the epidermis. bar = 2µm b) Higher magnification of mature 85 nm virion (C-capsid). bar = 100nm c) Large numbers of herpesvirus particles (arrows) in degenerating nucleus of affected cell in cell culture. bar = 1µm d) Higher magnification of mature 85 nm virion. bar = 100nm.

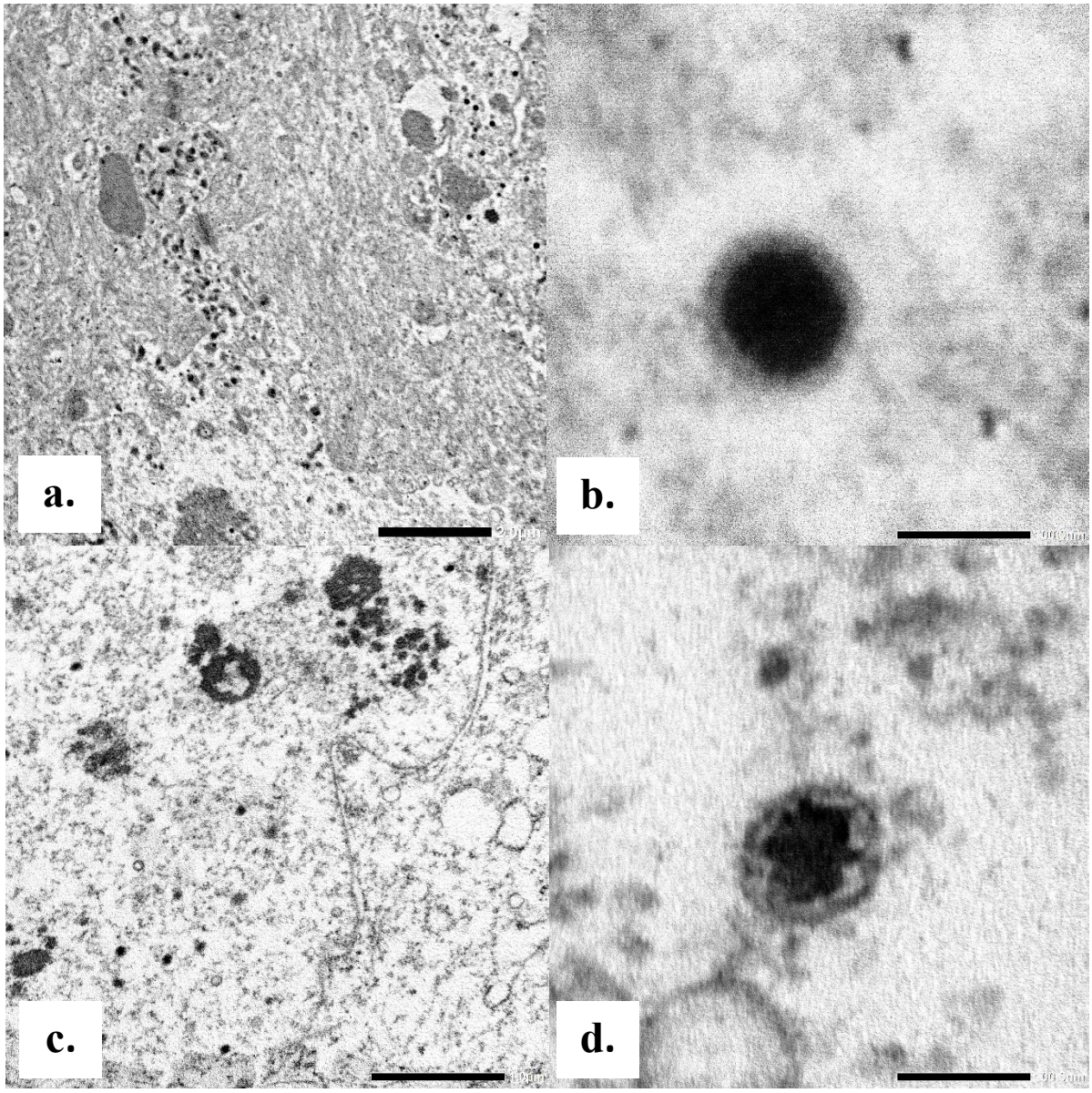

Figure 5. Dendrogram depicting the relationships of the lake Sturgeon herpesvirus sequences (n=19, denoted with solid squares) generated in the current study to other representative alloherpesviruses. The tree was generated in MEGA X (Kumar et al. 2018) using the Neighbor-Joining method, whereby evolutionary distances were determined via the Kimura 2-parameter method (Kimura 1980) gamma distribution for site rate variation; complete deletion option) and are in the units of the number of base substitutions per site. A bootstrap test (n=1000 replicates) was completed and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are displayed adjacent to branches (only bootstrap values > 70 are shown). A total of 418 nucleotides comprised the final dataset, and Silurid Herpesvirus 1 served as the outgroup.

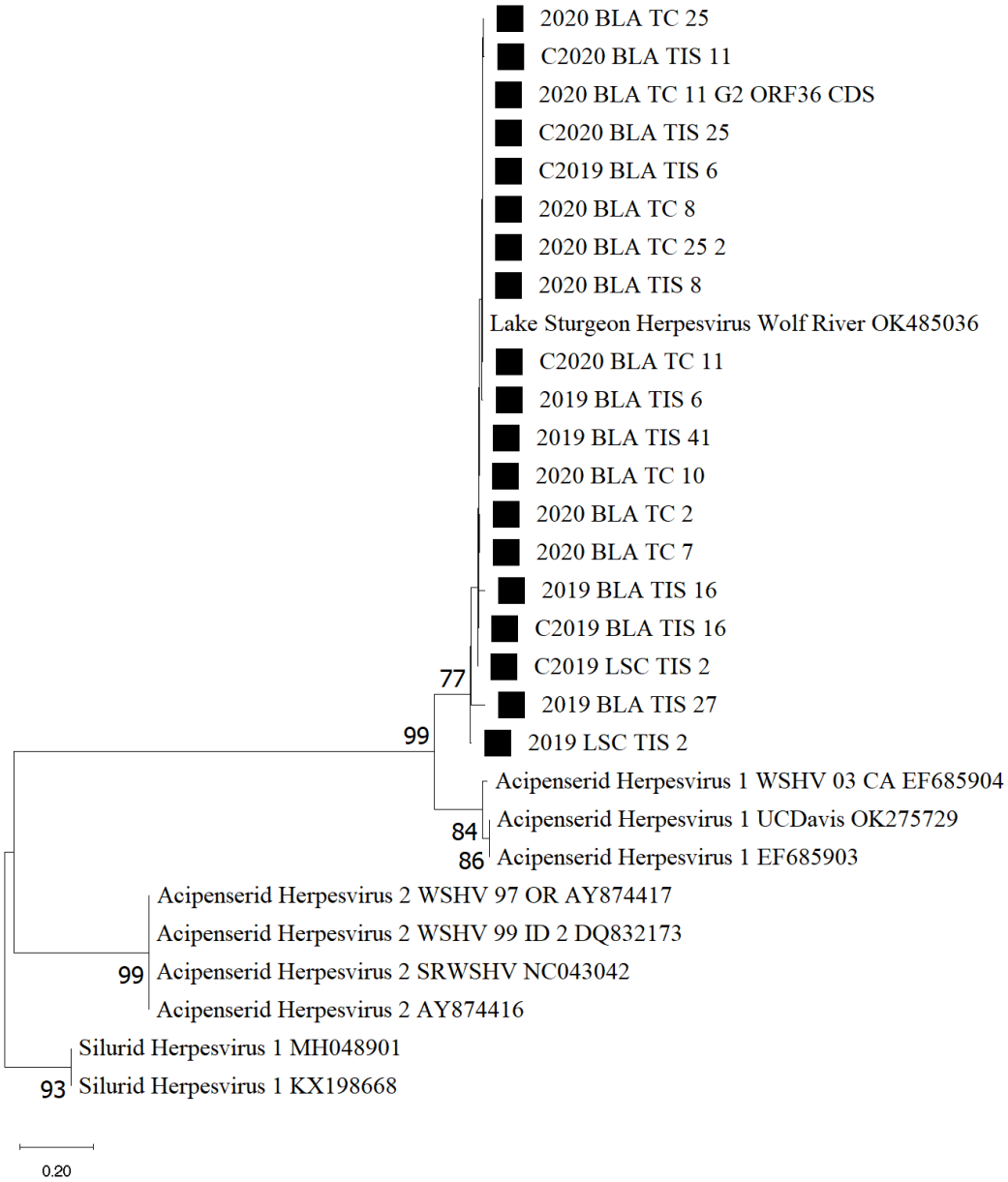

Figure 6. Gross signs of disease that were observed in juvenile lake sturgeon (*Acipenser fulvescens*) that were exposed to the newly isolated lake sturgeon herpesvirus (isolate 200413-11TC). a) Hemorrhage surrounding the oral siphon extending linearly to the barbels indicated with the arrow. b) and c) Focal ulceration of the skin extending into the underlying musculature indicated with arrows. d) Severe erosion of the caudal fin into the caudal peduncle, indicated with the arrow, contiguous with marked pallor extending anteriorly to the trunk.

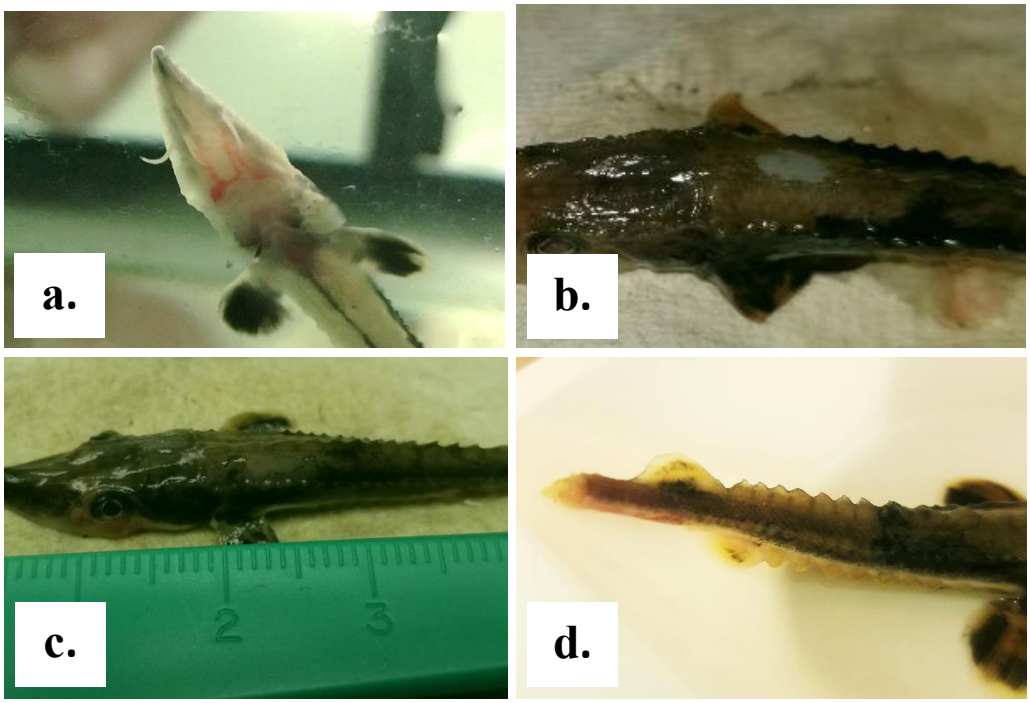

Figure 7. Gross signs of disease that were observed in juvenile lake sturgeon (*Acipenser fulvescens*) that were exposed to the newly isolated Lake Sturgeon herpesvirus (isolate 200413-11TC) via immersion. a) Hemorrhage surrounding the oral siphon extending linearly to the barbels 10 days post-exposure (PE). b) Loss of the caudal fin and ulceration into the caudal peduncle (19 days PE). c) Diffuse hemorrhage on the dorsal aspect of a lake sturgeon, along with deep ulceration of the caudal peduncle, 23 days PE. d) Complete loss of the caudal fin and complete loss of the caudal fin and posterior portions of the caudal peduncle 34 days PE.

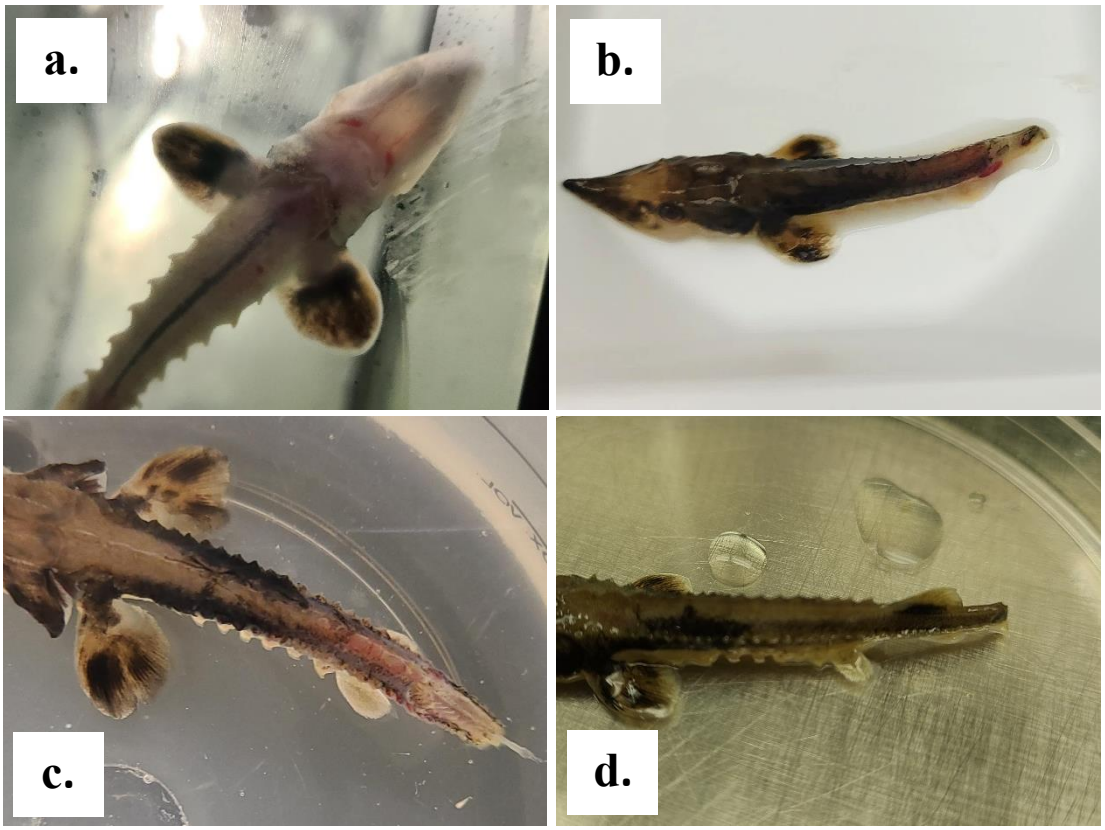

Figure 8. Cumulative percent mortality in juvenile Great Lakes lake sturgeon experimentally immersed in a suspension of the newly isolated lake sturgeon herpesvirus ($1.28x10^6$ $TCID_{50}$/mL). Error bars indicate standard error for every ten days post-infection.

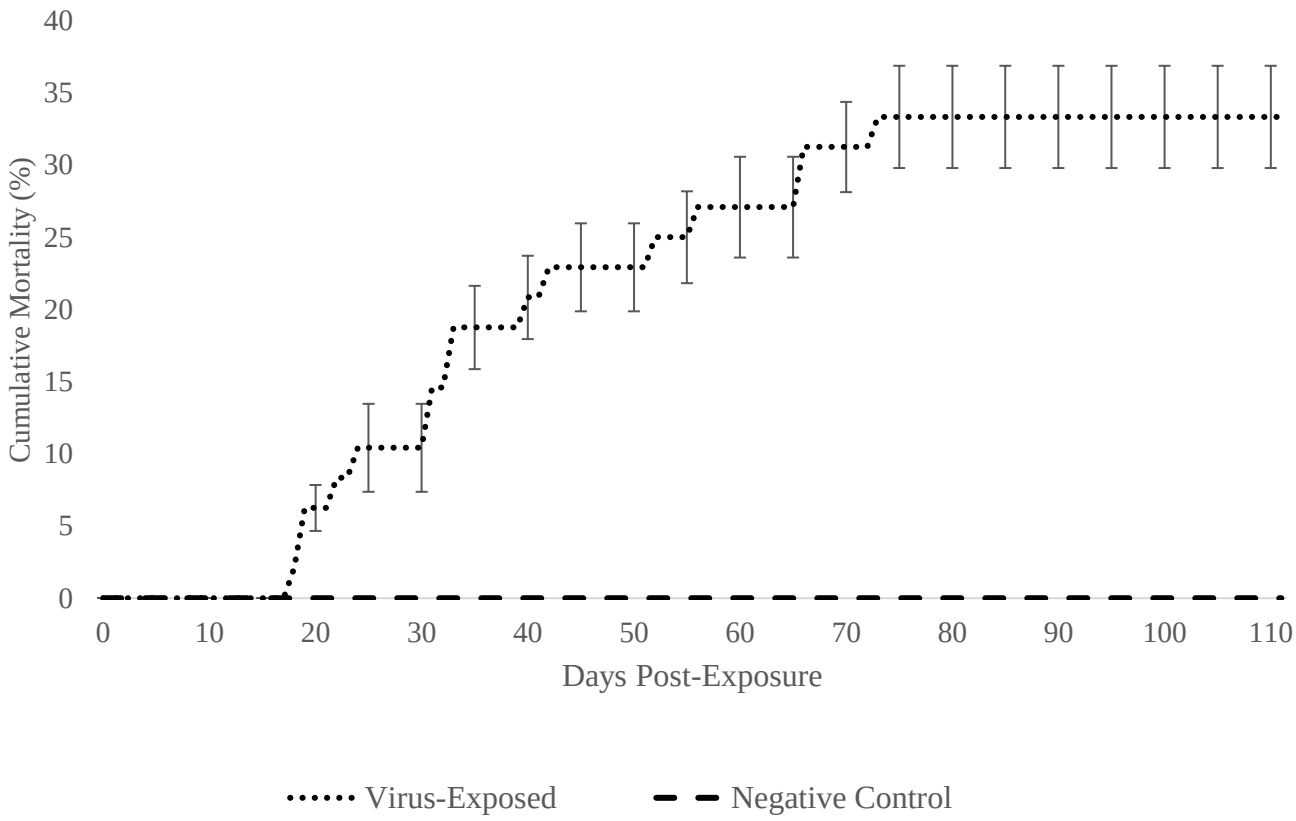

Figure 9. White sturgeon x lake sturgeon (WSxLS) hybrid spleen cells 8 days post-infection with tissue collected from a representative caudal peduncle lesion. Arrows indicate large, multinucleated giant cells, or syncytia.

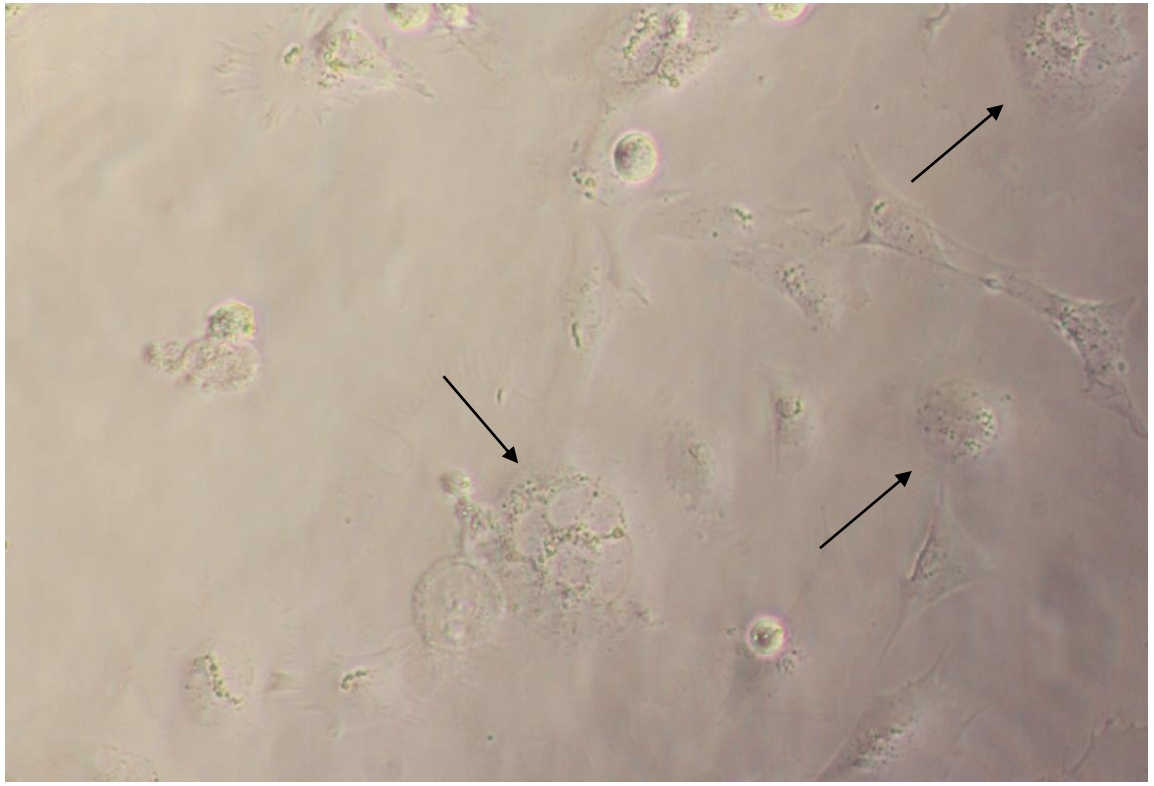

Table 1. Summary data for pilot *in vivo* experiments in juvenile Great Lakes lake sturgeon (*A. fulvescens*; n=5/treatment/pilot experiment) that were exposed to the newly isolated Lake Sturgeon herpesvirus under multiple conditions and exposure routes. $TCID_{50}$, Tissue culture infectious dose 50; IC, intracelomic; FT, flow-through; RC, Re-circulating.

								Cumulative Mortality (%)	
Fish Age (Weeks)	Mean Weight (g)	Tank Type	Water Temperature (°C)	Exposure Route	$TCID_{50}$/mL	Challenge Volume (µL)	Duration (Days)	Exposed	Mock-Exposed
10.5 weeks	1.2	FT	10 ± 1	IC Injection	$1.53x10^2$	50	295	80%	20%
10.5 weeks	1.2	FT	10 ± 1	IC Injection	$1.53x10^2$	25	295	0%	20%
23.2 weeks	2.1	FT	10 ± 1	Immersion	$6.40x10^5$	NA	202	20%	0%
23.2 weeks	2.1	FT	10 ± 1	Immersion	$3.20x10^5$	NA	202	0%	0%
24 weeks	2.1	RC	22 ± 1	IC Injection	$1.53x10^6$	50	208	20%	20%

Table 2. Biometric, lesion prevalence, and packed cell volume (PCV, σ = Standard deviation) data collected from adult lake sturgeon in year one and two from 2 rivers within the Lake Huron (LH) and Lake Erie (LE) watersheds. Means and ranges are shown for biometric data.

Site	Lake Sturgeon Sampled	Total Length (cm)	Weight (kg)	Girth (cm)	Lesion prevalence	PCV	PCV w/ skin lesions
Black River Year 1 (LH)	137	148.6 (112-192)	24.5 (9.8-58.6)	57 (39-90)	6.6%	33.6% σ=5.5	33.3% σ=8.7
Females	26	154.1 (140-192)	40.3 (22.4-58.6)	73.1 (52-90)	15.4%	30.7% σ=6.0	26.0% σ=11.3
Males	111	147.55 (112-168)	20.8 (9.8-44.5)	53.2 (39-83)	4.5%	34.5% σ=5.3	35.2% σ=5.7
Black River Year 2 (LH)	79	150.45 (117-190)	26.62 (13.6-49.7)	55.05 (25-82)	15.2%	37.6% σ=7.9	34.4% σ=5.9
Females	25	160 (137-190)	33.3 (18.2-49.7)	63.8 (49-82)	24.0%	35.7% σ=7.4	37.0% σ=3.6
Males	54	145.9 (117-166)	23.0 (13.6 -38.2)	51 (25-70)	11.1%	38.2% σ=8.1	32.7% σ=6.4
St. Clair River Year 1 (LE)	76	102.9 (27.2-176.1)	15.4 (0.6-29.9)	42.6 (9.5-78.1)	1.1%	25.6% σ=7.6	38.0%

Table 3. Sequences producing significant (e-value < 1.0E-03) alignments (Blastn) with the 2,100 bp complete CDS of the Great Lakes lake sturgeon herpesvirus DNA packaging terminase subunit I.

Description	Acc. No.	% identity	alignment length (bp)	mismatches	e-value	bit score
Acipenserid herpesvirus 1 strain UC Davis	OK275734	85.7	2088	289	0	2422
Lake sturgeon herpesvirus strain Wolf River	OK485036	86.7	825	110	0	993
Lake sturgeon herpesvirus strain Wolf River	OK485036	93.3	570	38	0	857
Lake sturgeon herpesvirus strain Wolf River	OK485036	82.7	681	118	0	697
Acipenserid herpesvirus 1 isolate Italy	EF535575	90.7	290	27	2.00E-106	402
Acipenserid herpesvirus 1	EF535573	90.0	290	29	1.04E-103	393
Lake sturgeon herpesvirus isolate WVL19064-03	OL440177	99.4	156	1	5.74E-69	278
Lake sturgeon herpesvirus isolate WVL18106-01A	OL440176	99.4	156	1	5.74E-69	278
Lake sturgeon herpesvirus isolate WVL18090-01A	OL440175	99.4	156	1	5.74E-69	278
Lake sturgeon herpesvirus isolate WVL18089-02A	OL440173	99.4	156	1	5.74E-69	278
Lake sturgeon herpesvirus isolate WVL21072-4	OL440170	99.4	156	1	5.74E-69	278
Lake sturgeon herpesvirus isolate WVL17229-01A	OL440174	96.8	156	5	1.54E-63	260
Lake sturgeon herpesvirus isolate WVL19055-03	OL440172	96.2	156	6	1.88E-62	255
Lake sturgeon herpesvirus isolate WVL19077-02	OL440179	95.5	156	7	7.98E-61	251
Lake sturgeon herpesvirus isolate WVL19077-01	OL440178	95.5	156	7	7.98E-61	251
Lake sturgeon herpesvirus isolate WVL21072-1	OL440171	95.5	156	7	7.98E-61	251
Tiger shark herpes-like virus	MZ381454	65.9	369	115	2.01E-11	86
Ranid herpesvirus 2 strain ATCC VR-568	DQ665652	72.4	123	34	1.54E-06	69.8

Table 4. Sequences producing significant (e-value < 1.0E-03) alignments (Blastn) with the 4,638 bp complete CDS of the Great Lakes Lake Sturgeon herpesvirus DNA polymerase catalytic subunit.

Description	Acc. No.	% identity	alignment (bp)	mismatches	e-value	bit score
Lake sturgeon herpesvirus strain Wolf River	OK485036	94.264	2859	143	0	4409
Lake sturgeon herpesvirus strain Wolf River	OK485036	90.777	1865	100	0	2631
Acipenserid herpesvirus 1 strain UC Davis	OK275729	80.449	4629	860	0	4250
Acipenserid herpesvirus 1 isolate WSHV-03-CA	EF685904	82.03	473	85	1.05E-126	471
Acipenserid herpesvirus 1 isolate AciHV1 DNA	EF685903	80.973	473	90	3.43E-120	448
Cyprinid herpesvirus 3 strain GY-01	MK260013	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 strain T	MG925491	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 strain M3	MG925490	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 strain I	MG925489	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 strain GZ11-SC	MG925488	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 strain FL	MG925487	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 strain E	MG925486	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 strain Cavoy	MG925485	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 isolate PP3_070411	KX544848	88.525	61	7	6.65E-09	79.7

Table 4 (cont'd)

Description	Acc. No.	% identity	alignment (bp)	mismatches	e-value	bit score
Cyprinid herpesvirus 3 isolate I_10_3	KX544847	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 isolate I_09_2i3	KX544846	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 isolate J1_101110	KX544845	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 isolate CB4_181110	KX544844	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 isolate J2_101110	KX544843	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 isolate PoB3	KX544842	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 isolate FL BAC revertant ORF56-57	KP343684	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 isolate FL BAC revertant ORF136	KP343683	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 strain KHV-GZ11	KJ627438	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 isolate MN	MT914509	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 DNA TUMST1	AP008984	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 strain KHV-U	DQ657948	88.525	61	7	6.65E-09	79.7
Koi herpesvirus strain KHV-I	DQ177346	88.525	61	7	6.65E-09	79.7

Table 4 (cont'd)

Description	Acc. No.	% identity	alignment (bp)	mismatches	e-value	bit score
Koi herpesvirus isolate 323909	DQ128163	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 KHV-JG01	AB196133	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 KHV-JG07	AB196131	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 KHV-JG05	AB195962	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 KHV NRIA 0301	AB195961	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 KHV-I	AB195960	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 3 KHV-U	AB195959	88.525	61	7	6.65E-09	79.7
Cyprinid herpesvirus 1 isolate Ma1	MK507842	83.333	72	12	2.32E-08	77
Cyprinid herpesvirus 1 strain NG-J1	JQ815363	83.333	72	12	2.32E-08	77
Anguillid herpesvirus 1 isolate HVA980811	KX027736	86.792	53	7	1.47E-04	65.3
Anguillid herpesvirus 1 strain UK N080	MW580855	86.792	53	7	1.47E-04	65.3
Anguillid herpesvirus 1 strain HVA 486123	MW580854	86.792	53	7	1.47E-04	65.3
Anguillid herpesvirus 1 strain DK-206116-1	MW580853	86.792	53	7	1.47E-04	65.3
Anguillid herpesvirus 1 strain DK-205223-2	MW580852	86.792	53	7	1.47E-04	65.3

Table 4 (cont'd)

Description	Acc. No.	% identity	alignment (bp)	mismatches	e-value	bit score
Anguillid herpesvirus 1 strain DK-200249	MW580851	86.792	53	7	1.47E-04	65.3
Anguillid herpesvirus 1 strain DK-2008-50-66-1	MW580850	86.792	53	7	1.47E-04	65.3
Anguillid herpesvirus 1 strain 500138	MW580849	86.792	53	7	1.47E-04	65.3
Anguillid herpesvirus 1 isolate C3P2	GU233800	86.792	53	7	1.47E-04	65.3

Table 5. Average nucleotide identity estimations for Wolf River Lake Sturgeon alloherpesvirus 1 (LSAHV1; OK485036) and the Michigan State Strain of putative Lake Sturgeon Alloherpesvirus 2 (LSAHV2).

LSAHV1 vs LSAHV2				
Minimum alignment	Minimum identity	One-way ANI 1	One-way ANI 2	Two-way ANI
125	70%	81.21% (SD: 6.62%), from 344 fragments.	81.36% (SD: 6.49%), from 342 fragments.	82.15% (SD: 6.96%), from 206 fragments.
125	75%	81.57% (SD: 6.41%), from 333 fragments.	81.67% (SD: 6.30%), from 333 fragments.	82.39% (SD: 6.80%), from 202 fragments.
125	80%	83.73% (SD: 5.53%), from 260 fragments.	83.64% (SD: 5.51%), from 264 fragments.	84.19% (SD: 6.13%), from 166 fragments.
125	85%	88.31% (SD: 5.05%), from 119 fragments.	88.17% (SD: 4.99%), from 123 fragments.	89.02% (SD: 5.64%), from 79 fragments.
250	70%	81.22% (SD: 6.61%), from 335 fragments.	81.27% (SD: 6.51%), from 338 fragments.	82.15% (SD: 6.96%), from 206 fragments.
250	75%	81.56% (SD: 6.42%), from 325 fragments.	81.61% (SD: 6.30%), from 328 fragments.	82.39% (SD: 6.80%), from 202 fragments.
250	80%	83.73% (SD: 5.55%), from 253 fragments.	83.61% (SD: 5.51%), from 259 fragments.	84.19% (SD: 6.13%), from 166 fragments.
250	85%	88.29% (SD: 5.10%), from 116 fragments.	88.12% (SD: 5.06%), from 119 fragments.	89.02% (SD: 5.64%), from 79 fragments.
500	70%	81.25% (SD: 6.65%), from 306 fragments.	81.31% (SD: 6.59%), from 305 fragments.	82.17% (SD: 6.97%), from 204 fragments.
500	75%	81.55% (SD: 6.48%), from 298 fragments.	81.65% (SD: 6.39%), from 296 fragments.	82.42% (SD: 6.81%), from 200 fragments.
500	80%	83.75% (SD: 5.64%), from 231 fragments.	83.64% (SD: 5.64%), from 234 fragments.	84.18% (SD: 6.15%), from 165 fragments.
500	85%	88.46% (SD: 5.23%), from 104 fragments.	88.34% (SD: 5.18%), from 106 fragments.	89.08% (SD: 5.65%), from 78 fragments.
750	70%	81.85% (SD: 6.59%), from 253 fragments.	81.99% (SD: 6.49%), from 255 fragments.	82.40% (SD: 6.85%), from 198 fragments.
750	75%	82.04% (SD: 6.46%), from 249 fragments.	82.19% (SD: 6.36%), from 251 fragments.	82.59% (SD: 6.72%), from 195 fragments.
750	80%	83.84% (SD: 5.80%), from 202 fragments.	83.72% (SD: 5.77%), from 210 fragments.	84.15% (SD: 6.16%), from 164 fragments.
750	85%	88.58% (SD: 5.47%), from 92 fragments.	88.38% (SD: 5.38%), from 97 fragments.	89.07% (SD: 5.69%), from 77 fragments.

Table 6. Average nucleotide identity estimations for channel catfish virus (CCV; NC_001493) and blue catfish alloherpesvirus (BCAHV; MK392382).

CCV vs BCAHV				
Minimum alignment	Minimum identity	One-way ANI 1	One-way ANI 2	Two-way ANI
125	70%	93.38% (SD: 4.79%), from 651 fragments.	93.42% (SD: 4.75%), from 651 fragments.	94.73% (SD: 3.49%), from 525 fragments.
125	75%	93.38% (SD: 4.79%), from 651 fragments.	93.42% (SD: 4.75%), from 651 fragments.	94.73% (SD: 3.49%), from 525 fragments.
125	80%	93.48% (SD: 4.59%), from 649 fragments.	93.52% (SD: 4.53%), from 648 fragments.	94.73% (SD: 3.49%), from 525 fragments.
125	85%	93.87% (SD: 3.96%), from 635 fragments.	93.90% (SD: 3.92%), from 636 fragments.	94.78% (SD: 3.37%), from 524 fragments.
250	70%	93.38% (SD: 4.79%), from 651 fragments.	93.41% (SD: 4.79%), from 650 fragments.	94.73% (SD: 3.49%), from 525 fragments.
250	75%	93.38% (SD: 4.79%), from 651 fragments.	93.41% (SD: 4.79%), from 650 fragments.	94.73% (SD: 3.49%), from 525 fragments.
250	80%	93.49% (SD: 4.59%), from 647 fragments.	93.51% (SD: 4.57%), from 647 fragments.	94.73% (SD: 3.49%), from 525 fragments.
250	85%	93.91% (SD: 3.96%), from 628 fragments.	93.96% (SD: 3.92%), from 627 fragments.	94.79% (SD: 3.37%), from 523 fragments.
500	70%	93.71% (SD: 4.52%), from 620 fragments.	93.69% (SD: 4.59%), from 623 fragments.	94.73% (SD: 3.49%), from 525 fragments.
500	75%	93.71% (SD: 4.52%), from 620 fragments.	93.69% (SD: 4.59%), from 623 fragments.	94.73% (SD: 3.49%), from 525 fragments.
500	80%	93.80% (SD: 4.34%), from 617 fragments.	93.82% (SD: 4.33%), from 619 fragments.	94.73% (SD: 3.49%), from 525 fragments.
500	85%	94.13% (SD: 3.82%), from 603 fragments.	94.14% (SD: 3.82%), from 605 fragments.	94.79% (SD: 3.37%), from 523 fragments.
750	70%	94.08% (SD: 4.16%), from 586 fragments.	94.12% (SD: 4.16%), from 584 fragments.	94.76% (SD: 3.46%), from 523 fragments.
750	75%	94.08% (SD: 4.16%), from 586 fragments.	94.12% (SD: 4.16%), from 584 fragments.	94.76% (SD: 3.46%), from 523 fragments.
750	80%	94.12% (SD: 4.09%), from 585 fragments.	94.19% (SD: 4.02%), from 582 fragments.	94.76% (SD: 3.46%), from 523 fragments.
750	85%	94.35% (SD: 3.67%), from 576 fragments.	94.39% (SD: 3.66%), from 574 fragments.	94.82% (SD: 3.34%), from 521 fragments.

Table 7. Average nucleotide identity estimations for Cyprinid herpesvirus 1 (CyHV1; JQ815363) and Cyprinid herpesvirus 3 (CyHV3; MK260013).

CyHV1 vs CyHV3				
Minimum alignment	Minimum identity	One-way ANI 1	One-way ANI 2	Two-way ANI
125	70%	74.15% (SD: 4.61%), from 78 fragments.	74.46% (SD: 4.43%), from 76 fragments.	Insufficient hits
125	75%	75.97% (SD: 3.86%), from 58 fragments.	76.42% (SD: 3.56%), from 55 fragments.	Insufficient hits
125	80%	Insufficient hits	Insufficient hits	Insufficient hits
125	85%	Insufficient hits	Insufficient hits	Insufficient hits
250	70%	72.90% (SD: 3.98%), from 57 fragments.	73.33% (SD: 4.02%), from 54 fragments.	Insufficient hits
250	75%	Insufficient hits	Insufficient hits	Insufficient hits
250	80%	Not tested	Not tested	Not tested
250	85%	Not tested	Not tested	Not tested
500	70%	Insufficient hits	Insufficient hits	Insufficient hits
500	75%	Insufficient hits	Insufficient hits	Insufficient hits
500	80%	Not tested	Not tested	Not tested
500	85%	Not tested	Not tested	Not tested
750	70%	Insufficient hits	Insufficient hits	Insufficient hits
750	75%	Not tested	Not tested	Not tested
750	80%	Not tested	Not tested	Not tested
750	85%	Not tested	Not tested	Not tested

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CHAPTER 3:

SURVEILLANCE FOR STURGEON-PATHOGENIC VIRUSES IN WILD ADULT AND HATCHERY-REARED GREAT LAKES LAKE STURGEON (*ACIPENSER FULVESCENS*)

1. Abstract

Relatively little is known about viral diseases that may be present in lake sturgeon (*Acipenser fulvescens*) within the Great Lakes basin despite being the only native sturgeon species therein. Globally, the propagation of sturgeon for human consumption continues to grow, as do reports of viruses linked to diseases in sturgeon. In North America, reports of viral disease outbreaks have increased and appear to be encroaching towards the Great Lakes. However, until the recent identification of a lake sturgeon herpesvirus (Chapter Two), there we're no reports of sturgeon pathogenic viruses in Great Lakes lake sturgeon. In other sturgeons, detection of many of the viruses causing these infections, including Nucleocytoplasmic Large DNA Viruses (NCLDVs) and frog virus 3 (FV3), requires specialized diagnostic assays, some of which are not routinely utilized in the Great Lakes. To this end, a two year disease surveillance study of both wild adult (Black River, Lake Huron watershed and St. Clair River, Lake Erie watershed) and hatchery-reared juvenile Great Lakes lake sturgeon (Cedar and Whitefish River, Lake Michigan watershed; Ontonagon River, Lake Superior watershed; Black River, Lake Huron watershed) was conducted using five cell lines (e.g., *Epithelioma papulosum cyprini*, EPC; Chinook salmon embryo-214, CHSE-214; white sturgeon skin, WSSK-1; white sturgeon x lake sturgeon hybrid, WSxLS; white sturgeon gonad, WSGO) coupled with quantitative and conventional polymerase chain reaction assays to detect NCLDVs and FV3. Tissues (e.g., fin, gill, lesion) and/or fluids (e.g., blood, reproductive fluids) were non-lethally collected from 291 adult lake sturgeon in two Great Lakes watersheds (Erie and Huron) and assayed for viral infections. Similarly, tissues (e.g., fin, gill, opercula, visceral organs) were collected from juvenile lake sturgeon from four streamside rearing facilities and screened for sturgeon pathogenic viruses. Following these analyses, neither FV3 nor NCLDVs were detected *in vitro* or via PCR in wild adult or juvenile

LST. Fortunately, findings suggest these sturgeon pathogenic viruses are not yet present in the Huron and Erie watersheds; however given recent host and geographic range expansions, their possible emergence in the Great Lakes basin should be monitored.

2. Introduction

Lake sturgeon (*Acipenser fulvescens*; Family *Acipenseridae*) are culturally and ecologically important fish within the GL, but their abundance is estimated to be <1% of historical values (Hay-Chmielewski et al. 1997). Numerous factors are proposed to be contributing to these substantial population declines (e.g., dam construction, habitat degradation, unique life history traits, overfishing; Crouse at al. 1999, Hayes et al. 2012, Pollock et al. 2015). Furthermore, some of these factors also place lake sturgeon at a heightened risk for experiencing losses due to infectious diseases. Among disease causing agents in sturgeons, viruses are particularly problematic (Mugetti et al. 2020).

In addition to herpesviruses (Family *Alloherpesviridae*; see Chapters One and Two), the Nucleocytoplasmic Large DNA Viruses (NCLDVs) and frog virus 3 (FV3) have proven to be particularly damaging to multiple sturgeon species (Kurobe et al. 2010, Clouthier et al. 2013, Waltzek et al. 2014, Clouthier et al. 2015). The NCLDVs hail from multiple virus families (e.g., *Poxviridae, Iridoviridae, Asfarviridae, Phycoviridae, Ascoviridae, Mimiviridae,* and the proposed family *Marseilleviridae;* Colson et al. 2012, Colson et al. 2013). These viruses (Phylum Nucleocytoviricota, proposed Order Megavirales; Colson et al. 2013) were given the name NCLDVs given their large genome size compared to other viruses and because they replicate in host cell nuclei and/or cytoplasm (Koonin et al. 2019). Among them are those that have been

linked to disease in sturgeon and thus collectively referred to as “sturgeon NCLDVs,” namely: white sturgeon iridovirus, Missouri River sturgeon iridovirus, British Columbia sturgeon iridovirus, Shortnose sturgeon virus, and Namao virus (Clouthier et al. 2015, Raverty et al. 2003, Watson et al. 1998).

One of the most well-known sturgeon NCLDVs is white sturgeon iridovirus (WSIV; proposed Family *Mimiviridae*; Clouthier et al. 2015). First detected in California, it was linked to significant disease and mortality in cultured white sturgeon (*A. transmontanus*; Hedrick et al, 1990). Virulence was further studied later in the decade, when controlled laboratory experiments demonstrated a clear ability to induce disease and mortality in cultured white sturgeon (Hedrick et al. 1990). WSIV remains a contemporary threat to sturgeon culture globally, where it continues to cause mortality in white sturgeon in the Western US and other sturgeons globally (Hofsoe-Oppermann et al. 2020, Drennan et al. 2006).

Missouri River sturgeon iridovirus, British Columbia sturgeon iridovirus, and shortnose sturgeon iridovirus have also been linked to mortality events in multiple sturgeon species (shortnose sturgeon, *A. brevirostrum*; pallid sturgeon, *Scaphirhynchus albus*; shovelnose sturgeon, *S. platorhynchus*; Raverty et al. 2003, Kurobe et al. 2011, LaPatra et al. 2014). Generally, NCLDVs are linked to a damaging wasting disease of sturgeons, where the viruses target the integument of infected sturgeon (Clouthier et al. 2013). Of particular concern to Great Lakes lake sturgeon is the recently described Namao virus (Clouthier et al. 2013). Namao virus was recovered from a lake sturgeon mortality event in Manitoba, Canada, where juvenile hatchery reared lake sturgeon exhibited significant disease and mortality (Clouthier et al. 2013). Not only is Namao virus the only sturgeon NCLDV documented in lake sturgeon, but the current understood geographic range is relatively close to the Great Lakes basin (Clouthier et al. 2013).

Another viral pathogen of concern for sturgeons in North American is frog virus 3 (Family *Iridoviridae*, Rakesh et al. 1999). Frog virus 3 is notorious for its wide host range, and in 2009, it was first documented in hatchery reared pallid sturgeon exhibiting gross signs of disease and mortality (*S. albus*) in a Missouri rearing facility (Waltzek et al. 2014). Alarmingly, frog virus 3 has been documented in states adjacent to the Great Lakes, and while there are currently no reports of the virus in Great Lakes fishes, it should not be ruled out as a possible threat to already endangered lake sturgeon. Furthermore, trials conducted in Waltzek et al (2014) demonstrated that under experimental conditions, frog virus 3 can induce disease and mortality in juvenile pallid sturgeon (Waltzek et al. 2014).

The diagnosis of NCLDVs and FV3 often requires specific *in vitro* and molecular techniques. FV3 can be isolated *in vitro* using amphibian/reptilian cell lines (Rakesh et al. 1999), and reports of its detection using fish-derived cell lines are sparse (*Epithelioma papulosum cyprini*; EPC; Waltzek et al. 2014). A PCR assay described in Waltzek et al (2014) also readily detects FV3; however this PCR assay is not routinely employed in the Great Lakes basin. Some NCLDVs are unable to be propagated *in vitro*. As a result, a series of molecular assays were designed by Clouthier et al. (2015). Two quantitative PCR (qPCR) assays (e.g., Q1 and Q2) target the highly conserved Major Capsid Protein of sturgeon NCLDVs, however this assay cannot be used to discern amongst sturgeon NCLDVs. To do so, a conventional PCR (cPCR) was also developed (C1; Clouthier et al. 2015). Hence, if virus is detected using the Q1 or Q2 qPCRs that target all sturgeon NCLDVs, follow-up testing using the C1 cPCR is deployed and followed by sequencing for virus identification (Clouthier et at. 2015). None of the above assays are in the suite of routinely employed diagnostic tests in the Great Lakes.

Given the detection of these viruses necessitates specialized diagnostic techniques, little is known regarding their presence in Great Lakes lake sturgeon. Likewise, the negative impacts these viruses could have in adult and juvenile Great Lakes lake sturgeon populations is largely unknown. Furthermore, given hatchery rearing of lake sturgeon for conservation has increased in the Great Lakes, and rearing facilities are populated with gametes or larvae from wild adults, understanding the pathogens within wild adult populations is highly important. With this in mind, a two-year disease surveillance study employing the specific *in vitro* and molecular assays above was undertaken to uncover the presence of sturgeon pathogenic viruses (e.g., NCLDVs and FV3) in two populations of adult Great Lakes lake sturgeon (Lake Erie and Huron watersheds). Additionally, and given the increase in the hatchery-rearing of lake sturgeon, juvenile lake sturgeon were collected, examined, and screened for these viruses from four streamside rearing facilities in three different Great Lakes watersheds (Lake Erie, Michigan, and Superior). This disease surveillance study marks the first-time sturgeon specific cell lines in conjunction with specialized molecular assays aimed at the detection of sturgeon pathogenic viruses have been employed on a large scale across the Great Lakes basin.

3. Materials and methods

3.1. Collection of adult lake sturgeon

During May and June of 2019, adult lake sturgeon were non-lethally sampled from two Great Lakes locations in the Black River (Cheboygan County, Michigan; Lake Huron watershed) and the St. Clair River (Macomb County, Michigan; Lake Erie watershed). Fish were sampled and captured via hand netting (Black River) or trot line (St. Clair River) in collaboration with

Michigan State University and Michigan Department of Natural Resources personnel. All fish were released after sampling.

3.2. Non-lethal sampling of adult lake sturgeon

Once captured, fish were gently restrained while immersed in water for gross clinical examination and tissue collection. Biometric data including length, weight, and girth were also collected at time of capture. Following restraint, during year one whole blood was collected via caudal venipuncture (<5 mL), where blood was divided between a heparinized tube for packed cell volume (1 mL; heparin sodium salt, ThermoFisher Scientific Waltham, MA, USA), a 1.5 ml tube for virus isolation (~250 µL), and a 5 mL tube for serum collection (3-4 mL) and placed into an ice-chilled cooler. If spawning, reproductive fluids and/or eggs were gently expressed and collected via syringe or Whirl Pak™ (VWR, Radnor, PA, USA). Following collection, gamete samples were maintained in an ice chilled cooler. Fin and gill biopsies were collected using a separate pair of clean scissors for each fish, and biopsied tissues (<250 mg) were placed into 1 mL RNA Later (Sigma-Aldrich, St. Louis MO, USA) and frozen for future analyses. In year one, all tissues were frozen at -20°C prior to analyses.

In year two, fish were sampled again at the Black River, however sample collection methods were modified. Gamete and whole blood were collected similarly, however samples for virus isolation were collected into a viral growth media containing Earle's salt-based minimal essential medium (EMEM; Invitrogen, Carlsbad CA, USA) supplemented with 10% BD BactoTM tryptose phosphate broth (TPB; ThermoFisher Scientific, Waltham MA, USA), 2% Fetal Bovine Serum (FBS; Gemini Bioproducts, Sacramento CA, USA), penicillin (100 IU mL-1),

streptomycin (100 µg mL-1; Invitrogen, Carlsbad CA, USA), gentamicin sulfate (0.1 mg mL-1; Sigma-Aldrich, St. Louis, MO, USA), amphotericin B (2.5 µg mL-1; Sigma-Aldrich, St. Louis MO, USA), and buffered with sodium bicarbonate (pH 7.4-7.6; Sigma-Aldrich, St. Louis MO, USA) at a ratio of 1:4 (V:V; gametes) or 1:10 (V:V; whole blood). Additionally, all samples for virus isolation were maintained at 4°C prior to inoculation onto cells. Fin and gill biopsies were also collected similarly to year one and frozen at -20°C.

3.3. Hematology for adult lake sturgeon

Within eight hours of collection, heparinized blood was aliquoted into a micro-hematocrit tube, spun at 16,000 rpm in a CritSpin™ Hematocrit Centrifuge (2 minutes; Iris Sample Processing, Westwood, MA, USA), after which the packed cell volume (PCV) was measured using the provided card-style reader (product #HR05; Grant et al, 2015).

3.4. Juvenile lake sturgeon collection

In collaboration with Michigan DNR, juvenile lake sturgeon were collected from four streamside rearing facilities in year one, some of which were populated with gametes collected from wild adult lake sturgeon. Namely, the Black River Sturgeon Hatchery (Cheboygan County, Michigan, Lake Huron watershed) populated with larvae and gametes collected from the Black River (Presque Isle County, Michigan, Lake Huron watershed), the West Branch Ontonagon River Streamside Rearing Facility (Baraga County, Michigan, Lake Superior watershed) populated with larvae collected from the Sturgeon River (Baraga County, Michigan, Lake Superior watershed), and the Cedar River and Whitefish River Streamside Rearing Facilities

(Menominee County, Michigan and Alger County, Michigan, Lake Michigan watershed) populated with gametes collected from the Peshtigo River (Marinette County, Wisconsin, Lake Michigan watershed) were sampled. Sixty fish from each facility were sampled once five-six weeks post-hatch and again just prior to stocking (approximately three months post-hatch).

In year two and as a result of the ongoing COVID-19 pandemic, the only sturgeon rearing facility populated out of the four mentioned above was the Black River Sturgeon Rearing Facility. Thus, sixty fish from this facility were examined six weeks post-hatch and again just prior to stocking (approximately three months post-hatch).

3.5. Juvenile lake sturgeon examination and sample collection

All juvenile lake sturgeon collected from all rearing facilities across both years were collected and driven live to the Michigan State University Research Containment Facility for clinical examination and tissue collection. Upon arrival, fish were euthanized with an overdose of tricaine methanesulfonate (MS-222; Syndel, Ferndale WA, USA; 250 mg/L) buffered with sodium bicarbonate (500 mg/L). Weight and length measurements were taken, and gross external and internal exams were performed. Next, multiple tissues were collected in pools (n= 5 fish/pool) for virus isolation, including visceral organs (kidney, spleen, and heart), pectoral fin, gill, barbel, and/or oral siphon. In 2020, tissues were collected directly into the viral growth media detailed in Section 2.2.

3.6. Maintenance of cultured cells

Five fish cell lines were utilized in this study for virus isolation including *Epithelioma papulosum cyprini* (EPC; Fijan et. al 1983), Chinook salmon embryo (CHSE-214; Lannan et. al 1984), white sturgeon skin (WSSK-1, Hedrick et al. 1991), white sturgeon gonad (WSGO, Watson et al. 1998), and a white sturgeon and lake sturgeon hybrid spleen cell line (WSxLS). All cell lines were maintained in 75 cm^2 flasks (Corning, Corning, NY, US) at 25°C (EPC) or 21°C (WSSK-1, WSGO, WSxLS, CHSE). EPC and all sturgeon-derived cell lines were maintained in a growth media consisting of EMEM supplemented with 10% TPB, 10% FBS, penicillin (100 IU mL^{-1}), streptomycin (100 µg mL^{-1}), amphotericin B (2.5 µg mL^{-1}), 2mM L-Glutamine, and buffered with sodium bicarbonate (pH 7.4-7.6), CHSE cells were grown in Eagle's MEM (with Earle's salts, nonessential amino acids, and sodium pyruvate; ATCC, Manassas, VA, USA), nystatin (20 µg mL^{-1}; Sigma-Aldrich, St. Louis, MO, USA), L-glutamine, FBS, penicillin, and streptomycin as above. Cells were prepared for virus isolation in flat-bottom 96-well plates and supplemented with media as further detailed in Chapter Two, Section 2.4.3.

3.7. Adult and juvenile lake sturgeon sample processing

During year one, samples collected from adult lake sturgeon were thawed from -80°C on ice and processed for virus isolation. Lesion and reproductive fluid samples were diluted 1:4 (W:V) with EMEM supplemented with TPB, penicillin (100 IU mL^{-1}), streptomycin (100 µg mL^{-1}), gentamicin sulfate (0.1 mg mL^{-1}), amphotericin B (2.5 µg mL^{-1}), and buffered with UltraPure Tris (to pH 7.4-7.6; Invitrogen, Carlsbad, CA, USA). Whole blood samples were diluted 1:10 (W:V) with the solution detailed above. Following dilution, all samples were vortexed or

homogenized, centrifuged (4°C, 5,000 RPM, 30 minutes), and clarified supernatant was aliquoted for virus isolation. During year two, samples collected from adult lake sturgeon were diluted upon collection detailed above and were vortexed, centrifuged (4°C, 5,000 RPM, 30 minutes), and clarified supernatant similarly aliquoted.

For juvenile lake sturgeon during year one, tissues (e.g., visceral mass for five-six week post-hatch fish; kidney, spleen, and heart, pooled in groups of five fish for approximately three month post-hatch fish; fin and gill or fin and opercula pooled in groups of five fish) were weighed and diluted 1:4 (W:V) with EMEM supplemented with TPB, penicillin (100 IU mL^{-1}), streptomycin (100 µg mL^{-1}), gentamicin sulfate (0.1 mg mL^{-1}), amphotericin B (2.5 µg mL^{-1}), and buffered with UltraPure Tris (to pH 7.4-7.6; Invitrogen, Carlsbad, CA, USA). During year two, samples collected for virus isolation and collected directly into media as detailed above were cut up using sterile scissors, vortexed, and centrifuged (4°C, 5,000 RPM, 30 minutes). Following separation of clarified supernatant for all adult and juvenile lake sturgeon samples, supernatant was incubated at 4°C for 24 hours or 15°C for 2 hours.

3.8. Virus isolation

After processing, clarified supernatant was centrifuged once more to remove any remaining tissue (4°C, 5,000 RPM, 15 minutes). Following centrifugation, 25 µL of supernatant was inoculated in triplicate onto selected (all samples were inoculated onto EPC, CHSE-214, WSSK-1, and WSxLS; select samples were inoculated onto WSGO) cell lines. Samples were passaged four to six times for a total observation period of 60 days. Following the 60-day observation period, all cell culture samples (i.e., regardless of the development of cytopathic effects; CPE)

were pooled (20/p) by original tissue type and preserved at -80 for NCLDV and FV3 molecular analyses.

3.9. Nucleic acid extraction

Pools of cultured cells and supernatant detailed above were thawed from -80°C on ice for nucleic acid (NA) extraction. Nucleic acid extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen Inc., Germantown, MD, USA) according to the manufacturers protocol for cultured cells.

Similarly, fin and gill biopsy samples collected from adult lake sturgeon and saved in RNALater (Sigma-Aldrich, St. Louis, MO, USA) were thawed on ice from -80°C. Tissues were removed from the solution using sterile forceps (one pair/sample) and NA was extracted with the DNeasy PowerLyzer PowerSoil kit (Qiagen Inc., Germantown, MD, USA) following the manufacturers protocol for 250mg soil samples with the exception that tissue samples were homogenized in a MiniBeadBeater-16 Model 607 (BioSpec Products Inc., Bartlesville, OK, USA) for five minutes at 3450 RPM, cooled and homogenized once more for five minutes. If needed, tissues were homogenized for a third five-minute cycle. Prior to the start of nucleic acid extraction and to quality check for PCR inhibition, 2 µL of VetMax Internal Positive Control DNA (20,000 copies; ThermoFisher Scientific, Waltham, MA, USA) was added to each sample. Following extraction, NA was quantified via a Qubit™ fluorometer using the double-stranded DNA broad range assay kit (dsDNA BR Assay Kit; Invitrogen, Carlsbad CA, USA) and diluted to 20ng/µL with sterile nuclease free water.

3.10. Endpoint PCR analysis for frog virus 3

Extract from tissue culture cell/supernatant pools from adults and juveniles and fin and gill tissue extract from adults were screened for frog virus 3 via the endpoint polymerase chain reaction targeting the full length major capsid protein gene (MCP) described in Hyatt et al. (2000) and as modified in Waltzek et al (2014). The PCR reaction consisted of 25 µL containing 2X GoTaq Green MasterMix (Fisher Scientific, Waltham, MA, US), 40 pmol of MCP-1 (modified to exclude the last three base pairs; 5'-CAC CGT GTA TCT TAT AAT AAA AAG GAA-3'; Waltzek et al, 2014), 40 pmol of MCP-6 (5'-AAA GAC CCG TTT TGC AGC AAA C-3'), and no more than 20 ng of DNA template. The cycling parameters were as follows: 95°C for 3 minutes, 35 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for two minutes, and a final extension step of 72°C for 10 minutes. All conventional PCR reactions were done in a Mastercycler Pro S 6325 Vapo protect PCR System (Eppendorf, Hamburg, Germany). Extracted NA from a previously confirmed Frog Virus 3 cell culture isolate was utilized as a positive control in every run, and nuclease free water as the negative control. Following amplification, PCR products were visualized via gel electrophoresis in a 1.5% agarose gel with SYBR Safe DNA Gel Stain (ThermoFisher Scientific, Waltham, MA, USA; 1 mM) at 100V for 30 minutes and then visualized via UV transillumination under a UVP DigiDoc-It Imaging System (UVP, Upland, CA, USA). A TrackIt™ 1 kb plus DNA ladder (Invitrogen, Carlsbad, CA, USA) was utilized for reference of amplicon size. The reported amplicon size for this assay is 1,511 bp (Waltzek et al. 2015)

3.11. Quantitative PCR analysis for Nucleocytoplasmic Large DNA Viruses

To screen for sturgeon NCLDVs, a quantitative PCR assay targeting the highly conserved major capsid protein and designed in Clouthier et al. (2015) was employed. The PCR reactions were 25 µL and comprised of 2X TaqMan Universal PCR Master Mix (Applied Biosystems, Waltham, MA, USA), 400nM of forward primer sNCLDV F21 (5'-CCT GAC GGT ATC AAC GTA-3'), 800nM of reverse primer sNCLDV R18 (5'-TCG ATG CGA ATC GAC TCA AA-3'), 250 nM Q2 assay probe sNCLDV P19 (5'-6FAM-CAC CAA CCT TCT GGA AGT GCC AAC TTT T-TAMRA-3'), 1 µL VetMax™ Xeno™ Internal Positive Control - VIC™ Assay (ThermoFisher Scientific, Waltham, MA, US), <40 ng DNA template, and nuclease free water. Reactions were performed using a QuantStudio™ 3 - 96-Well (ThermoFisher Scientific, Waltham, MA, USA) in MicroAmp™ Fast Optical 96-well plates (ThermoFisher Scientific, Waltham, MA, USA) with the following cycling parameters: 50°C for two minutes, 95°C for ten minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Extracted NA from a previously confirmed white sturgeon iridovirus cell culture isolate was utilized as a positive control for every run, nuclease free water as the negative control, and IPC DNA as the IPC control. Results were analyzed using the Design and Analysis application on the ThermoFisher Connect platform (ThermoFisher Scientific, Waltham, MA, US).

If the IPC quantification value (Cq) was outside of the manufacturer's suggested range (Cq of 17-34), samples were re-run with two-fold diluted DNA template. If following the second run with diluted template IPC values were still outside of the expected range, DNA template was diluted 10-fold and re-run again.

3.12. Conventional PCR analysis for Nucleocytoplasmic Large DNA Viruses

Following analysis with the Q2 qPCR assay (Clouthier et al. 2015), suspect samples (e.g., those with an IPC value within manufacturer detailed limits and amplification for sturgeon NCLDVs within positive detection limits) were analyzed using the conventional PCR assay also designed in Clouthier et al. (2015) that targets a fragment of the major capsid protein. Each 25 µL reaction consisted of 100nM forward primer ginF (5'-GGT ATC AAC GTA TAT TCG TTT GC-3'), 100 nM reverse primer glaR (5'-GCA AAC GAA TAT ACG TTG ATA CC-3'), 1X PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, Coralville, IA, USA), <40 ng of DNA template, and nuclease free water. PCR reactions were performed in a Mastercycler Pro S 6325 Vapo protect PCR System (Eppendorf, Hamburg, Germany) with the following cycling parameters: 5 minutes at 95°C, 40 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30s, and a final extension step of 72°C for 10 minutes (Clouthier et al. 2015). Extracted NA from a previously confirmed white sturgeon iridovirus cell culture isolate was utilized as a positive control for every run and nuclease free water as the negative control.

Following amplification, DNA was visualized via gel electrophoresis in a 1.5% agarose gel supplemented with SYBR Safe DNA Gel Stain (ThermoFisher Scientific, Waltham, MA, USA; 1 mM). Each gel was processed at 100V for 30 minutes and visualized via UV transillumination under a UVP DigiDoc-It Imaging System (UVP, Upland, CA, USA). DNA band size was determined via comparison to the TrackIt™ 1 kb plus DNA ladder (Invitrogen, Carlsbad, CA, USA). The reported amplicon size for this assay is 219 bp (Clouthier et al. 2015).

3.13. Gene sequencing and analysis

Representative PCR amplicons generated via the C1 PCR (Clouthier et al. 2015; one from each site and year) were purified using the QIAquick Spin Kit (Qiagen Inc., Germantown, MD, USA) and cloned using the TOPO-TA cloning kit with TOP-10 *Escherichia coli* chemically competent cells (Qiagen Inc. Germantown, MD, USA) following manufacturer's protocols with the modification that clones were left to transform for 12 hours (37°C, 200 RPM). Following transformation, *E. coli* cell suspension was plated onto Luria-Bertani (LB) plates (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 50mg/mL of kanamycin (MilliporeSigma, St. Louis, MO, USA) and incubated for <24 hours. Individual colony forming units were suspended in 10 µL of LB broth and analyzed via PCR where 25 µL reactions consisted of 12.5 µL 2X GoTaq Green (ThermoFisher Scientific, Waltham, MA, USA), 0.5 µL of each primer (M-13 Forward-20: 5' GTA AAA CGA CGG CCA G 3' and M 13 Reverse: 5' CAG GAA ACA GCT ATG AC 3'), 2 µL of broth/bacteria suspension, and nuclease free water. Cycling parameters were as follows: 94°C for 10 minutes, 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds, and a final extension at 72°C for 10 minutes. Following amplification, PCR products were visualized via gel electrophoresis and purified as described above and clones which were found to have been the expected band size (219 bp target plus plasmid DNA; ~300 bp) were submitted to the Michigan State University Genomics Technical Support Facility for bi-directional Sanger sequencing.

Once sequenced, resultant chromatograms were quality checked, trimmed, and converted to .FASTA files using Chromatogram Explorer (version 5.0.2.3). Following this, contigs were assembled using the BioEdit sequence alignment editor (version 7.2.5; Hall 1999) via the Contig Assembly Program. Generated sequences were analyzed using the nucleotide Basic Local

Alignment Search Tool (BLASTn) software from the National Center for Biotechnology Information (NCBI) nucleotide database using the megablast algorithm for Highly Similar Sequences.

4. Results

4.1. Adult lake sturgeon sampling

During year one and year two, a total of 292 adult lake sturgeon were non-lethally sampled. In year one, 137 fish from the Black River and 76 fish from the St. Clair River were collected whereas in year two, 79 fish were sampled from the Black River. Biometric data separated by sex is detailed in Table 8.

4.2. Adult lake sturgeon hematology

Fish from all sites in year one and two had a mean PCV of 32.2%, with some variation by sampling site and year (Table 8). No clear trends in PCV were appreciable by site or year (Table 8); however, female lake sturgeon had slightly lower mean PCVs in both year one and two at the Black River site when compared to males (Table 8). PCV measurements were comparable to reference intervals established by DiVincenti et. al and Cassle et. al (2013).

4.3. Juvenile lake sturgeon sampling

During year one, 120 juvenile lake sturgeon were collected from each rearing facility, wherein 60 fish were collected five-six weeks post-hatch and 60 more approximately three

months post-hatch (Table 9). Upon clinical examination, the majority of fish appeared apparently healthy with no external signs of disease. However, a few (<5) fish from each facility collected just prior to stocking presented with mild congestion at the base of pectoral and/or pelvic fins and mild peri-oral hemorrhaging (Table 2). No gross signs of disease were noted in fish collected five-six weeks post-hatch (Table 2). Similarly, 120 juvenile lake sturgeon were collected from the Black River SRF in 2020 as a result of the COVID-19 pandemic hampering sturgeon spawning and rearing efforts. No gross signs of disease were noted in fish from each age groups (Table 9).

4.4. *In vitro* virus isolation

4.4.1 Wild adult lake sturgeon

During year one, 333 tissue samples for virus isolation were collected from adult lake sturgeon from the Black River and St. Clair River (Table 10). During year two, 137 samples for virus isolation were collected from adult lake sturgeon from the Black River (Table 10). Following 60-day observation periods, no samples resulted in cytopathic effects on any of the five cell lines (e.g., EPC, CHSE, WSSK, WSxLS, and/or WSGO) other than those previously reported in Chapter Two (i.e., those associated with the presence of a lake sturgeon herpesvirus).

4.4.2. Juvenile lake sturgeon

During year one and year two, 120 pools of visceral organs or kidney, spleen, and heart (12 pools of tissue from five fish, per 60 fish examined) and 120 pools of fin, gill, and/or opoercula

tissue (12 pools of tissue from five per 60 fish examined) were collected for virus isolation from juvenile lake sturgeon being reared at the Cedar, Whitefish, Ontonagon, and Black Rivers. Following 60-day observation periods, no samples resulted in cytopathic effects on any of the five cell lines (e.g., EPC, CHSE, WSSK, WSxLS, and/or WSGO).

4.5. Molecular and gene sequence analysis

4.5.1. Frog virus 3 conventional PCR

Following analyses of 282 fin and gill tissues collected from adult lake sturgeon using the FV3 assay detailed above (Waltzek et al. 2014), FV3 was not detected (Table 3). Additionally, no evidence of FV3 was detected molecularly in any pools of cell culture supernatant from adult and juvenile lake sturgeon (33 pools from adults, 60 pools from juveniles).

4.5.2. Sturgeon Nucleocytoplasmic Large DNA Virus qPCR

Following completion of the 60-day observation period on four-five cell lines, cells and supernatant were pooled by original tissue type (<20 fish/pool) and analyzed for the presence of sturgeon NCLDVs via the Q2 assay described above (Clouthier et al. 2015). Extract from 93 cell culture cell/supernatant samples (pooled <20 fish/pool; 33 from adults, 60 from juveniles; Table 2 and 3) were all negative for sturgeon NCLDVs via the Q2 PCR (Clouthier et al. 2015). Additionally, extract from 282 fin and gill tissues collected from adult lake sturgeon were analyzed for the presence of sturgeon NCLDVs via the Q2 assay (Clouthier et al. 2015). Following screening, samples were either marked as “NCLDVs not detected”, suspect, or “potential PCR inhibition” (need to be re-ran). Samples that were deemed “NCLDVs not

detected" had IPC Cq values within the 17-35 Cq range and no amplification indicative of sturgeon NCLDVs. Suspect samples had IPC values within the expected range described above and also had positive amplification indicative of sturgeon NCLDVs. Samples that had evidence of potential PCR inhibition had IPC values <17 or >35 and thus were rerun. Following one or two re-runs wherein template was decreased each run (detailed in Section 3.11), any samples where the IPC fell outside of normal range were excluded from the data set. Samples where the IPC value was within 17-35 Cq and yielded a Cq of <37 were deemed suspect and, as suggested in Clouthier et al. (2015), were screened using the NCLDV C1 conventional PCR.

From the Black River during year one, 35 samples were omitted from the data set, 97 were negative for NCLDVs, and 4 were suspect for NCLDVs. From the St. Clair River during year one, 2 samples were omitted from the data set, 56 were negative, and 18 were deemed suspect for NCLDVs. Lastly, during year two and from the Black River, 13 samples were excluded from the data set, 55 were negative, and 2 were deemed suspect for NCLDVs. Select suspect samples (n=1 per site) were screened using the conventional PCR described in Clouthier et al (2015).

4.5.3. Sturgeon Nucleocytoplasmic Large DNA Virus cPCR

Three representative samples that were suspect for sturgeon NCLDVs following screening with the Q2 PCR assay were tested using the C1 cPCR assay (Clouthier et al. 2015). An initial run using the cycling parameters described in Clouthier et al. (2015) resulted in amplification of non-target-band-sized amplicons when sturgeon NCLDV suspect samples were visualized via gel electrophoresis (two bands approximately 650 and 800 bp in length; target band size of 219 bp not detected). Of note, positive control DNA (white sturgeon iridovirus extract) and the

negative control (nuclease free water) results were as expected when visualized via gel electrophoresis (~200 bp amplicon from WSIV, no amplification in nuclease free water). Following an initial run and no target band detection, a second PCR was run with varying annealing temperatures (43.9°C-60°C). All other parameters were unchanged. At the lowest gradient annealing temperature (43.9°C), a band size of ~200 bp was visualized on the gel in the selected NCLDV suspects, however two non-target bands were noted once again (650 and 800 bp). The same three samples were cloned as described above, and a total of 11 clones (five from the year one Black River sample, five from the year one St. Clair River sample, and one from the year one Black River sample) which contained the target PCR fragment band-size (e.g., ~300 bp following TOPO TA cloning) were then Sanger sequenced.

4.5.4. Sequence Analysis

Following contig assembly detailed above (Section 3.13), all sequences exhibited a 94.98-97.04 percent similarity to *Acipenser ruthenus* genome assembly, chromosome: 5 (Accession # OV754674.1; Table 11). This was the highest sequence similarity observed, and no similarity to any sturgeon viruses was found.

5. Discussion

A primary goal of this study was to determine the prevalence of viruses that negatively affect sturgeon species across North America in Great Lakes lake sturgeon. Herein, frog virus 3 was undetected using cell culture and PCR in all adult and juvenile samples screened. While this

could be indicative of the virus truly not being yet present in the Great Lakes basin, it should be noted that in Waltzek et al. (2014), virus was detected in visceral organs, though other tissues were not screened. Given the nature of the lake sturgeon populations in the Great Lakes, we were limited to non-lethal sampling, and while we successfully and minimally invasively sampled two-four different tissues and/or fluids from every fish handled, we were unable to screen any visceral organs, which may be a tropism of FV3 (Forzan et al. 2017). Nevertheless, given the successful replication of frog virus 3 in EPC cells (Waltzek et al. 2014) and lack thereof herein, I posit that FV3 was not present in adult and/or juvenile samples collected from regions of the Lake Erie, Huron, Michigan, and Superior watersheds. However, given the wide host range and significant losses it has caused in other sturgeons, it will be important to continue periodic surveillance to detect a potential future emergence in Great Lakes lake sturgeon.

Sturgeon NCLDVs are notoriously damaging sturgeon pathogens affecting sturgeon species across North America, however prior to this study their presence in the Great Lakes was largely unknown. Fortunately, there was no molecular evidence of a sturgeon NCLDV in Great Lakes lake sturgeon (adults and juveniles) samples. Likewise, there were no gross signs of disease suggestive of a sturgeon NCLDV disease process, which, detailed in Clouthier et al. (2013, 2015) consists of damage to the olfactory organs and oral-pharyngeal tissues, often resulting in a deadly wasting syndrome. Moreover, no evidence of a replicating agent was detected on cell culture. Although many NCLDVs cannot be grown *in vitro* (Clouthier et al. 2015), some have been successfully isolated, namely, WSIV, which was recovered on WSSK-1 (Hedrick et al. 1992). Thus, given no molecular evidence of a sturgeon NCLDV in 208 adult tissues or any cell culture supernatant pools (93 pools encompassing 700 inoculated tissue samples), no evidence of a replicating agent in cell culture, and no gross signs of disease noted consistent with an NCLDV

disease course, I posit that sturgeon NCLDVs are not yet present in the adult and juvenile lake sturgeon populations sampled which encompassed regions of the Erie, Huron, Michigan, and Superior watersheds.

Some adult tissue samples from the Black and St. Clair Rivers across both years did show amplification indicative of a sturgeon NCLDV using the Q2 primer set. When screened with the C1 cPCR and visualized via gel electrophoresis, samples all had multiple bands. Initial screening yielded no bands of the expected size (219 bp), however subsequent analyses, including the adjusting of the annealing temperature, resulted in a target-sized amplicon at the lowest tested annealing temperature (43.9°C). However, following TOPO TA cloning and sequence analysis of amplicons generated via the C1 cPCR, the target sized sequences generated via the cPCR seemed most genetically similar to *A. ruthenus* genome sequence fragments (Table 4). These results so far suggest potential PCR non-specificity in the employed assays, and how this may or may not affect future sturgeon NCLDV detections warrants further research.

Furthermore, a subset of fin and gill tissue samples from adult lake sturgeon were omitted from this data set given no or poor amplification of the IPC. The likely cause behind the poor IPC amplification in these samples is PCR inhibition, and despite diluting DNA template, some samples still yielded results outside of the manufacturers detailed range and were discarded from the data set. The reason for this inhibition is unknown, however the presence of organic compounds such as ethanol and proteins such as melanin, and collagen are all known PCR inhibitors (Schrader et al. 2012). In this vein, a potential cause of inhibition in these reactions could have happened during extraction, where ethanol is used to wash NA and though unlikely, residual ethanol could have contaminated the sample. Furthermore, proteins such as melanin and collagen are present in fin tissues, and perhaps may have had negative downstream effects if not

all eliminated through NA extraction. Overall, the exact cause behind this inhibition remains unknown, however further research could be done to assess the effects of using different extraction methods.

While covered in depth in the context of adult lake sturgeon in Chapter Two, juvenile lake sturgeon were also screened for the novel lake sturgeon herpesvirus using similar *in vitro* techniques. Despite detections in reproductive fluids raising suspicions of vertical transmission with other sturgeon herpesviruses (Watson et al 1995) and the known presence of a lake sturgeon herpesvirus in Wisconsin lake sturgeon (Walker et al. 2022), there was no evidence of a replicating agent indicative of a lake sturgeon herpesvirus infection in any juvenile lake sturgeon screened herein. This finding was highly encouraging for Great Lakes lake sturgeon, although risk potential is there, and warrants continued monitoring.

6. Conclusions

Herein we detail the first glimpse into the presence of sturgeon NCLDVs in Great Lakes lake sturgeon populations. Fortunately, current results indicate that through extensive screening spanning four Great Lakes watersheds, there is no evidence of sturgeon NCLDVs or frog virus 3 infections. These results are dually supported with *in vitro* cell culture techniques as well as specialized molecular assays. Herein I also found possible evidence of non-specificity in assays designed to screen for NCLDVs, and the effects this finding could have on future NCLDV screening warrants further research. Despite not being detected in the present study, given the vast and expanding host range of FV3 and wide geographic range of NCLDVs, I suggest continued monitoring for these pathogens. Furthermore, sturgeon NCLDVs and FV3 should

remain on the potential list of causative agents should disease and/or mortality be observed in wild or hatchery reared Great Lakes lake sturgeon.

APPENDIX

Table 8: Biometric data and packed cell volume of adult lake sturgeon sampled from two sites (St. Clair River, Lake Erie watershed; Black River, Lake Huron watershed) during year one (2019) and year two (2020) of this study. σ indicates standard deviation.

Site	Fish Sampled	Total Length (cm)	Weight (kg)	Girth (cm)	Packed Cell Volume (%)
Black River Year 1 (LH)	137	148.6 (112-192)	24.5 (9.8-58.6)	57 (39-90)	33.6% σ=5.5
Females	26	154.1 (140-192)	40.3 (22.4-58.6)	73.1 (52-90)	30.7% σ=6.0
Males	111	147.55 (112-168)	20.8 (9.8-44.5)	53.2 (39-83)	34.5% σ=5.3
Black River Year 2 (LH)	79	150.45 (117-190)	26.62 (13.6-49.7)	55.05 (25-82)	37.6% σ=7.9
Females	25	160 (137-190)	33.3 (18.2-49.7)	63.8 (49-82)	35.7% σ=7.4
Males	54	145.9 (117-166)	23.0 (13.6 -38.2)	51 (25-70)	38.2% σ=8.1
St. Clair River Year 1 (LE)	76	102.9 (27.2-176.1)	15.4 (0.6-29.9)	42.6 (9.5-78.1)	25.6% σ=7.6

Table 9: Details of fish collected and examined from streamside rearing facilities (SRFs) in the Great Lakes basin during year one (2019) and year two (2020). Black River SRF is in the Lake Huron watershed, Cedar and Whitefish River SRFS are in the lake Michigan watershed, and the Ontonagon River SRF is in the Lake Superior watershed. Additionally, results after screening tissues for Nucleocytoplasmic Large DNA Viruses and Frog Virus 3 using *in vitro* and molecular analyses. * indicates samples were screened using the following cell lines: Epithelioma papulosum cyprini, EPC; Chinook salmon embryo-214, CHSE-214; white sturgeon skin, WSSK-1; white sturgeon x lake sturgeon hybrid, WSxLS; select samples were inoculated onto white sturgeon gonad, WSGO). ≠ Indicates samples were screened using the conventional PCR detailed in Waltzek et al. (2014). # Indicates samples were screened using the Q2 PCR assay described in Clouthier et al. (2015). ND indicates no gross signs of disease were present. a: mild congestion of pelvic and/or pectoral fins; b: mild hemorrhaging around the oral siphon.

		Time of Collection				Diagnostic Testing		
		5-6 weeks post hatch		Pre-Stocking		*In vitro* Detections	Molecular Analyses	
Year	Facility	Number of Fish	Signs of Disease	Number of Fish	Signs of Disease	Virus Detections via Tissue Culture*	Frog Virus 3≠	NCLDVs#
2019	Black River SRF	60	ND	60	a, b	0	0	0
	Cedar River SRF	60	ND	60	a, b	0	0	0
	Whitefish River SRF	60	ND	60	a, b	0	0	0
	Ontonagon River SRF	60	ND	60	a, b	0	0	0
2020	Black River SRF	60	ND	60	ND	0	0	0

Table 10. Samples collected from two adult lake sturgeon populations during year one (2019) and year two (2020) of this study. Samples were collected for either molecular analyses or virus isolation. * indicates samples were screened using the following cell lines: Epithelioma papulosum cyprini, EPC; Chinook salmon embryo-214, CHSE-214; white sturgeon skin, WSSK-1; white sturgeon x lake sturgeon hybrid, WSxLS; select samples were inoculated onto white sturgeon gonad, WSGO). [≠] Indicates samples were screened using the conventional PCR detailed in Waltzek et al. (2014). [#] Indicates samples were screened using the Q2 PCR assay described in Clouthier et al. (2013). Numbers in parentheses indicate the number of samples excluded from the data set following analysis via the Q2 PCR.

		Virus Isolation			Molecular Analyses
Year	Site	Blood*	Reproductive Fluids*	Lesions*	Fin and Gill[≠, #]
2019	Black River	119	91	52	136 (35)
	St. Clair River	57	3	11	76 (2)
2020	Black River	67	47	23	70 (13)

Table 11. Percent sequence similarity of cloned sequences from a ~200 bp fragment recovered when sturgeon Nucleocytoplasmic large DNA virus (NCLDV)-suspect samples were screened using the conventional PCR assay of Clouthier et al. (C1; 2013). BR, Black River; SCR, St. Clair River; Y1, year one; Y2, year two. Five clones from both sites during year one were sequenced (C1-C5) and one from year two (C1).

Sample	Query Coverage (%)	Percent ID (%)	Description
BR Y2 C1	92	97.04	Acipenser ruthenus genome assembly, chromosome: 5 (Accession # OV754674.1)
SCR Y1 C1	93	95.89	Acipenser ruthenus genome assembly, chromosome: 5 (Accession # OV754674.1)
SCR Y1 C2	93	95.45	Acipenser ruthenus genome assembly, chromosome: 5 (Accession # OV754674.1)
SCR Y1 C3	93	95.45	Acipenser ruthenus genome assembly, chromosome: 5 (Accession # OV754674.1)
SCR Y1 C4	93	95.45	Acipenser ruthenus genome assembly, chromosome: 5 (Accession # OV754674.1)
SCR Y1 C5	93	95.89	Acipenser ruthenus genome assembly, chromosome: 5 (Accession # OV754674.1)
BR Y1 C1	93	95.45	Acipenser ruthenus genome assembly, chromosome: 5 (Accession # OV754674.1)
BR Y1 C2	93	95.45	Acipenser ruthenus genome assembly, chromosome: 5 (Accession # OV754674.1)
BR Y1 C3	93	95.45	Acipenser ruthenus genome assembly, chromosome: 5 (Accession # OV754674.1)
BR Y1 C4	93	95.91	Acipenser ruthenus genome assembly, chromosome: 5 (Accession # OV754674.1)
BR Y1 C5	93	94.98	Acipenser ruthenus genome assembly, chromosome: 5 (Accession # OV754674.1)

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CHAPTER 4:

ASSESSING THE EFFICACY OF MULTIPLE HATCHERY DISINFECTANTS AGAINST A NEWLY-ISOLATED LAKE STURGEON HERPESVIRUS

1. Abstract

Infectious diseases are a leading cause of losses in the aquaculture industry globally, and simultaneously pose substantial risks to hatchery-reared fishes for conservation, including Great Lakes lake sturgeon (*Acipenser fulvescens*). Lake sturgeon rearing facilities across the Great Lakes are at a heightened risk for infiltration of infectious diseases, given they are populated with gametes collected from wild adults and/or wild larval fish collected post-hatch. A primary means of preventing and controlling both horizontal and vertical pathogen transmission is through the use of disinfectants. Recently, an alloherpesvirus was detected in two adult lake sturgeon populations, one of which serves as the gamete source to populate the largest lake sturgeon rearing facility in Michigan. The newly isolated virus (lake sturgeon herpesvirus 2; LSHV-2; Chapter Two) is also capable of inducing mortality in juvenile lake sturgeon, raising concerns for its implications for conservation. To reduce risk of virus transmission and develop efficacious protocols for disease prevention and control, *in vitro* experiments were designed to elucidate the susceptibility of LSHV-2 to three routinely employed hatchery/aquaculture facility disinfectants (e.g., Virkon™-Aquatic, Ovadine®, and Perox-Aid®). Lake sturgeon herpesvirus-2 isolate 200413-11TC propagated on a white sturgeon x lake sturgeon hybrid cell line (WSxLS; 15°C) was exposed to each disinfectant at two concentrations (e.g., Virkon™-Aquatic, 1% and 2%; Ovadine, 50 and 100 ppm; Perox-Aid®, 500 and 1,000 ppm) in duplicate for durations of 1, 10, and 30 minutes, after which percent reduction in infectious virus was determined. After 14 days of incubation on the WSxLS cell line, the tissue culture infectious $dose_{50}$ of control (i.e., non-disinfectant exposed) virus was $2.39x10^5$. When LSHV-2 isolate 200413-11TC was exposed to Perox-Aid® (500 and 1,000 ppm) for 1, 10, and 30 minutes, the percent reduction was 58.66%, 92.32%, and 97.13% (500 ppm) and 92.95%, 95.98%, and 99.51% (1,000 ppm). When

exposed to Ovadine® (50 and 100 ppm) for 1, 10, and 30 minutes, the percent reduction was 99.35%, 99.90%, and 100% (50 ppm) and 99.42%, 99.90%, and 100% (100 ppm). Lastly, percent reduction when exposed to Virkon™-Aquatic was 100% for all concentrations and timepoints. Based upon these results from *in vitro* experiments, Virkon™-Aquatic, Ovadine®, and Perox-Aid® represent potential means to reduce the risk of LSHV-2 transmission under field and hatchery conditions.

2. Introduction

Infectious disease outbreaks are not infrequent amongst hatchery reared and farmed (i.e., aquaculture) fishes. In the 2018 Census of Aquaculture, disease was listed as the leading cause of production losses in aquaculture facilities (FAO 2020). A cornerstone for preventing pathogen transmission amongst and within aquaculture facilities and hatcheries is biosecurity, generally defined as preventative measures aimed at reducing risk associated with infectious agents in a facility, including their transmission within and between facilities and/or dissemination into the environment (Faisal et al. 2017).

Alloherpesviruses, including the lake sturgeon herpesvirus-2 (LSHV-2) that was recently detected in Great Lakes lake sturgeon (Chapter Two), are a group of viruses known to be damaging to captive reared-fishes (Hedrick et al. 1991, Michel et al. 2010, Faisal et al. 2017). Given the negative effects that alloherpesviruses have on cultured fishes, understanding the disinfectants capable of preventing spread to and within rearing facilities is important. Indeed, a recent study showed the efficacious potential of Virkon™-Aquatic (Syndel, Ferndale, Washington, USA) to reduce horizontal transmission of salmonid herpesvirus 3, where net

disinfection between the handling of infected fish to the handling of naïve fish prevented transmission of the virus (Purbayu et al. 2021). Vertical transmission via eggs and reproductive fluids is also a transmission risk. Indeed, viruses in the family Alloherpesviridae are known to be transmitted vertically (Wise et al. 1988), while others, including those affecting sturgeons, have been detected in the reproductive fluids (Watson et al. 1995). In the context of Great Lakes lake sturgeon, facilities are populated exclusively with gametes collected from wild adults or wild larvae harvested post-hatch, meaning vertically transmitted pathogens pose significant risks. Furthermore, the recent detection of LSHV-2 in a wild adult lake sturgeon population raises concerns of the negative impacts of vertical transmission, especially given it was detected in an adult lake sturgeon population used to populate the largest lake sturgeon rearing facility in the state of Michigan (Black River Sturgeon Rearing Facility, Cheboygan County, Michigan, USA). Unfortunately, the effect of routinely employed disinfectants for hatchery use on LSHV-2 remains unknown.

Three commonly deployed and approved disinfectants in hatchery/aquaculture settings to reduce risk of horizontal and/or vertical transmission of pathogens are potassium peroxymonosulfate (e.g., Virkon™-Aquatic), polyvinylpyrrolidone-iodine complex (e.g., PVP iodine; Ovadine®; Syndel, Ferndale, Washington, USA), and hydrogen peroxide (e.g., Perox-Aid®; Syndel, Ferndale, Washington, USA). Virkon™-Aquatic is a virucidal and bactericidal used for the disinfection of rearing unit tools, field equipment, and also serves as a means to control what comes in and out of facilities through personnel traffic via the use of footbaths (Hernandez et al. 2000). Additionally, PVP iodine is widely utilized in the USA to disinfect eggs during the fertilization process in an effort to prevent pathogens from being transmitted vertically from parents to progeny, including for fish-pathogenic viruses (Batts et al. 1991; USFWS, ND).

Disinfection with PVP Iodine is also routinely employed in lake sturgeon rearing facilities (Chalupnicki et al. 2014). Furthermore, hydrogen peroxide (Perox-Aid®) is routinely used as treatment for infectious diseases emerging in the various life stages, including in lake sturgeon rearing facilities (Pedersen and Pedersen 2012).

Given the novelty and many unknowns surrounding LSHV-2, susceptibility of this virus to routinely employed hatchery disinfectants, including potassium peroxymonosulfate (Virkon™-Aquatic), PVP iodine (Ovadine®), and hydrogen peroxide (Perox-Aid®) has yet to be determined. Furthermore, all three of these disinfectants are already approved for use in hatcheries in the USA, and have been successful in the prevention and control of other fish pathogenic viruses; therefore, their effects against LSHV-2 necessitated further research. Thus, I conducted the first *in vitro* disinfectant efficacy experiments with novel LSHV-2 as a critical first step towards equipping fishery personnel who rear Great Lakes lake sturgeon with readily applicable means of preventing and controlling LSHV-2.

3. Materials and methods

3.1. Maintenance and preparation of cells

In these experiments, the white sturgeon x lake sturgeon (WSxLS) cell line was used. Growth media for WSxLS cells was comprised of Eagle's minimum essential media (with Earle's salts, nonessential amino acids, and sodium pyruvate; ATCC, Manassas, VA, USA) supplemented with 10% tryptose phosphate broth (ThermoFisher Scientific, Waltham MA, USA), 10% fetal bovine serum (Gemini BioProducts, Sacramento CA, USA), penicillin (100 IU mL^{-1}), streptomycin (100 µg mL^{-1}), amphotericin B (2.5 µg mL^{-1}), 2mM L-Glutamine, and buffered

with sodium bicarbonate and HEPES (Fisher Scientific, Waltham MA, USA; pH 7.4-7.6). Herein, this medium is referred to as MEM-10-SBH. For virus propagation, cells were grown in 96-well, flat bottom plates (Corning, Corning, NY, USA) to 80%-90% confluency (<48 hours) in a medium similar to MEM-10-SBH but modified to contain 2% FBS and buffered with HEPES and UltraPure Tris (MEM-2-TH; Invitrogen, Carlsbad, CA, USA) to pH 7.4-7.6 (AFS-FHS 2016).

3.2. Origin of virus isolate

Lake sturgeon herpesvirus-2 isolate 200413-11TC, originally isolated from a skin lesion on an adult lake sturgeon from the Black River on WSxLS cells (Chapter Two), was utilized for all disinfectant efficacy experiments. Isolate 200413-11TC was passaged five times prior to freezing in liquid nitrogen in a solution of 20% FBS and 20% glycerol.

3.3. Preparation of virus for disinfectant efficacy assessments

Lake sturgeon herpesvirus-2 isolate 200413-11TC was thawed from -193°C rapidly at 25°C. The virus stock was gently mixed and inoculated onto multiple wells of a 96 well plate (Corning, Corning, NY, USA) with an 80-90% confluent monolayer of WSxLS cells incubated at 15°C. Following the development of cytopathic effects (CPE), cells and supernatant were harvested and passaged into a 25cm^2 cell culture flask (Corning, Corning NY, USA) with an 80-90% confluent monolayer of WSxLS cells, incubated at 15°C, and observed for CPE. Eight days post-

inoculation into the 25cm^2 flask, ~90% of cells were exhibiting CPE and cells/supernatant/virus suspension was harvested.

3.4. Preparation of disinfectants

The three disinfectants (e.g., Virkon™-Aquatic, Ovadine®, and Perox-Aid®) were prepared at 2X the working concentration for use in this experiment. Two solutions of each disinfectant were mixed at a high concentration and a low concentration, and all disinfectant solutions were prepared using ultrapure water. For Virkon™-Aquatic, solutions of 1% and 2% were prepared using manufacturer's instructions to obtain a working concentration of 0.5% and 1%. Similarly, for Ovadine®, 200 ppm and 100 ppm solutions were prepared to obtain working concentrations of 100 and 50 ppm, in accordance with manufacturer's protocols for the surface disinfection of eggs. For Perox-Aid®, 2,000 ppm and 1,000 ppm solutions were prepared (working concentrations of 1,000 ppm and 500 ppm), these were also chosen based on manufacturer's protocols for the treatment of diseases in early life stages. All disinfectant solutions were filter sterilized prior to use and prepared just prior to experiments being conducted.

3.5. Virus exposure to disinfectant

Prior to the start of experiments and to check for any cytotoxicity induced by the disinfectants directly to the WSxLS cells, all disinfectant working concentrations were inoculated onto WSxLS cells and examined for cytotoxicity via light microscopy periodically for 14 days. Following that initial test, and for each of the two disinfectant concentrations (Virkon™-Aquatic [0.5%, 1.0%], Ovadine® [50 ppm, 100 ppm], and Perox-Aid® [500 ppm,

1,000 ppm]; Table 12) two replicate aliquots of 500 µL of virus cell/supernatant suspension were prepared. Next, 500 µL of 2X disinfectant stock (preparation detailed in Section 3.3) was aliquoted into each tube containing the 500 µL of virus suspension. Immediately after addition of disinfectant to the replicate, a timer was started. Three disinfectant exposure time points were assessed per disinfectant concentration and replicate, including 1, 10, and 30 minutes (Table 12). Following exposure and for each respective time point, the disinfectant was deactivated by diluting the virus suspension 1:10 then 1:5, ending in a 1:50 dilution with MEM-10-SBH, following protocols detailed by Amend and Pietch (1972). In brief, 50 µL of virus/disinfectant suspension was aliquoted into 450 µL of MEM-10-SBH and gently mixed. Next, 100 µL of the 1:10 diluted virus/disinfectant suspension was aliquoted into 400 µL of MEM-10-SBH, for a final dilution of 1:50. Virus un-exposed to disinfectant was also diluted 1:50 with MEM-10-SBH.

Following disinfectant deactivation and to quantify virus concentration, a tissue culture infectious dose$_{50}$ ($TCID_{50}$) was prepared, whereby seven 10-fold serial dilutions were prepared and inoculated onto 96-well plates (four wells per dilution) and examined for development of CPE via light microscopy. To serve as a positive control, virus not exposed to any disinfectant was diluted 1:50, serially diluted 10-fold seven times, and a $TCID_{50}$ prepared. To ensure dilution with MEM-10-SBH did not affect virus growth due to a 10% FBS concentration, virus was diluted 1:50 with MEM-2-TH and another $TCID_{50}$ was prepared. On the day the experiment was performed, 1X concentrations of each disinfectant (50 and 100 ppm for Ovadine®, 500 and 1,000 ppm for Perox-Aid®, and 0.5% and 1% Virkon™-Aquatic) were inoculated in quadruplicate onto WSxLS cells. All $TCID_{50}$ plates were incubated at 15°C and checked periodically for 14 days. At 14 days, $TCID_{50}$ plates were examined for CPE and wells exhibiting

CPE consistent with that of LSHV-2 (Chapter Two) were deemed positive for active virus. All $TCID_{50}$ values were calculated using the methodology outlined in Reed and Muench (1938). Virus concentrations ($TCID_{50}$) for each disinfectant concentration at each time point (Table 12) were calculated by averaging the $TCID_{50}$ values for each replicate.

3.6. Calculation of percent reduction of active virus

To calculate the percent reduction in active virus for each disinfectant concentration, time-point, and replicate, $TCID_{50}$ values for each disinfectant exposed group were compared to the $TCID_{50}$ of the 1:50 diluted, non-disinfectant exposed control. The two replicates of each disinfectant concentration and timepoint were averaged to calculate percent reduction.

4. Results

4.1. Positive and disinfectant control results

Lake sturgeon herpesvirus-2 that was unexposed to any disinfectant was determined to have a $TCID_{50}$ of $2.39x10^5$ (Table 12). When virus diluted with MEM-10-SBH was compared to virus diluted with MEM-2-TH, there were no differences in $TCID_{50}$ values. No cytotoxic effects were noted on cells inoculated with the 1X concentration of any disinfectant at any concentration concentrations (Virkon™-Aquatic [0.5%, 1.0%], Ovadine® [50 ppm, 100 ppm], and Perox-Aid® [500 ppm, 1,000 ppm]).

4.2. Virus exposure to Virkon™-Aquatic

When LSHV-2 isolate 200413-11TC was exposed to Virkon™-Aquatic at a final concentration of 0.5%, 100% active virus percent reduction was observed at timepoints of 1, 10, and 30 minutes (Tables 12, 13). Likewise, when exposed at a concentration of 1%, 100% reductive was observed following 1, 10, and, 30 minutes post-exposure (Tables 12, 13).

4.3. Virus exposure to Ovadine®

When LSHV-2 isolate 200413-11TC was exposed to Ovadine® at a concentration of 50 ppm, a 99.35% reduction was noted just one minute post exposure (Tables 12, 13). At 10 and 30 minutes post exposure, 99.9% and 100% reduction occurred (Tables 12, 13). Furthermore, when exposed to a final concentration of 100 ppm Ovadine®, a 99.42% reduction was noted after one minute of exposure. After 10 and 30 minutes of exposure, 99.9% and 100% reduction was observed (Tables 12, 13).

4.4. Virus exposure to Perox-Aid®

When LSHV-2 isolate 200413-11TC was exposed to hydrogen peroxide at a concentration of 500 ppm, a 58.7% reduction of active virus was observed after 1 minute of exposure (Tables 12, 13). At 10 and 30 minutes of exposure, a 92.3% and 97.13% decrease was observed (Tables 12, 13). At 1,000 ppm, a 92.6% reduction in active virus was observed after just one minute of exposure, while 95.9% and 99.5% reductions were observed after 10 and 30 minutes of exposure (Tables 12, 13).

5. Discussion

Disinfectants for use in field settings and aquaculture facility/hatchery conditions are crucial for the prevention and control of disease (Faisal et al. 2017). However, when new pathogens are discovered, their susceptibility to disinfectants is unknown and hampers disinfectant-based biosecurity efforts employed to reduce transmission. In this context and following the discovery of a novel virus (LSHV-2) infecting and causing disease in Great Lakes lake sturgeon, determining the susceptibility of LSHV-2 to hatchery disinfectants is a crucial first step to developing efficacious methods of prevention and control. Results from the *in vitro* experiments herein revealed that three compounds (Virkon™-Aquatic, Ovadine®, and Perox-Aid) hold promise in mitigating the risk of LSHV-2 transmission in the field and in hatcheries. This is also encouraging, given all three disinfectants are already approved for use in aquaculture/hatchery settings.

Surface disinfection of eggs via PVP iodine is currently an efficacious method of disease prevention used in the USA (USFWS ND). Current results show that this compound likewise holds promise for similarly reducing the transmission if LSHV-2 is indeed being transmitted via reproductive fluids. Currently, lake sturgeon eggs are disinfected at a concentration of 50 or 100 ppm for 30 or 10 minutes (Chalupnicki et al. 2014), which is comparable to my testing of both concentrations at both timepoints, where a 100% (50 ppm for 30 minutes) and 99.9% (100 ppm for 10 minutes) reduction was noted. However, if pathogens are transmitted intra-ovum, or inside of the egg, surface disinfection may not be efficacious. Indeed, some fish pathogens are transmitted intra-ovum (Kohara et al. 2012). Unfortunately, the exact mechanism of vertical transmission of many fish viruses, including alloherpesviruses, isn't completely understood, as is the case with LSHV-2, warranting further research.

Hydrogen peroxide (Perox-Aid®) is approved for use by the Food and Drug Administration to treat external manifestations of disease in different species of fish at various life stages (Yanong 2011). It is commonly used in lake sturgeon rearing facilities to treat eggs and is approved for use on eggs at concentrations of 500-1,000 ppm for 15 minutes (Yanong 2011; Bauman 2016). Similarly, I assessed the efficacy of Perox-Aid® against LSHV-2 at 500 and 1,000 ppm for durations of 1, 10, and 30 minutes. While results showed that the other disinfectants worked more rapidly and induced higher percent reductions overall, this compound could potentially hold promise for mitigating losses during an outbreak, especially given it is the only of the three tested herein also approved for use in fish post-hatch. Ultimately, Perox-Aid® appeared most effective at 1,000 ppm for 30 minutes, however percent reduction was >92% at both concentrations after 10 minutes.

The most effective disinfectant tested against this virus in these experiments was Virkon™-Aquatic. After just one minute of exposure at both 0.5% and 1%, no active virus was detected, as was also the case at 10 and 30 minutes of exposure. Although Virkon™-Aquatic is not used to treat fish or eggs directly, it is widely used in hatchery and field settings for gear and is also safe for use in environments housing fish (Stockton-Fiti and Moffitt 2017). The virucidal effects of Virkon™-Aquatic and similar disinfectants are widely studied, and it is efficacious in reducing transmission risk of a diversity of viruses (Rohaim et al. 2015; Tsujimura et al. 2015), including an alloherpesvirus (Purbayu et al. 2021). Results herein and previous literature support the potential Virkon™-Aquatic has for reducing transmission risk of LSHV-2 in hatchery and field conditions.

Virkon™-Aquatic, Ovadine®, and Perox-Aid® can serve different and critical rolls in fish hatchery and aquaculture facility environments. Acknowledging that additional studies under *in*

vivo conditions are needed, based upon results from current study, it appears all three compounds hold promise for mitigating LSHV-2 transmission and disease-associated losses. For example, and to deactivate LSHV-2 potentially harbored on tools or other surfaces in hatcheries, experimental evidence herein suggests it may prove fruitful to use Virkon™-Aquatic at a concentration of 0.5% for a contact time of at least 1 minute. Similarly, for disinfection of egg surfaces using Ovadine®, these *in vitro* experiments suggest a contact time of 10 minutes at 50 or 100 ppm is highly (>99%) effective in virus reduction; however how this translates to *in vivo* hatchery settings warrants further study. Lastly, while Perox-Aid® was not as effective as others in the deactivation of LSHV-2, when at concentrations of 500 or 1,000 ppm for 30 minutes, it showed great promise in reducing active virus (97.13% and 99.51% reduction). Similar to the other disinfectants, whether it could help with disease outbreaks in *in vivo* hatchery settings warrants further study.

6. Conclusion

Herein, we describe the first *in vitro* disinfectant efficacy experiments conducted with an alloherpesvirus recovered from Great Lakes lake sturgeon, LSHV-2. Given the ability of other alloherpesviruses to induce disease and mortality in other hatchery reared fishes, in addition to the recent isolation and proven pathogenic effects of LSHV-2 (Chapter Two), arming those in the Great Lakes basin rearing lake sturgeon with efficacious methods of disease prevention and control is critical. While the *in vitro* results detailed herein may not directly translate to hatchery and field settings, Virkon™-Aquatic, Ovadine®, and Perox-Aid® appear to be highly effective in deactivation of LSHV-2. Furthermore, all are promising candidates for minimizing transmission risks associated with LSHV-2 in hatchery and field environments via the

disinfection of eggs and equipment, or mitigating disease-associated losses via treatment of live fish.

APPENDIX

Table 12. Tissue culture infectious dose$_{50}$ ($TCID_{50}$) results in each disinfectant concentration at each time point. Values are an average of two replicates, except for the "no disinfectant" row.

		Time Points		
Disinfectant	Concentration	1 minute	10 minutes	30 minutes
Ovadine®	50 ppm	$1.66x10^3$	$1.44x10^2$	0
	100 ppm	$1.38x10^3$	$2.36x10^2$	0
Perox-Aid®	500 ppm	$9.88x10^4$	$1.84x10^4$	$6.86x10^3$
	1,000 ppm	$1.76x10^4$	$9.60x10^3$	$1.12x10^3$
Virkon™-Aquatic	0.5%	0	0	0
	1.0%	0	0	0
No Disinfectant	NA		$2.39x10^5$	

Table 13. Percent reduction of active virus for each disinfectant concentration at each time point. Values are an average of two replicates.

		Time Points		
Disinfectant	Concentration	1 minute	10 minutes	30 minutes
Ovadine®	50 ppm	99.35%	99.9%	100%
	100 ppm	99.42%	99.9%	100%
Perox-Aid®	500 ppm	58.66%	92.32%	97.13%
	1,000 ppm	92.95%	95.98%	99.51%
Virkon™-Aquatic	0.5%	100%	100%	100%
	1.0%	100%	100%	100%

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CHAPTER 5:

CONCLUSIONS AND FUTURE STUDIES

1. Conclusions

Great Lakes lake sturgeon (*Acipenser fulvescens*) populations are remnant of what they once were, and substantial resources are being allocated to their conservation and population recovery. A possible contributing factor, of which little is known, is the presence of infectious diseases in adult populations and what negative effects they could be having on Great Lakes lake sturgeon populations. Thus, the studies conducted herein encompass the largest lake sturgeon disease surveillance study done to date. Through the course of this study, I non-lethally collected an array of tissues and fluids (e.g., blood, reproductive fluids, fin, gill, and lesions) from 292 adult lake sturgeon in two distinct Great Lakes lake sturgeon populations (Black River, Cheboygan County, MI; St. Clair River, Macomb County, MI), resulting in the collection of 752 samples for *in vitro* virus isolation and molecular analyses. Furthermore, I performed clinical examinations and collected tissues (fin, gill, visceral organs) from 600 juvenile lake sturgeon from four different lake sturgeon rearing facilities in the Great Lakes basin encompassing the Lake Erie, Huron, and Michigan watersheds. Herein, I describe one of the first glimpses into the presence of sturgeon pathogenic viruses in Great Lakes lake sturgeon and detail the first detection of a novel alloherpesvirus (LSHV-2) from Great Lakes lake sturgeon, and the first isolation of an alloherpesvirus from Great Lakes lake sturgeon ever. These findings provide insight into viral pathogens in Great Lakes lake sturgeon, pave the way for further LSHV-2 research, and provide a foundation for future studies into efficacious methods of prevention and control against LSHV-2.

In my second chapter, I detail the first isolation and detection of the novel LSHV-2. While just prior to this study, a LSHV was molecularly detected in adult lake sturgeon in the Lake Michigan watershed, this study describes the first isolation of an alloherpesvirus from Great

Lakes lake sturgeon ever, and first detection in the Huron and Erie watersheds. Alloherpesviruses are notoriously difficult to isolate, and in my second chapter, I describe methodology for potentially improving the recovery of alloherpesviruses, including the development of a viral transport media. I also outline other factors such as avoidance of freezing and use of host specific cell lines, both of which likely contributed to the successful recovery of this virus *in vitro*. Furthermore, LSHV-2 was recovered from adult lake sturgeon showing gross signs of disease in the skin, epithelial changes were also noted histologically, and virions were found in association with skin lesions. Given the crucial roll the skin plays in host osmoregulation and immune response, skin damage could pose health risks to already imperil adult lake sturgeon. This virus was detected molecularly in two Great Lakes watersheds (Huron and Erie) and isolated from one (Huron). The spawning population it was isolated from (Black River, Cheboygan County, Michigan) is the adult population from which gametes and larvae are collected to populate the largest lake sturgeon rearing facility in the state. Furthermore, while vertical transmission risk remains unknown, should this virus be transmitted from infected adults to juveniles, I concluded LSHV-2 is capable of inducing serious disease and mortality in juvenile lake sturgeon. The negative effects this virus could be having on already struggling adult lake sturgeon populations has only begun to be uncovered, however the risk it poses to juveniles is unfortunately clear and should remain a top management priority for rearing lake sturgeon in the Great Lakes.

While alloherpesviruses are some of the most reported viruses in other sturgeon populations, especially in north America, other viruses including sturgeon nucleocytoplasmic large DNA viruses (NCLDVs) and frog virus 3 (FV3) have demonstrated apparent host and range expansions. These expansions have brought these sturgeon pathogenic viruses dangerously close

to the Great Lakes basin, and unfortunately, given they are only detected via specialized and specific diagnostic tests, some of which are not often deployed in the Great Lakes, little is known about their presence in Great Lakes lake sturgeon populations. In my third chapter, I deploy a series of *in vitro* and molecular diagnostic techniques aimed at the detection of sturgeon NCLDVs and FV3 and for the first time screened for their presence in Great Lakes lake sturgeon. Fortunately, active virus (NCLDVs or FV3) was not detected via *in vitro* methods in adult or juvenile Great Lakes lake sturgeon sampled herein. Furthermore, no genetic traces of NCLDVs or FV3 were detected via the use of specialized and specific PCR tests. However, we did uncover what appears to be non-specificity in the Q2 and C1 PCR assays designed for the detection of sturgeon NCLDVs (Chapter Three) and the potential implications this finding has on future detection of and screening for NCLDVs warrants future research. Lastly, despite no positive detection events herein, the fact remains that NCLDVs and FV3 are encroaching upon the Great Lakes basin. Resultantly, these viruses should be periodically screened for, and should remain on the list of potential etiologies should mortality events occur in adult or juvenile Great Lakes lake sturgeon populations.

Furthermore, and following the isolation and detection of the novel LSHV-2 from adult lake sturgeon, I determined the efficacy of three approved and routinely used hatchery disinfectants against LSHV-2. *In vitro* experiments with potassium peroxymonosulfate (Virkon™-Aquatic; Syndel, Ferndale, Washington, USA), polyvinylpyrrolidone-iodine complex (PVP) iodine (Ovadine®; Syndel, Ferndale, Washington, USA), and hydrogen peroxide (Perox-Aid®; Syndel, Ferndale, Washington, USA) revealed high percentages of virus reduction at safe-to-use concentrations and timepoints. Therefore, all are promising options for potential prevention and control of LSHV-2.

Collectively, findings of my thesis research answered many questions regarding the presence of sturgeon pathogenic viruses in Great Lakes lake sturgeon populations yet, given the detection of a never-before-described alloherpesvirus, created many more unknowns. Given the novelty of LSHV-2, and my development of successful methods of cryopreservation and revival, I have paved the way for critically needed future studies into pathogenesis, characterization, and biology of LSHV-2. Lastly, disinfectant efficacy experiments have fortunately highlighted that under *in vitro* laboratory conditions, three common hatchery disinfectants are effective against LSHV-2, providing a baseline for future *in vivo* studies to determine efficacy under hatchery and field conditions.

2. Future studies

Research conducted herein has not only provided a baseline regarding virus prevalence in Great Lakes lake sturgeon by screening for an array of sturgeon pathogenic viruses via specialized and specific diagnostic techniques, but has also resulted in the detection, isolation, and preservation of novel LSHV-2 for future studies. Furthermore, I've generated a suite of tissue samples and DNA extracts from 292 adult and 600 juvenile lake sturgeon from the Great Lakes that could potentially be screened for genetic traces of other possible pathogens of concern retroactively.

With the isolation of LSHV-2, I've opened the door for a diversity of viral studies. One potential area for future research is investigating vertical transmission of LSHV-2 from parents to offspring. While studies herein screened reproductive fluids via *in vitro* methods, further work screening reproductive fluids molecularly may aid in uncovering virus presence, given molecular

assays are often more sensitive to mild or potentially latent infections. Additionally, reproductive fluids could be collected from adult lake sturgeon showing gross signs of disease suspect for LSHV-2, fertilized and eggs hatched, and resultant progeny screened for molecular or *in vitro* evidence of LSHV-2. Also related to potential vertical transmission, results detailed in Chapter Four revealed the possible efficacy of surface disinfection with Ovadine® against LSHV-2, and armed with this information, future *in vivo* studies could involve the screening of fertilized eggs from infected adults before and after disinfection. This would potentially gain insight into 1) Ovadine® efficacy under hatchery conditions and 2) mechanism of vertical transmission if applicable (intra-ovum vs. via reproductive fluids).

In Chapter Four, I detailed *in vitro* experiments to determine the efficacy of Virkon™-Aquatic, Perox-Aid®, and Ovadine® against LSHV-2. While all three disinfectants demonstrated promise as detailed above, future studies could focus on evaluating the efficacy of these disinfectants under hatchery conditions. Experiments along these lines could be conducted via looking for active virus in eggs, on equipment, or in infected juvenile lake sturgeon pre- and post-disinfection. By addressing the *in vivo* efficacy of Virkon™-Aquatic on hatchery and field tools/equipment, Ovadine® as detailed above, and Perox-Aid® as a disease treatment during the early life stages, lake sturgeon rearing facility personnel would be armed with critical information needed to best decrease the transmission risk of LSHV-2.

While optimization of *in vitro* diagnostic techniques for the isolation of LSHV-2 was discussed in Chapter Two, future studies could supplement this via investigation of the effect of different temperatures on the growth of LSHV-2 *in vitro*. Many pathogens have optimal growth temperatures, and while 15°C worked well for the present study, other temperatures should be evaluated, perhaps guided by river temperatures associated with populations where LSHV-2 was

detected and/or isolated. Further along the lines of *in vitro* diagnostics, the development of lake sturgeon specific cell lines and subsequent studies determining if LSHV-2 is better propagated therein would be beneficial to further improving the isolation of LSHV-2.

In vivo experiments in Chapter Two provided compelling evidence that LSHV-2 is capable of inducing serious disease and mortality in juvenile LST. To build off of these results, future research could focus on determining a median lethal dose (LD_{50}) of LSHV-2. Additionally, further experiments investigating the effect of different temperatures on naïve host disease progression and mortality would be beneficial. By understanding temperatures which LSHV-2 appears to be most lethal, hatchery managers could monitor facility water temperatures and potentially predict disease outbreaks.

Additionally, I have created a clear and well supported link between the presence of unique, grossly apparent skin lesions (Chapter Two) and LSHV-2, meaning that even if resources for extensive diagnostics are not available, those handling adult lake sturgeon could potentially track prevalence over time via the monitoring of LSHV-2 linked skin lesions. Furthermore, if these lesions have been tracked in previous years, which is the case for adult lake sturgeon collected from the Black River for years prior to this study, investigations into historical prevalence could be done. This would potentially provide a glimpse into fluctuations in virus prevalence over time.

Furthermore, given the difficulty of propagating sturgeon viruses in vitro and the apparent rapid evolution of sturgeon viruses (i.e. iridoviruses and alloherpesviruses; Donohoe et al. 2021), prevalence of viral infection in sturgeon may be greater than I observed herein. In this regard, it is possible that some viruses were missed in culture or that the PCR primers didn't match. Therefore, efforts should be directed to developing sturgeon cell lines capable of propagating a

greater number of relevant viruses. Additionally, efforts should be directed on non-targeted metagenomic sequencing of suspected sturgeon viral lesions to further elucidate the extent of viral infections in sturgeon and generate sequence data for aide in the development of novel diagnostic assays. Nonetheless, In the current study, we generated a genome assembly of a novel alloherpesvirus infecting sturgeon that will aid future development of molecular diagnostic assays for the surveillance of LSHV-2 infection in sturgeon.

Lastly, a top priority building off of what I have conducted herein should be the continued disease surveillance and monitoring of adult and juvenile lake sturgeon. While sturgeon pathogenic viruses such as NCLDVs and FV3 were not detected in the present study, more surveillance could be done in other Great Lakes watersheds and spawning adult lake sturgeon populations. Additionally, surveillance efforts for LSHV-2 could be deployed more widely across the Great Lakes basin to begin piecing together a geographic range, especially given virus can be recovered from adults with lesions via non-lethal methods. Furthermore, this study marks one of few conducted investigating the presence of disease in Great Lakes lake sturgeon, and future studies could consider screening for other viral, bacterial, and/or fungal pathogens of concern across the Great Lakes basin, all with an eye towards continuing to uncover the unknowns associated with Great Lakes lake sturgeon health.

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