# Analytical Performance Characteristics and Application of Diagnostic Tests for Namao Virus in Experimentally Infected and Wild Manitoba Lake Sturgeon (*Acipenser fulvescens*)

By

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## Abstract

Namao virus (NV) was associated with mortality in lake sturgeon *Acipenser fulvescens* reared as part of a conservation stocking program for this endangered species in Manitoba, Canada. The virus itself was large, doubly encapsidated and icosahedral-shaped. Phylogenetic analyses using the major capsid protein showed that NV and other epitheliotropic sturgeon nucleo-cytoplasmic large DNA viruses shared a common evolutionary past and formed a distinct evolutionary lineage within *Megavirales*. Three PCR tests were developed and their analytical performance was validated for detection of these viruses. Testing of wild sturgeon revealed that NV is endemic in the Nelson River water basin in Manitoba. Bath exposure resulted in transmission of NV to healthy sturgeon. The gills appeared to be the initial site of infection with virus persisting in the head skin tissue for up to 62 days. The molecular tests will be useful tools for disease management in sturgeon conservation stocking programs.

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# 1.0 Introduction

## 1.1 Lake Sturgeon

### 1.1.1 Taxonomy

Lake sturgeon, *Acipenser fulvescens*, belong to the genus *Acipenser* which, along with the genera *Huso, Scaphirhynchus and Pseudoscaphirhynchus,* are part of the Acipenseridae family. The family classification encompasses about 24 species of sturgeon native to North America and Eurasia (Stewart and Watkinson 2004).

### 1.1.2 Biology

Lake sturgeon are unique freshwater fish as they are particularly large (reaching lengths up to 1.5m) and long-lived with some lake sturgeon living up to 100 years (Scott and Crossman 1973). They have distinctive bony ridged plates composed of ganoine scales, known as scutes, along their back and a heterocercal tail (Harkness and Dymond 1961). They are bottom feeders and rely on four sensory barbels located on the lower side of the snout to detect and locate food (Scott and Crossman 1973). Adult lake sturgeon are toothless and feed opportunistically on benthic organisms including molluscs, small fish and insects by extending a protrusible mouth (Smith and Hobden 2011).

The habitat range of lake sturgeon extends from the North Saskatchewan River in Alberta, to the western coast of the Hudson Bay, east to the St. Lawrence river basin and then southward through to the lower Mississippi drainage basin (Harkness and Dymond 1961, Scott and Crossman 1998, DFO 2010). Within Manitoba, the lake sturgeon population is divided into five distinct biogeographically separate groups referred to as Designated Units (DUs) (DFO 2011). DU1 covers the Western Hudson Bay region including the Churchill River system of

northern Manitoba and Saskatchewan. The Saskatchewan River system in Alberta, Saskatchewan and Manitoba is included in DU2. DU3 encapsulates the Nelson River as it flows from Lake Winnipeg to Hudson's Bay and DU4 includes the Assiniboine and Red Rivers, Lake Winnipeg as well as eastern tributary rivers running from Northwestern Ontario. The Winnipeg River is included in DU5 along with English-Wabigoon River system in Northwestern Ontario (Cleator 2010a-e).

As with many long-lived species, lake sturgeon take time to reach sexual maturity. Females are often not able to reproduce until they reach their second decade of life and males age 15 to 20 years before participating in spawning (Harkness and Dymond 1961). Another factor that limits reproduction is lake sturgeon spawn intermittently. Females may follow a three to seven year reproductive cycle while males may take two to four years between spawning events (Auer 1999).

### 1.1.3 Status

Sturgeon meat and roe for caviar were in high demand at the turn of the 20th century which led to intense overfishing throughout the continent (Dick et al. 2006). Exploitation throughout the historical range led to a crash in sturgeon populations and the subsequent closure of the commercial fishery in Manitoba (Harkness and Dymond 1961). Since then, sturgeon populations have struggled to recover due to a combination of late sexual maturity, overfishing and habitat loss (Auer 1996).

In 2006, lake sturgeon within Manitoba river systems (DU1 to DU5) were designated as endangered (COSEWIC 2006, 2011) and are currently being considered for inclusion into Schedule 1 of the federal Species at Risk Act (SARA registry 2010).

### 1.1.4 Management Strategies

The province of Manitoba assumed responsibility for fisheries management in 1930, after most provincial sturgeon stocks had been depleted. A lake sturgeon management strategy was implemented by the Manitoba Conservation and Water Stewardship (MCWS) in 1992, updated in 1997 and again in 2012. The management strategy involves multiple approaches to conservation that include: protecting existing sturgeon stocks by closing the commercial fishery, introducing conservation closures on harvesting in regions where stock levels are critically low, performing stock assessments of sturgeon populations and developing sturgeon management boards in partnership with First Nations in order to protect local sturgeon populations. Important research has been undertaken in the areas of lake sturgeon genetics, behaviour, habitat preference and diseases. A conservation stocking program was implemented in 2003 to replenish stocks that were severely depleted (Manitoba Lake Sturgeon Management strategy 2012).

Conservation stocking involves the collection of gametes from wild adult broodstock during spawning. The eggs are fertilized and placed into incubation jars in a hatchery setting. Once the incubation period is complete, larval sturgeon hatch and are reared until they can be released into the wild (Smith and Hobden 2011). The risks of reducing genetic variability, selecting artificial characteristics during mating and the introduction of disease into the native population need to be mitigated (LaPatra et al 1999).

The white sturgeon *Acipenser transmontanus* population within the Kootenai River in the northern United States is declining steadily and under the threat of extinction. The Kootenai Conservation Aquaculture Program was implemented to mitigate population decline and aid in

recovery (KTOI 2007). Wild broodstock chosen are representative of the natural spawning geography and multiple adult sturgeon contribute gametes. Disease testing methods have been implemented to monitor the health of the fish. The objective of conservation stocking is to re-establish natural recruitment while maintaining genetic variability and health in sturgeon populations (LaPatra et al. 1999). Similarly, a concerted effort is taking place to conserve and manage Manitoba's lake sturgeon populations. Culturing lake sturgeon in hatcheries is a valuable tool necessary to replenish dwindling wild stocks and recover the population. Focusing on disease prevention, development of diagnostic tests as well as assessing wild populations for specific pathogens of concern will help create and maintain a sustainable lake sturgeon population (Manitoba Lake Sturgeon Management strategy 2012).

## 1.2 Nucleocytoplasmic Large DNA Viruses

### 1.2.1 Nucleocytoplasmic Large DNA Virus Defining Characteristics.

Nucleocytoplasmic large DNA viruses (NCLDVs) currently comprise a putative monophyletic class of viruses grouped into the families of *Poxviridae, Asfarviridae, Iridoviridae, Ascoviridae, Marseilleviridae, Phycodnaviridae* and *Mimiviridae* as well as the recently discovered Pandoraviruses and Pithoviruses (Iyer et al. 2001, 2006, Yutin et al. 2009, 2014). NCLDVs share complex virion structures with capsids measuring greater than 120nm in diameter and extensive double-stranded DNA genomes larger than 100 kbp (Clouthier et al. 2013, Colson et al. 2013). The capsid proteins are folded in a jelly-roll configuration and often times the virion is icosahedral in shape (Klose and Rossmann 2014). NCLDV replication takes place exclusively in the host cell cytoplasm or the virion lifecycle begins in the host nucleus and concludes in the cytoplasm (Iyer et al. 2001, 2006, Koonin and Yutin 2010). Cytoplasmic

replication takes place in viral factories where cellular components are concentrated and virions are assembled (Iyer et al. 2001, Kuznetsov et al. 2010, Kuznetsov and McPherson 2011).

Conservative phylogenetic reconstructions have mapped approximately 50 core genes to a common NCLDV ancestor (Koonin and Yutin 2010). Using computational methods, 1445 nucleocytoplasmic virus orthologous groups (NCVOGs) were identified, 177 of which were found in two or more NCLDV families and five protein coding sequences are conserved across all NCLDV families (Koonin and Yutin 2010, Yutin et al. 2009, 2013). These include conserved genes encoding for the major capsid protein (MCP), helicase-primase, a DNA polymerase elongation subunit, DNA-packaging ATPase and a late transcription factor (Yutin et al. 2009). Double stranded DNA viruses with a large particle size, such as herpesviruses or baculoviruses, are excluded from the NCLDV classification as they do not contain shared ancestral genes nor do they have a cytoplasmic stage in their replication cycle (Iyer et al. 2001). The complexity and size of these large particles call into question our understanding of virus origins and their evolution.

### 1.2.2 NCLDV Background

Historically, viruses were viewed exclusively as small unfilterable entities and virus isolation involved passing aquatic samples through a 0.2 to 0.45 µm filter prior to examination by cell culture. As a result, viruses with capsid diameters greater than 150 nm were not discovered till 1992 (LaScola et al. 2003). Discovery of the uniquely large *Acanthamoeba polyphaga mimivirus* (APMV) from amoebas in a water-cooling tower in Bradford, England proved to be a breakthrough in the field of virology as the virus size was unprecedented. APMV was originally mistaken for a gram-positive coccus bacterium and named mimivirus for its

ability to “mimic” microbes (La Scola et al. 2003, Iyer et al. 2006). Its genome measures 1,181,404 bp, which is larger than those of parasitic bacteria and archaea (Raoult et al 2004). This led to the creation of a family called *Mimiviridae*. Several other large DNA viruses have been identified either through inoculation of amoeba monolayers with potential virus samples and monitoring for cytopathic effect or by isolating protists and searching for virus within them (La Scola 2014). Pandoraviruses, with genomes reaching lengths up to 2.5 Mb, were discovered in sediment off the coast of Chile and in an Australian freshwater pond (Philippe et al. 2013). The Pithovirus, recovered from permafrost sediment older than 30,000 years, has virions 1.2 uM in size containing a genome of 600 kb (Legendre et al. 2014). The NCLDV “super-group” is not a formally recognized group by the International Committee on Taxonomy of Viruses (ICTV). The new order *Megavirales* has been proposed as a formal taxonomic classification encompassing the seven NCLDV families (Colson et al. 2012, 2013, Yutin et al 2014).

In 2008, a small 50nm icosahedral virus dubbed Sputnik was identified in association with the APMV virus. This pathogen appeared to have deleterious effects on APMV virion production and was termed a virophage due to its analogy with bacteriophages (La Scola et al. 2008). Ten additional virophages have since been uncovered; all infect members of the *Mimiviridae* family (LaScola et al. 2010, Fischer et al. 2011, Zhou et al. 2013, Gaia et al. 2013, 2014, Kutikhinm et al. 2014). These viral parasites further complicate metagenomic analysis as they could be the source of widespread horizontal gene transfer (HGT) (Colson et al. 2012, Filee 2013). This relationship between phage and host may be a contributing factor in the complexity of NCLDV evolution.

### 1.2.3 NCLDV Evolution

NCLDVs have a biological sophistication similar to complex cellular life forms and may evolve by similar mechanisms including HGT, lineage-specific gene expansion and gene duplication (Boyer et al. 2009, Filee 2013). Amoeba of the genus *Acanthamoeba* act as a virus host and provide an ideal environment for exchange and gain of genes between pathogen, bacteria, virophage and host (Boyer et al. 2009, Colson et al. 2012). Among NCLDVs, viruses infecting Metazoa or free living algae that do not feed on bacteria have a higher ratio of host-derived genes. Those NCLDVs that regularly infect or live symbiotically with eukaryotes ingesting bacteria have greater potential to acquire bacterial genes (Iyer et al. 2006, Filee et al. 2008, Filee 2009). Mobile genetic elements (MGEs) including virophages and transpovirions (small mobile units of DNA) also play a role in gene transfer by shuttling DNA between host and pathogen (Filee 2013). The resulting picture of NCLDV evolution is a complex process resulting in a high degree of genome plasticity and mosaicism.

Genes related to translation, such as aminoacyl-tRNA synthetases and translation factors, are shared among Eukarya, Bacteria, Archaea and the NCLDVs. These shared proteins, though not found in other viruses, are universal in cellular life and led to the proposed delineation of NCLDVs as a fourth domain of life (Raoult et al. 2004, Boyer et al. 2011). Recent phylogenetic reconstruction that takes into account sequence homoplasy and heterogeneity indicates that it may be inappropriate to place NCLDVs into a separate fourth domain (Williams et al 2011, Yutin et al. 2014). The universal genes present in members of the NCLDVs were likely acquired from their eukaryotic hosts rather than by independent evolution (Yutin et al. 2014).

Though NCLDV members may not qualify for a separate domain of life they are fundamentally distinct organisms.

NCLDVs are exceptional in that they are relatively independent from host cells and encode several proteins used in viral reproduction. These include genes for DNA polymerase, DNA clamps used in replication, Holliday junction resolvases and topoisomerases, helicases, transcription factors, ATPase pumps and chaperones mediating capsid assembly (Iyer et al. 2001, 2006). This relative autonomy indicates that NCLDVs descended from a common ancestor that likely had the ability to replicate with some independence from a host (Iyer et al. 2001, 2006). Though some virus groups have lost shared ancestral genes, approximately 50 core genes encoding for crucial viral functions have been mapped to a common NCLDV ancestor (Koonin and Yutin 2010, Yutin et al. 2009, 2013). Phylogenetic reconstructions of shared gene complements suggest that giant viruses may have evolved from smaller simpler agents via extensive gene gain from eukaryotes, bacteria and each other (Iyer et al. 2006, Yutin et al 2014). This evolutionary process probably occurred on at least three different occasions because even though giant viruses Pandoravirus, Pithovirus and Mimiviruses do share some core genes, phylogenomic analysis suggests they evolved from independent ancestors within the NCLDV group. Each of these giant viruses can be placed into separate subtrees within the proposed *Megavirales* order (Yutin et al. 2014). A clearer understanding of the evolutionary process and common ancestor will be revealed with further virus discovery and genome sequencing.

### 1.2.4 Host Range

NCLDVs have a broad host range. *Ascoviridae* members infect insects, mainly those of the *Noctiudae* family (Federici 1983). Viruses belonging to the family *Iridoviridae* have the capability to infect insects, several fish species, amphibians as well as snails (Williams et al. 2005). The *Iridoviridae* family member, Frog-virus 3 (FV3), is commonly found in amphibians and turtles; however, it was recently identified as the agent responsible for mass mortality of juvenile pallid sturgeon *Scaphirhynchus albus* (Waltzek et al. 2014). *Phycodnaviridae* members infect algal hosts, with the exception of Pandoraviruses which infect amoeba (Philippe et al. 2013, Yutin et al. 2014). The Marseillevirus has also been reported to replicate in amoeba (Boyer et al. 2009). Members of *Ascoviridae* or *Poxviridae* infect mammals, whereas viruses in the *Poxviridae* family are also found in insects, reptiles and birds (Koonin and Yutin 2010). Members of the *Mimiviridae* family have been found in amoeba, microzooplankton as well as algae (ex: green algae, heterokonts and haptophyta) (Fischer et al. 2010, Colson et al. 2013). Furthermore, Mimivirus relatives have been isolated from invertebrates such as corals, marine sponges, leeches and oysters, as well as from human and animal samples (Claverie et al. 2009, Boughalmi et al. 2013, Saadi et al. 2013, Dornas et al. 2014). The sturgeon viruses discussed in this thesis may be members of a new, as yet, unrecognized virus genus and family within the order *Megavirales*.

## 1.3 NCLDVs Found in Sturgeon

### 1.3.1 Host and Geographic Range

A growing number of NCLDVs are associated with members of the Acipenseridae family. Manitoban lake sturgeon mortalities have been associated with Namao virus (NV) (Clouthier et al 2013). White sturgeon iridovirus (WSIV) has been isolated from white sturgeon populations in California, Oregon and Idaho (Hedrick et al. 1992, LaPatra et al. 1994) and an unique WSIV isolate has been found in white sturgeon originating from the Fraser River in British Columbia (BCWSIV) (Raverty et al. 2003). Lake sturgeon are susceptible to WSIV infection, though they show resistance to serious manifestation of disease (Hedrick et al. 1992). The Missouri River sturgeon iridovirus (MRSIV) is associated with disease and mortality among pallid and shovelnose sturgeon *Scaphirhynchus platorynchus* from the Missouri River within North Dakota, South Dakota and Montana (Kurobe et al. 2010, 2011). An iridovirus was isolated from Russian sturgeon *Acipenser guldenstadi* cultured in Northern Europe (Adkinson et al. 1998). Classifying the latter virus as a NCLDV member is tentative as genetic information for it is not available. A similar virus has been uncovered in shortnose sturgeon *Acipenser breviro* originating from the St. John River in Atlantic Canada. Preliminary phylogenetic analysis, discussed in chapter 3, suggests that the shortnose sturgeon iridovirus is a member of the sturgeon NCLDV group within the superfamily.

### 1.3.2 Disease Dynamics

Sturgeon NCLDV infections are epitheliotropic in nature and are regularly found in epithelia tissues of the nose, mouth, gills and barbels (Watson et al. 1998b, Kurobe et al. 2011). Histological examination of infected tissues reveals hypertrophied amphophilic staining epithelial cells surrounded by a translucent pericellular cisterum (Hedrick et al 1990, 1992,

Watson et al. 1998b). These damaged cells lead to the impairment of osmoregulation organs and may explain the compromised swimming behavior and erratic buoyancy of infected sturgeon. Also, the impairment of sensory and olfactory organs, such as the barbels, could lead to changes in feeding behavior and cause the cessation of feeding and eventual starvation observed in sturgeon suffering with NCLDV diseases (Watson et al. 1998b).

NCLDV-associated mortality events tend to impact juvenile sturgeon less than one year in age and smaller than 15 cm in length. Disease outbreaks have resulted in upwards of 95% mortality in captive juvenile sturgeon populations (Hedrick et al. 1990, Kurobe et al. 2011). Infections are also associated with a chronic wasting syndrome responsible for impairing fry and fingerling growth and reducing sturgeon survival (Hedrick et al. 1990, Watson et al. 1998b). WSIV disease is manifested as respiratory dysfunction, anorexia, poor osmoregulation and susceptibility to secondary microbial infections (Watson et al 1998b, Drennan 2006). Water-borne transmission of WSIV and MRSIV has been demonstrated, in experimental trials, by cohabitation of infected sturgeon with naïve fish (Hedrick et al. 1990, Drennan 2006, Kurobe et al 2011). Furthermore, adult sturgeon have been shown to act as asymptomatic carriers of sturgeon NCLDV and have the capacity to transmit the virus when not presenting symptoms (Drennan et al. 2005, Kurobe et al. 2011). WSIV and MRSIV infections may be chronic and, therefore, it is possible that adult broodstock may transmit virus to progeny during spawning (LaPatra et al. 1994, Georgiadis et al 2001, Drennan et al 2006). Infectious agents can be passed to offspring through eggs via either egg-surface-associated or intra-ova situated mechanisms. Viruses shown to be associated with egg-surface transmission include the infectious hematopoetic necrosis virus (IHNV) (Golds and Mead 1995, Drennan et al. 2006). Disinfection of

the eggs using iodine and/or ozonated seawater reduces or prevents transmission of egg-associated pathogens (Arimoto et al. 1996, Grotmol and Totland 2000). However, pathogens carried intra-ovum cannot be effectively eliminated by egg disinfection (Bootland et al. 1991, Drennan et al. 2006). Currently, there is no direct evidence demonstrating that the sturgeon NCLDVs are intra-ovum or egg-surface associated.

Several risk factors contribute to the onset and virulence of sturgeon NCLDV outbreaks. Stressors such as high stocking density, handling, transport, and fluctuations in water temperature and water quality can induce WSIV disease in asymptomatic carriers (LaPatra et al. 1994, Watson et al. 1998b, Giorgiadis et al 2001, Drennan et al 2005, 2006). Familial genetic susceptibility of sturgeon stocks to the virus may also influence the mortality and morbidity of an outbreak (Giorgiadis et al. 2001, Drennan et al. 2006).

### 1.3.3 Sturgeon NCLDV Biological Characteristics

Enlarged cells found in the epithelia of compromised sturgeon contain cytoplasmic inclusion bodies. Electron microscopy of these hypertrophic cells reveals the presence of multiple virions within the inclusion bodies. The virions appear icosahedral in shape, are doubly encapsidated and contain a bar shaped nucleoid (Hedrick et al 1990, Watson et al. 1998, Kurobe et al. 2011, Clouthier et al. 2013). They range from 242 to 262 nm in diameter when measured from apex to apex. Preliminary analysis of the NV genome suggests that it could be greater than or equal to 350 kb (Clouthier unpublished results). Some but not all of the WSIV isolates can been successfully grown in cell culture with white sturgeon spleen or skin cell lines

(Watson et al. 1998). Neither MRSIV nor NV has been successfully cultured *in vitro* despite use of primary cell lines derived from sturgeon hosts (Kurobe et al. 2011, Clouthier et al. 2013).

### 1.3.4 Diagnostic Assay Development and Validation

Diagnosis of sturgeon NCLDVs has historically been made using a combination of histology and electron microscopy. Presumptive diagnosis involves observation of pathognomonic cellular changes in hematoxylin and eosin (H&E) stained tissue sections. Confirmatory diagnosis involves observation of characteristic virus particles within the infected cells by electron microscopy (Hedrick et al. 1990, 1992, Kurobe et al. 2011, Adkison et al. 1998, Clouthier et al. 2013). The sturgeon NCLDVs are difficult to grow in cell culture despite the development of cell lines derived from lake sturgeon, white sturgeon or pallid and shovelnose sturgeon (Hedrick et al. 1991, Watson et al. 1998a, Kurobe et al. 2011, Clouthier et al. 2013). Some of these are permissive to WSIV amplification but they do not support growth of MRSIV (Kurobe et al. 2011). Conventional and quantitative PCR tests have been developed for detection of WSIV (Kwak et al. 2006a, 2006b) or MRSIV (Kurobe et al. 2010). The assays target viral DNA encoding the serpin protein or the major capsid protein. These molecular diagnostic tests have a greater sensitivity and have a higher throughput compared to histological analysis (Kwak et al. 2006a, Drennan et al. 2007, Kurobe et al. 2011). Neither of these assays is capable of detecting NV (Clouthier et al. 2013).

Diagnostic assays must perform consistently and reliably over time. A set of criteria is applied to assay development and validation to establish the fitness of a diagnostic test. The intended purpose of the test will influence its design, development, and optimization. The analytical validation process involves assessing the inter– and intra–assay repeatability,

analytical specificity and analytical specificity of a test. International guidelines for this process are provided by the World Organization for Animal Health (OIE). Validation is an ongoing process requiring continued monitoring of an assay's performance characteristics to ensure consistent relevant results.

### 1.3.5 Study Objectives

Viruses are ubiquitous in the natural aquatic environment and have a direct impact on the health and management of lake sturgeon populations (Manitoba Lake Sturgeon Management Strategy 2012). In this study, specific knowledge and technology gaps pertaining to NV in lake sturgeon will be addressed; diagnostic molecular tests will be designed, developed and optimized for the intended purpose of detecting and diagnosing sturgeon NCLDVs. The analytical performance characteristics of these tests will be evaluated including their analytical specificity, the capacity of an assay to differentiate between target and non-target DNA, and their analytical sensitivity, the detection limit of an assay (OIE 2014). These diagnostic tests will be used to assess NV distribution, disease causality as well as infection transmission dynamics and tissue tropism in juvenile lake sturgeon in Manitoba. Ultimately, this research project's aim is to create a body of knowledge that will aid in the design and development of a lake sturgeon infectious disease management plan.

## 2.0 Identification of a New Unclassified Virus Associated with Mortalities in Manitoba Lake Sturgeon *Acipenser fulvescens*

Based on Clouthier et al. (2013) Dis Aquat Org 102:195-209. Figures and tables used with permission.

### 2.1 Introduction

Lake sturgeon *Acipenser fulvescens* populations in Manitoba were designated as endangered by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) in 2006 and are currently being assessed for inclusion in Schedule 1 of the federal Species at Risk Act (SARA). Potential development of hydroelectric generating stations on the lower Nelson River (Manitoba, Canada) and their subsequent operation for power production may further negatively impact resident lake sturgeon populations through fundamental changes in their physical habitat and biological ecosystem. A conservation stocking program has been implemented as a possible mitigation measure to supplement current lake sturgeon recovery efforts. Through this initiative, juvenile lake sturgeon will be reared in a hatchery setting and then released for the purpose of replenishing natural resident populations. Unusually high mortalities have been observed in populations of juvenile lake sturgeon reared at the Grand Rapids Hatchery (GRB) or the University of Manitoba's Aquatic Animal Holding Facility (UMAAHF).

A virus associated with these mortality events may belong to a monophyletic class of viruses referred to as nucleocytoplasmic large DNA viruses (NCLDVs) (Table 2.1) (Iyer et al. 2001, 2006, Koonin and Yutin 2010). The name is derived from the fact that NCLDVs either start their life cycle in the nucleus and complete it in the cytoplasm or replicate exclusively in the

cytoplasm of the host cell (Iyer et al. 2001, 2006, Koonin and Yutin 2010). They have a virion structure that is exceptionally large (120 to 440 nm), so large that they that they may not be able to pass through a 0.2 – 0.3 µm pore-sized filter historically used to separate bacteria from viruses (Claverie et al. 2006). NCLDVs also have large (0.1 – 1.3 Mb) double stranded DNA genomes, indeed the genome of several NCLDVs exceeds that of the smallest free-living bacteria *Mycoplasma genitalium* (580 Kb, Claverie et al. 2006).

This group of viruses potentially inherited approximately 50 conserved genes from a common virus ancestor (Koonin and Yuitn 2010). They also share 5 core signature genes encoding proteins involved in basic functions such as structure, replication, transcription and chromosome segregation (Yutin et al. 2009). A large repertoire of genes affords NCLDVs relative autonomy from the host cell. As a group, NCLDVs also have the capacity to infect a broad host range that includes amoeba, insects, algae, birds, reptiles, mammals and fish (table 2.1) (Van Etten 2010, 2011).

A subset of NCLDVs infects members of the sturgeon Acipenseridae family. Sturgeon NCLDVs pose a disease risk to fish reared as part of conservation efforts meant to supplement sturgeon populations in North America. These viruses include WSIV which is found primarily in white sturgeon *Acipenser transmontanus* (Hedrick et al. 1990, LaPatra et al. 1994, Rafferty et al. 2003) and MRSIV which is known to infect pallid sturgeon *Scaphirhynchus albus* and shovelnose sturgeon *S. platorynchus* (Kurobe et al. 2010, 2011).

Table 2.1 Members of the nucleo-cytoplasmic large DNA virus (NCLDV) super-family

| Virus family[a] | Host range | Genome size (kb) | Capsid diameter (nm) |
|---|---|---|---|
| Iridoviridae | Insects, cold blooded vertebrates | 100-220 | 120-350 |
| Ascoviridae | Insects, mainly Noctuids | 150-190 | 130 |
| Asfarviridae | Mammals | 170 | 170-190 |
| Poxviridae | Animals, insects, reptiles, birds, mammals | 130-380 | 200 |
| Phycodnaviridae | Green algae; algal symbionts of paramecia & hydras | 150-400 | 130-200 |
| Marseillevirus | Amoeba | 370 | 250 |
| Mimiviridae | | | |
| CroV | Marine microzooplankton | 730 | 300 |
| Mimivirus | Amoeba | 1,181 | 390-400 |
| Mamavirus | Amoeba | 1,191 | Not available |
| Megaviruses | Unknown | 1,259 | 440 |
| Sturgeon viruses | | | |
| NV | Lake sturgeon | unknown | 225-263 |
| WSIV | White sturgeon | unknown | 262 |
| MRSIV | Pallid and shovelnose sturgeon | unknown | 254 |
| RSIV | Russian sturgeon | unknown | 283 |

[a] See Appendix 1 for virus abbreviations; MRSIV, Missouri River sturgeon iridovirus, RSIV, Russian sturgeon iridovirus

The transmission dynamics of sturgeon NCLDVs is complex and still not fully understood. Epizootics attributed to WSIV and MRSIV infection affect white sturgeon, pallid or shovelnose sturgeon during their first year of life and can have mortality rates up to 95% (Hedrick et al. 1990, 1992, Kurobe et al. 2011). If infection is not lethal, a chronic debilitating wasting syndrome may develop leading to inhibited growth and reduced survival in young sturgeon (Hedrick et al. 1990, LaPatra et al. 1994, Kurobe et al. 2011). These viruses are also associated with adult sturgeon which can act as asymptomatic subclinical carriers that intermittently shed virus (Hedrick et al. 1990, LaPatra et al. 1994, Kurobe et al. 2011). Sturgeon NCLDVs are

epitheliotrophic in nature (Hedrick et al. 1990, Drennan et al. 2007) therefore shedding of the epidermis can lead to dispersing the pathogen into the water column and potential horizontal transmission (Hedrick et al. 1990, Watson et al. 1998a, Drennan et al. 2006, 2007, Kurobe et al. 2011). There have been some instances in which egg-associated virus transmission from adult to offspring has been tentatively tied to disease outbreaks (Hedrick et al 1992, LaPatra et al 1994, Georgiadis et al 2001). The severity and onset of a disease outbreak is influenced by the health of the sturgeon which may be negatively impacted by high stocking densities, handling, fluctuations in water level or changes in water temperature (LaPatra et al 1994, 1996, Watson et al 1998b, Georgiadis et al 2000, 2001, Drennan et al 2005, 2006).

The detection and diagnosis of sturgeon NCLDVs are crucial for ensuring the success of sturgeon conservation stocking programs. As mentioned above, WSIV and MRSIV are localized primarily in epithelial cells of the integument, but are also found in gill epithelia (Hedrick et al. 1990, Watson et al. 1998a, Drennan et al. 2007, Kurobe et al. 2011). Examination of infected epithelial tissues, through histology and electron microscopy, reveals enlarged hypertrophied amphophilic staining cells with inclusion bodies containing icosahedral virions (Hedrick et al. 1990, 1992, Kurobe et al. 2011). WSIV can be amplified in cell culture, however, only some isolates have been successfully isolated and cytopathic effects appear inconsistently (Hedrick et al. 1991, Watson et al. 1998a). Efforts to grow MRSIV on cell lines derived from shovelnose and pallid sturgeon tissue have been unsuccessful (Kurobe et al. 2011). Conventional polymerase chain reaction (cPCR) and/or quantitative PCR (qPCR) have been developed to diagnose WSIV and MRSIV infections. Both the qPCR and cPCR assays designed to detect WSIV amplify the MCP of the virus (Kwak et al. 2006a, 2006b) and the qPCR assay used for MRSIV detection relies on

targeting the serpin gene (Kurobe et al. 2010). Both of these tests were unable to detect the virus associated with lake sturgeon mortalities that took place within Manitoba hatcheries in 2009 and 2010.

A new emerging pathogen associated with lake sturgeon mortality is described in this chapter. Preliminary phylogenetic testing reveals the relationship of this novel NCLDV to other viruses within the taxonomic group.

## 2.2 Materials and Methods

### 2.2.1 Case History

In 2009 and 2010, groups of juvenile lake sturgeon reared and held at the Grand Rapids Hatchery (GRH, Grand Rapids, MB) or the University of Manitoba Aquatic Animal Holding Facility (UMAAHF, Winnipeg MB) began to experience unusual mortalities (Fig 2.1).

Lake sturgeon reared at UMAAHF originated from multiple gamete crosses. Gametes were collected from wild broodstock (16 males; 8 females) sourced from the Winnipeg River (MB) at Slave falls in May 2008. Fertilized eggs were hatched at a temporary Hatchery in Pinawa (MB). They were reared until fry reached lengths of 15-20 mm at which time they were transferred to the UMAAHF (June 12th, 2008). Tissue samples were collected from seven lake sturgeon perceived to be healthy on Dec 7, 9 11 and 17$^{th}$, 2008. Approximately 1758 lake sturgeon were transferred to successively larger tanks until they were moved to four 600 gallon tanks on the 22$^{nd}$ of December, 2008 (5.7g, length not recorded). They were held in de-chlorinated 16˚C municipal water at 1 to 2 L min$^{-1}$. Mortalities began to appear amongst the 606 lake sturgeon held in tank 600-1 (3.0 g, cumulative percent mortality CPM of 64%) in February of 2009 (Fig. 2A). Mortalities then began to occur in May 2009 in tanks 600-3, 600-4 and 600-5 stocked with

569, 195 and 388 lake sturgeon (19.3 g, 174 mm), respectively. Cumulative mortalities in these three tanks ranged from 62 to 99%. Tissue was collected from dead or moribund fish and submitted for diagnostic testing.

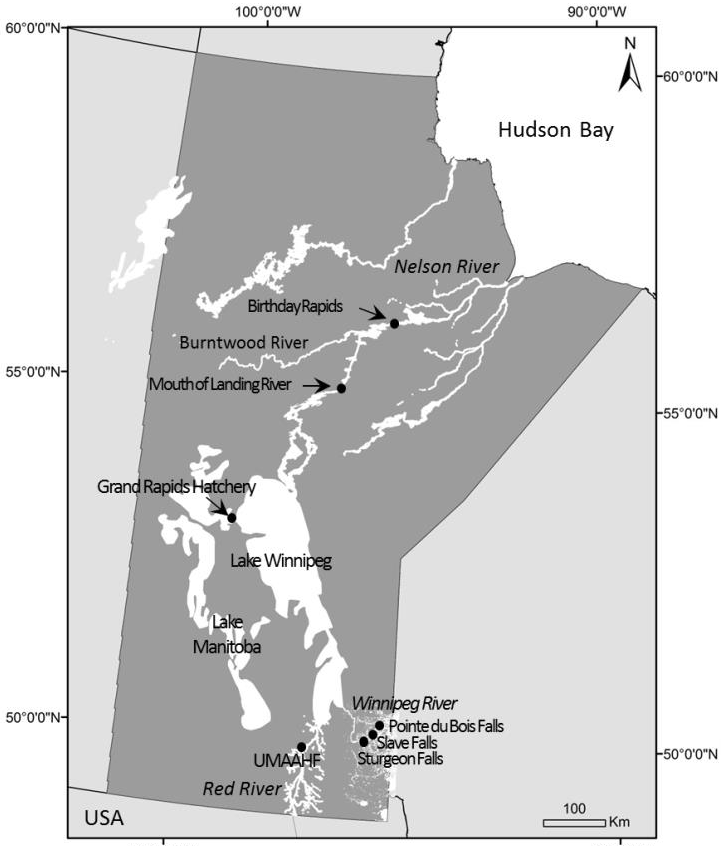



Figure 2.1 Location of rivers in Manitoba (dark grey shading), Canada where wild lake sturgeon *Acipenser fulvescens* broodstock were collected and hatcheries where progeny were held. UMAAFH: University of Manitoba Aquatic Animal Holding Facility

The GRH received fertilized eggs collected from wild Nelson River (MB) lake sturgeon stock (6 males, 4 females) on June 15$^{th}$, 2009. The hatchery also received fry derived from wild Winnipeg River sturgeon (17 males, 5 females) on July 9$^{th}$, 2009. The two populations were reared in separate long, shallow troughs (12”w x 17’ l x 10” d) and received untreated water

from the Saskatchewan River (Cross Bay, Cedar Lake) (10.1˚C average temperature June through September; 4.9˚C average temperature October through May). Unusual mortalities began to appear among the 1,974 Nelson River stock (Nov 4, 2009: 17.3 g, 15.5 cm) in late

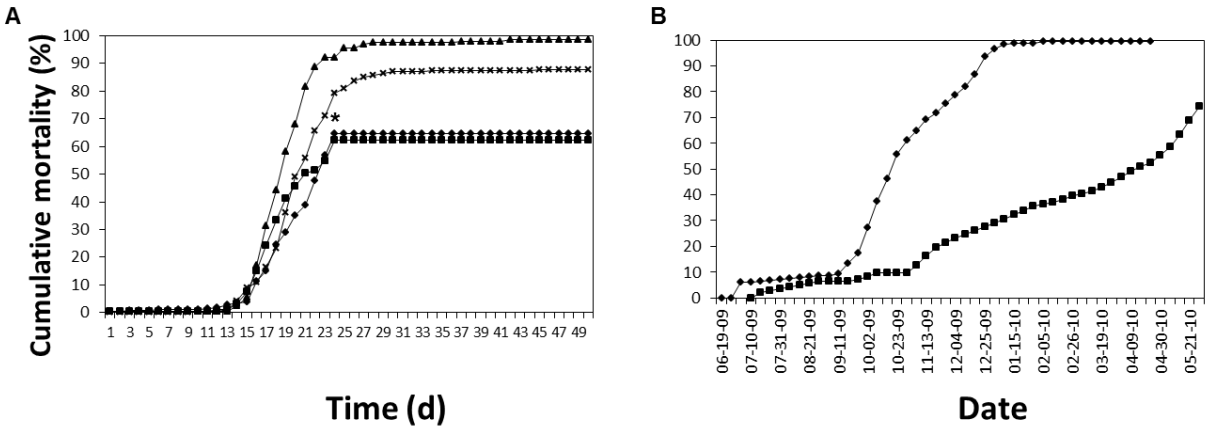



Figure 2.2 Cumulative mortality among juvenile lake sturgeon. (A) Sturgeon held at University of Manitoba Aquatic Animal Holding Facility: progeny (2008 year class) from wild Winnipeg River broodstock collected at Slave Falls. Time zero was normalized to 16 d prior to onset of mortality within each of 4 tanks: (♦) Tank 600-1, Day 16 = Feb 11, 2009; (■) Tank 600-3, Day 16 = May 9, 2009; (▲) Tank 600-4, Day 16 = May 27, 2009; (x) Tank 600-5, Day 16 = May 12, 2009. (*) Date when fish remaining in tanks 600-1 and 600-3 were removed. (B) Sturgeon held at Grand Rapids Hatchery: progeny (2009 year class) from (♦) wild Nelson River broodstock collected near Landing River and (■) Winnipeg River broodstock collected at Pointe du Bois

September 2009 and continued until February 19th, 2009 when CPM reached 99.6% (Fig. 2B). Mortalities also began to increase in approximately 14,608 Winnipeg River lake sturgeon held at the GRH (11 g, 13.9 cm) in early November 2009 and reached a CPM of 74% on May 28, 2010. On this date, all the remaining fish were euthanized. Sample of tissues were submitted from each group of lake sturgeon for diagnostic evaluation.

The UMAAHF also received wild Winnipeg River (Pointe du Bois) lake sturgeon progeny described in the previous paragraph on October 8th, 2009. The fish were held in 600 gallon tanks receiving dechlorinated 16˚C municipal water at 1 to 2 L / min. Intermittent mortality

occurred in these tanks throughout 2010 (11 to 32 g, lengths not recorded). Tissue samples were collected from dead or moribund fish and submitted for diagnostic evaluation.

### 2.2.2 Bacteriology, Virology, Histology and Electron Microscopy

Bacteriology was performed on dead or moribund juvenile lake sturgeon collected from the mortality events at UMAAHF (N = 10; Winnipeg River stock) or GRH (N = 15; 10 Winnipeg River stock, 5 Nelson River stock). Gill, spleen and kidney tissue were used to inoculate tryptic soy agar (TSA) supplemented with 5% sheep blood or MacConkey agar. Samples were incubated at 22˚C and 35˚C under aerobic as well as anaerobic conditions. Chocolate agar as well as TSA containing 5% sheep blood was incubated with gill tissue and incubated at 22˚C and 35˚C in a $CO_2$ candle jar. Further, skin, gill and intestinal wet mounts as well as Gram stains of kidney and gill smears were performed for dead or moribund fish from the UMAAHF (N = 15; Winnipeg River stock) and GRH (N = 15; 10 Winnipeg River stock, 5 Nelson River stock).

Barbels, gill arches, operculum, head skin, anal and/or pectoral fins as well as kidney, liver, spleen and/or pyloric caeca tissues were collected from Winnipeg River (N = 33) and Nelson River (N = 15) lake sturgeon. A portion of samples were sent to British Columbia Freshwater Fisheries Society (BCFFS) in Duncan, British Columbia. Epithelioma papillosum cyprini (EPC), Chinook salmon *Oncorhynchus tshawytscha* embryo 214 (CHSE-214), white sturgeon skin (WSSK-1), white sturgeon spleen (WSS-2), lake sturgeon gill (LSGI) and/or lake sturgeon gonad (LSGO) cell lines were seeded into 24-well plates. A homogenate was generated from tissues collected from a single fish (1:50 dilution) and 0.1 mL was used to inoculate the cell monolayers (Fijan et al. 1983, Lannan et al. 1984, Hedrick et al. 1991). Cell lines were propagated at 16˚C in minimum essential medium with Earle's salts (MEM-E) or Hanks salts

(MEM-H) containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine (Life Technologies). After 1 h of absorption, 1 ml of MEM-E or MEM-H supplemented with 2% FBS, 2 mM L-glutamine and 1 x antibiotic/antimycotic (Life Technologies) was added to each well. Cells were incubated at 16˚C and the monolayers were observed daily for evidence of CPE. At the time of harvest, each inoculated monolayer was scraped into suspension and transferred to a storage vial and placed at -80˚C

Moribund lake sturgeon from the Winnipeg River stock (5 from GRH, 4 from UMAAHF) and 5 from the Nelson River stock were prepared for histological examination. Each fish had an incision made in the abdomen and the whole fish was placed in 10% buffered formalin. Tissues were fixed for 24 to 48 hours and then processed for light microscopy into paraffin blocks. The blocks were cut into 5 µm sections, deparaffinised and stained with hematoxylin and eosin (H&E).

Gill from a single juvenile Winnipeg River lake sturgeon (UMAAHF) was examined with transmission electron microscopy (TEM). The tissue was formalin fixed, rinsed in buffer and post-fixed in 1% aqueous osmium tetroxide, dehydrated through a graded ethanol series, infiltrated and then embedded in Araldite 502/Embed 812 resin. Thin sections, approximately 100 nm, were stained with uranyl acetate and lead citrate prior to being examined with a Philips CM 120 transmission electron microscope at 80 kV. Digital images were taken with an AMT XR-611 camera system.

### 2.2.3 Establishment of Lake Sturgeon Cell Lines

A cell suspension was created by aseptically removing gill and gonad tissue from two juvenile sturgeon (52.5 and 62.7 g). Tissues were rinsed five times with sterile phosphate

buffered saline (PBS), cut into smaller pieces and resuspended in 0.25% porcine trypsin/EDTA (HyClone). Once suspended, cells were further separated from tissue through mechanical agitation for 20 min at 22 ˚C. The suspension was diluted 1:1 (v/v) with Dulbecco's Modified Eagles Medium (DMEM)/Ham's F-12 supplemented with 10% FBS, 200 IU penicillin and 200 µg streptomycin / ml (Life Technologies). The cell solution was centrifuged at 1228 x *g* for 15 min at 4˚C, resuspended in fresh media and used to seed 25 $cm^2$ flasks. Lake sturgeon cell lines were maintained in in MEM-H supplemented with 10% FBS, 2 mM L-glutamine and 2x antibiotic/antimycotic (Life Technologies) at 16 ˚C.

### 2.2.4 DNA Synthesis and Plasmid Purification

The DNA sequence encoding the MCP of white sturgeon iridovirus (GenBank accession number DQ897645) was synthetically constructed and ligated into a pJ204 cloning vector (DNA2.0, California, USA). The plasmid was transformed into *Escherichia coli* DH5$\alpha$ competent cells. The cells were lysed and the plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

### 2.2.5 DNA Extraction

Dead, moribund or frozen lake sturgeon were collected from mortality events occurring at GRH and University of Manitoba's UMAAHF. Tissue samples including ventral head skin, abdominal skin, gill arches, barbells, operculum, kidney, liver as well as pectoral and anal fins were homogenized in 50 mg portions. Samples were combined with lysis buffer (Qiagen buffer ATL), a 5 mm stainless steel bead (Qiagen) and pulverized in a TissueLyser (Qiagen) for 2 min at 30 Hz twice. DNA was extracted from the tissue homogenates using the DNeasy Blood and

Tissue Kit (Qiagen) following the manufacturer instructions and quantified using the Nanodrop 8000 (Nanodrop Technologies).

### 2.2.6 PCR Amplification, Cloning & DNA Sequence Analysis

Extracted DNA was tested with the WSIV and MRSIV qPCR protocols described by Kwak (2006b) and Kurobe et al. (2010), respectively. Additional samples were sent to the Animal Health Center (AHC) in Abbotsford, BC for molecular testing.

Primers designed to target the MCP of the Namao virus were chosen based on conserved regions shared among representative members of the NCLDV family of viruses (Appendix 1). Using pairwise combinations of primers (Table 2.2), a portion of Namao virus MCP gene was amplified by cPCR using DNA extracted from lake sturgeon experiencing mortality at either GRH or UMAAHF was used as a template. Naïve lake sturgeon tissue as well as white and lake sturgeon cell lines were also tested to determine the origin of the amplicon. Each 25 µl reaction contained 1 x PCR buffer (Applied Biosystems), 1.5 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates, 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.8 µM of each primer and between 100 to 500 ng of DNA. Thermocycling parameters were as follows: 95˚C for 5 min, 40 cycles of (95˚C for 30 s, 52 or 53˚C for 30 s, 72˚C for 1 or 1.5 min) followed by a post-cycle extension at 72˚C for 10 min.

Table 2.2 Oligonucleotides used in the present study for amplification of DNA encoding lake sturgeon Namao virus major capsid (partial). Nucleotide positions are shown relative to white sturgeon iridovirus major capsid protein coding region (GenBank accession no. DQ897645)

| Primer | Sequence (5' to 3') | Nucleotide position (bp)[a] | Source |
|---|---|---|---|
| speF | CCA GAA ATG ACT TAC TTC AAG | 64 | Present study |
| ginF | GGT ATC AAC GTA TAT TCG TTT GC | 1369 | Present study |
| ginR | GCA AAC GAA TAT ACG TTG ATA CC | 1391 | Present study |
| pekR | CCA GAA GGT TGG TGC TTT TCA GG | 1421 | Present study |
| glaR | GTA TGC CAG ACC CGC TAG ACC | 1587 | Present study |

[a] Nucleotide position relative to white sturgeon iridovirus major capsid protein coding region, DQ897645

PCR amplification products were analyzed for purity and size by electrophoresis in 1% agarose gels and then gel purified with the QIAquick Gel Extraction Kit (Qiagen) as outlined by the manufacturer. The amplicons were TA cloned into vector pGEM-T Easy (Promega). As outlined above (section 1.1.4), plasmid DNA was isolated from cultures of transformed *E. coli.* The plasmid DNA was screened for inserts using the relevant cPCR primer pairs. At least three positive clones per amplicon were selected and both DNA strands were sequenced by the dideoxynucleotide chain termination method with an automated sequencer (Sanger et al. 1977). DNA sequences were evaluated using BioEdit v7.0.9.0 software (Hall 1999).

### 2.2.7 BLASTP, Sequence Alignment & Phylogenetic Analysis

Amino acid sequences similar to the deduced amino acid sequence of the partial NV MCP were identified from searching protein databases using the BLASTP program (Altschul et al. 1990, 1997). The alignment of multiple protein sequences was performed by TCoffee (Notredame et al. 2000, Di Tommaso et al. 2011) with sequences that were trimmed to the first and last amino acids of the Namao virus MCP. A percent identity and similarity matrix

comparing the trimmed MCP sequences was created using the Matrix Global Alignment Tool (MatGAT 2.02) software (Campanella et al. 2003). Conserved domains in the partial protein sequences were annotated through the Conserved Domain Database using the National Center for Biotechnology Information's (NCBI) Conserved Domain Search service (Marchler-Bauer et al 2010).

Phylogenetic tree reconstructions were projected using Bayesian Inference analysis as implemented by MrBayes v3.2.1 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) and maximum-likelihood as implemented by PHYML 3.69 (Guidon et al. 2010). Bayesian Inference used multiple independent runs of aamodelpr = mixed and default settings for 1,500,000 generations until the average deviation of the split frequencies was <0.002. For maximum-likelihood trees, clade confidences were estimated from 1,000 bootstrap replicates. The resulting output trees from each method were visualized using FigTree v1.3.1 software (Rambaut 2008).

## 2.3 Results

### 2.3.1 Disease Signs and Bacteriology

Two separate facilities, the GRH and UMAAHF, experienced lake sturgeon mortality events in 2009 and 2010 (Fig.2.2). Affected fish stopped eating, became anorexic and presented erratic swimming behaviour such as swimming with the tail up and head down in the water column, unstable equilibrium and gasping at the water surface. Some sturgeon presented exaggerated opercular movement, dark red gills, pinpoint foci and accumulations of gill mucus. Petechial haemorrhaging was visible in some of the fish, particularly at the base of the pectoral

and anal fins as well as around the mouth and barbel area. Bacteriological testing of samples derived from moribund fish revealed no bacterial growth.

### 2.3.2 Virology and PCR

Namao virus was detected, by amplification of a 219 bp region of the NV MCP, in almost 94% (59/63) of dead or moribund lake sturgeon originating from the Nelson or Winnipeg Rivers (Table 2.3) tested in this study. The cPCR ginF and glaR primer pairs generated an amplicon of the expected size (data not shown) and sequence (described below). Neither the WSIV MCP-based nor the MRSIV serpin-based qPCR tests tested positive with these samples. Our Namao virus MCP-based cPCR test, which is able to detect WSIV including synthetically constructed WSIV MCP DNA, MRSIV and a recently diagnosed virus from white sturgeon cultured in British Columbia, will be described in further detail in Chapter 3. Lake sturgeon samples collected in December 2008 (before the UMAAHF 2009 outbreak) tested negative for the presence of Namao virus nucleic acid using the ginF/glaR cPCR assay.

Table 2.3 Diagnostic test results (no. positive/no. tested) from samples taken during disease outbreaks in the 2008 and 2009 year classes of juvenile lake sturgeon, sourced from the Winnipeg (WR) and Nelson River (NR), and raised at Grand Rapids Hatchery (GRH) and the University of Manitoba Aquatic Animal Holding Facility (UMAAHF). cPCR: conventional polymerase chain reaction; qPCR, quantitative PCR. See Appendix 1 & Table 2.1 for virus abbreviations

| Molecular test method[a] | 2008 YC | 2009 YC | | |
|---|---|---|---|---|
| | UMAAHF | Grand Rapids Hatchery | | UMAAHF |
| | Winnipeg River | Nelson River | Winnipeg River | Winnipeg River |
| NV cPCR | 7/8 | 3/3 | 35/37 | 14/15 |
| WSIV qPCR | 0/8 | 0/3 | 0/35 | 0/12 |
| MRSIV qPCR | 0/8 | 0/3 | 0/35 | 0/12 |

[a] See Appendix 1 for virus abbreviations; MRSIV, Missouri River sturgeon iridovirus

Inoculation of primary lake sturgeon gill and gonad cell lines with NV-positive tissue homogenates did not cause noticeable CPE after 21 to 60 days or after blind passage on the same cell lines. Culture fluid containing suspended cells from CPE-negative monolayers did test positive for Namao virus after the first passage (the second passage was not tested for NV DNA). The cell monolayers from white sturgeon (WSSK, WSS-2), Chinook salmon (CHSE-214) and common carp *Cyprinus carpio* (EPC) did not display CPE within 21 days post- inoculation using tissue homogenates derived from the same infected populations of lake sturgeon. The WSS-2 cells were inoculated with four samples, two of which tested positive for Namao virus by cPCR. The WSS-2 cells tested negative for the presence of Namao virus after a single passage. The cPCR did not amplify the NV 219 bp DNA product from any of the naïve cell lines used in this study.

### 2.3.3 Histology and TEM

Hypertrophic cells were identified in low numbers in H&E stained histological samples of gill and skin epithelia tissues collected from moribund lake sturgeon (Fig. 2.3). Cytoplasmic inclusion bodies often associated with sturgeon NCLDV infections of white-, pallid- and shovelnose sturgeon were observed in cytomegalic cells scattered throughout the epithelial layer of the gill lamellae. Kidney, heart, brain lower intestine, pancreas or nervous tissues of the same fish did not show evidence of infection. Quantification of inclusion bodies in longitudinal sections of 14 juvenile lake sturgeon collected from GRH in May 2009 revealed a viral tissue tropism for epithelial cells of the integument (Table 2.4).

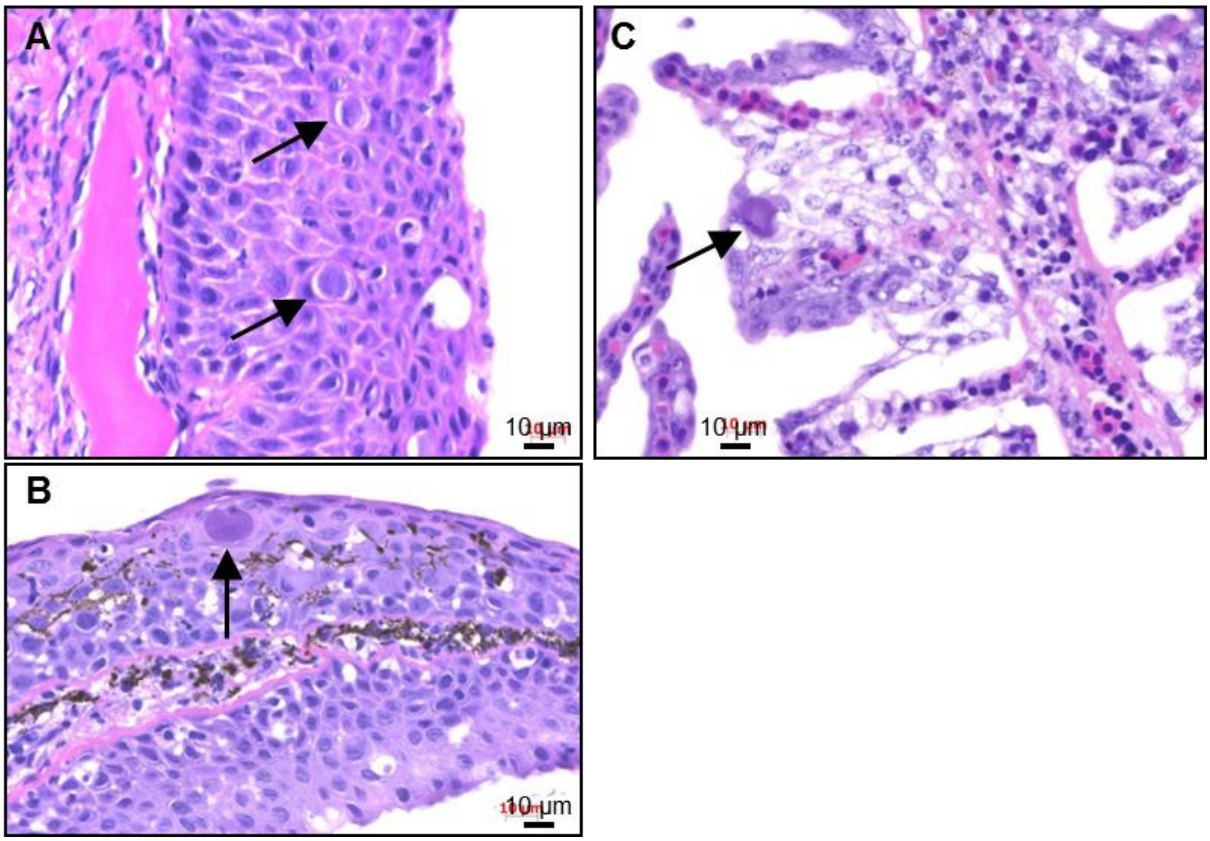



Figure 2.3 Light photomicrographs of H&E stained epithelial tissue sections from moribund juvenile lake sturgeon. (A) Skin tissue from Winnipeg River lake sturgeon (2008 year class) housed at the University of Manitoba Aquatic Animal Holding Facility. (B) Skin and (C) gill tissue from Winnipeg River lake sturgeon (2009 year class) housed at Grand Rapids Hatchery. Large amphophilic intracytoplasmic inclusions are evident (arrows)

Transmission electron microscopy of cytomegalic cells in the gill lamellae epithelium revealed icosahedral-shaped virus particles in the cell cytoplasm (Fig. 2.4). Virions containing a condensed bar-shaped core, amorphous virus-like particles and empty or incomplete capsids were present. The intact virions had two capsids and were approximately 242 nm in diameter as measured from side to side and 282 nm vertex to vertex (Fig. 2.4, Table 2.1).

Table 2.4 Enumeration of inclusion bodies (average no.) in longitudinal sections of moribund or mildly infected juvenile lake sturgeon from the Winnipeg River or Nelson River.

| Site | Average number of inclusion bodies | | |
|---|---|---|---|
| | Moribund sturgeon Winnipeg River (n=5) | Moribund sturgeon Nelson River (n=4) | Juvenile sturgeon Winnipeg River (n=5) |
| Nasal pits | 8.5 | 4 | 1 |
| Snout | 3.5 | 2.25 | 0 |
| Mouth | 4.3 | 1.75 | 0 |
| Gill | 3.6 | 3.5 | 0.4 |
| Epidermis | | | |
| Cranial | 14.1 | 8.45 | 2.0 |
| Abdominal – mid | 9.4 | 3.5 | 0.8 |
| Abdominal - distal | 4.5 | Not done | 0.5 |
| Fins | | | |
| Pectoral | 12.3 | 2.75 | 0 |
| Dorsal | 17 | Not done | Not done |
| Pelvic | 4.5 | 3.5 | 0 |

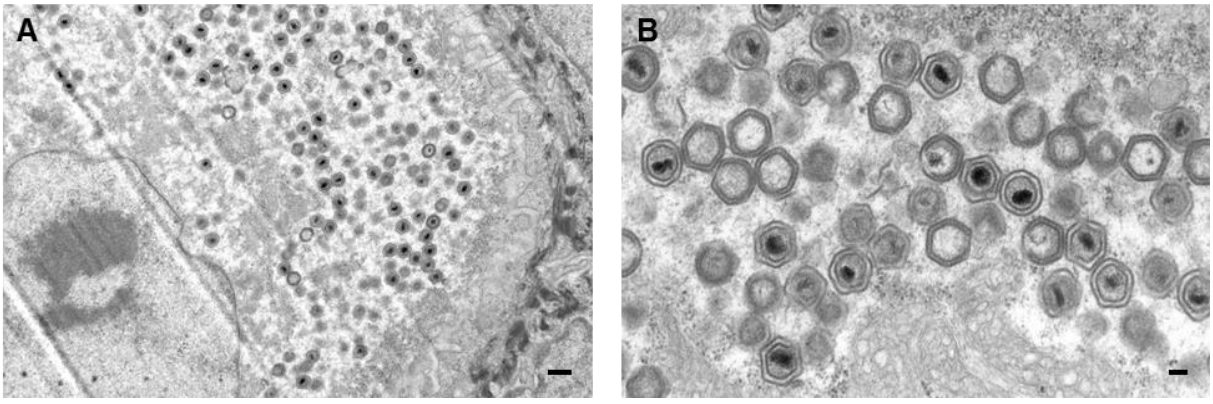



Figure 2.4 Transmission electron micrographs of cytoplasmic hexagonal virus particles in epithelial cells of gill lamellae from Winnipeg River, juvenile lake sturgeon (2008 year class) held at the University of Manitoba Aquatic Animal Holding Facility. Intact virions were 225 to 263 nm (n = 18, mean ± SD = 242 ± 11 nm) in diameter, as measured from side to side, and 263 to 300 nm from vertex to vertex (n = 18, mean ± SD = 282 ± 13 nm). Scale bars: (A) 500 nm; (B) 100 nm

### 2.3.4 MCP Alignment and Analysis

Based on the WSIV MCP sequence, approximately 95-96% of the Namao virus MCP open reading frame or its inferred amino acid sequence was captured (GenBank accession number JX155659). The amino acid sequences of the NV and WSIV MCPs were 83.5% identical (Table 2.5). They shared the smallest E-value (7e-99) with viruses grouped in the *Mimiviridae* family and to a lesser extent *Phycodnaviridae* (3e-46) and *Iridoviridae* (1e-15), according to BLASTP analysis (Altschul et al. 1990, 1997). Domains conserved among the NV and WSIV MCP are presented in Appendix 2.

Table 2.5 Analysis of the major capsid protein from Namao virus and other nucleo-cytoplasmic large DNA viruses. Values are expressed as a range of % identity / % similarity of amino acid sequences. WSIV: white sturgeon iridovirus; IAMV: Irido-asco-Marseilleviruses

| | Sturgeon viruses | | Mimi-Mama-Megaviruses | Phycodnaviruses | IAMV |
|---|---|---|---|---|---|
| | Namao virus | WSIV | | | |
| Namao virus | | | | | |
| WSIV | 83.5/94.1 | | | | |
| Mimi-Mama-Megaviruses | 20.6-34/ 41.6-54.7 | 20.8-32/ 40-54.1 | 22.1-99.1/ 43.5-99.1 | | |
| Phycodnaviruses | 20.5-29.8/ 40.2-53.2 | 19.1-30.2/ 38.4-51.9 | 20.3-42.3/ 37.4-60.0 | 25-77.4/ 39.1-86.7 | |
| Irido-Asco-Marseille viruses | 17.8-21.6/ 35.8-37.4 | 17.7-20.9/ 33.9-38.0 | 15.4-22.2/ 29.8-40.0 | 16.8-26/ 32.3-48.2 | 27.7-60.1/ 47.2-73.4 |
| Asfarviridae | 17.8/33.9 | 16.9/32.9 | 15.8-17.7/ 30.7-38.3 | 14.4-18/ 25.6-34.6 | 14.5-17.7/ 29-33.2 |

### 2.3.5 Phylogenetic Analyses

Phylogenetic analysis of the MCP amino acid sequence grouped NV and WSIV closer to each other than to any of the other NCLDVs (Fig 2.5). This was true for both tree reconstructions using Bayesian Inference (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) or maximum-likelihood methods (Guindon et al. 2010). The sturgeon viruses were in the *Mimiviridae* and *Phycodnaviridae* lineage but partitioned into the

*Mimiviridae* clade. Both sturgeon viruses were definitely separate from and not members of the clade containing Marseillevirus and the *Iridoviridae* and *Ascoviridae* families. Within the *Mimiviridae* family, Mimiviruses (APMV-1, -2) clustered together with two Megaviruses (proposed genera; MVChile, MVCour) and shared a common ancestor with Mamavirus (ACMV), CroV and sturgeon viruses. The branching topology of ACMV, CroV and sturgeon viruses was inconsistent between the two methods of tree reconstruction. In the maximum-likelihood tree, CroV and ACMV formed a lineage together that split from the sturgeon viruses after the node representing the *Mimiviridae* ancestor. Bootstrap values were too low (221/1000) to resolve the phylogenetic position of the sturgeon viruses relative to CroV and Mamavirus.

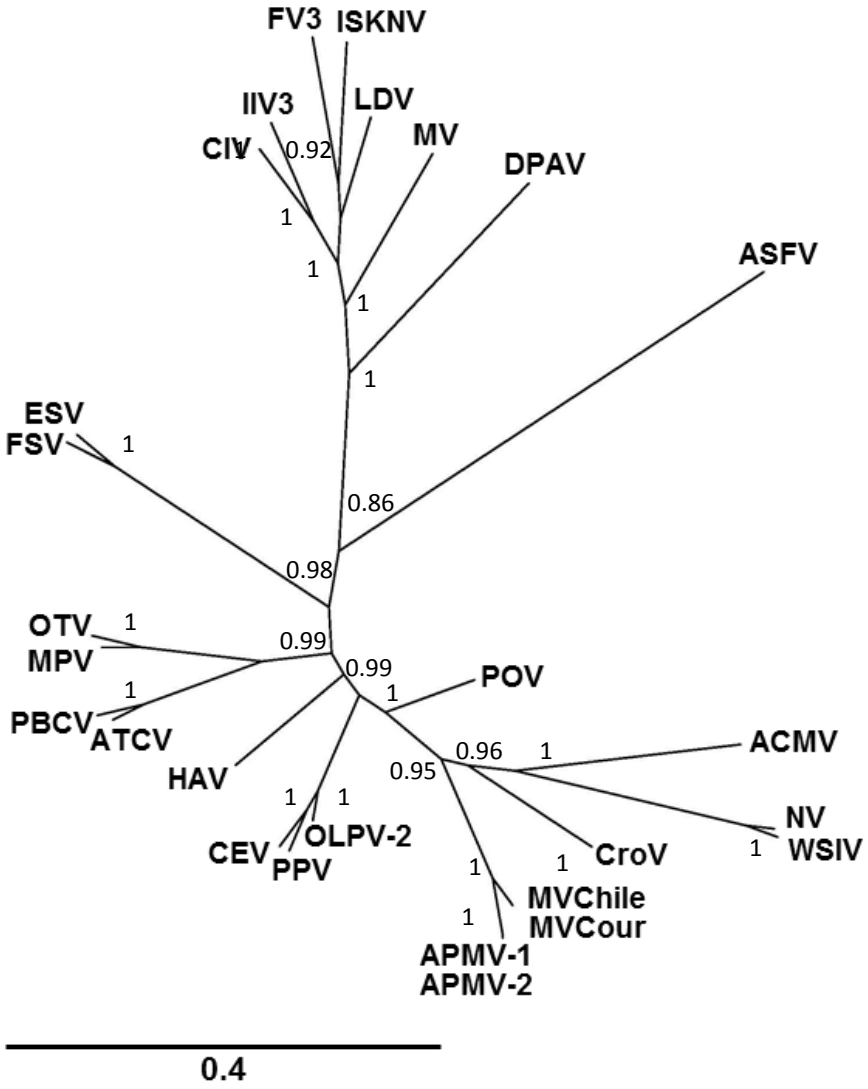



Figure 2.5 Phylogenetic tree reconstruction of the major capsid protein (MCP) of 27 nucleo-cytoplasmic large DNA viruses, including Namao virus (NV) and white sturgeon iridovirus (WSIV). The tree was generated using Bayesian inference (BI) from MCP sequences with nodes labeled with BI posterior probabilities (Materials and methods). Abbreviations of other viruses and accession numbers are shown in Appendix 3

## 2.4 Discussion

Namao virus is an emerging pathogen associated with mortalities among juvenile sturgeon reared as part of a conservation stocking program designed to supplement endangered populations of lake sturgeon in Manitoba. The virus is relatively large in diameter (242nm), icosahedral in shape and contains a condensed nucleoid core. Virus particles are more commonly found in epithelial cells of the integument and gills of infected sturgeon. NV and related sturgeon viruses, WSIV MRSIV and RSIV, share a similar particle size, conformation and tissue tropism (Hedrick et al. 1990, 1992, Kurobe et al. 2011, Adkinson et al. 1998). Phylogenetic analyses of sturgeon virus MCP sequences indicate the viruses may share a common evolutionary ancestor and may be part of a new unrecognized virus family.

Mortality events occurred in two geographically separate sturgeon rearing facilities and have been associated with NV. The cumulative mortality of sturgeon held at the GRH and UMAAHF reached close to 100%. Of the tissues collected during the mortality events and screened with cPCR, 93.6% tested positive for NV DNA. Further tests are needed to establish the causality between NV infections and lake sturgeon mortality and morbidity. However, the link is coherent based on the presence of NV DNA in moribund and dead sturgeon and the infection dynamics NV shares with other lethal sturgeon NCLDVs (ex: WSIV and MRSIV). The timelines of the outbreaks were variable. Mortalities in the UMAAHF happened over a range of 2 to 4 weeks and the GRH die-offs occurred over the course of one to six months. Similar infection kinetics have been observed in WSIV outbreaks in white sturgeon and MRSIV infections in pallid and shovelnose sturgeon (Watson et al. 1995, Drennan et al. 2005, Kurobe et al. 2010, 2011). Exposure to stressful events may trigger a switch between the chronic and

acute life stages in the virus life-cycle. Risk factors include handling, high stocking densities, fluctuations in water temperature and poor water quality (LaPatra et al. 1994, 1996, Watson et al. 1998b, Drennan et al. 2005, 2006). Fire damaged the UMAAHF in the spring of 2009 and disrupted water flow and air supply to tanks holding juvenile sturgeon for at least 72 hours. This event likely triggered NV disease outbreaks in the residing lake sturgeon populations. Risk factors in the GRH were not as apparent but conditions in these two facilities varied resulting in different infection dynamics.

The virus disease outbreaks were acute implying that a large percentage of fish were acting as carriers of the virus before the onset of mortality or the fish were exposed to a high dose of virus while residing in four separate tanks. Exposure to NV may have occurred at the time of fertilization either horizontally or vertically or at the time of rearing. For example, the 2008 year class may have been exposed at Slave Falls, the broodstock collection site, or at the Pinawa Hatchery, where the young larvae were raised. Both these sites incorporate untreated water from the Winnipeg River. There is a small possibility that the filtered and dechlorinated water at the UMAAHF may have carried the virus, however, this is unlikely. The 2009 GRH outbreak could be attributed to lake sturgeon eggs brought into the facility or, perhaps, the water from the Saskatchewan River which is used in the hatchery tanks. The exact cause is unknown and further study is warranted. Untreated river water has proven to be a potential source of WSIV affecting juvenile white sturgeon (LaPatra et al. 1994, Drennan et al. 2005, 2006).

The cPCR developed in this study may not be sensitive enough to amplify NV DNA from every lake sturgeon screened during this study. Therefore, fish testing negative with this assay

may still carry the virus. Variables such as the localized infection characteristic of sNCLDVs as well as the type of tissue sampled or the susceptibility of the host could potentially influence the outcome of diagnostic testing. A confirmatory diagnostic assay such as virus isolation by cell culture does not exist.

Isolating NV and other sturgeon NCLDVs on cell culture has proven to be difficult. Furthermore, little is known about the different steps of their virus replication cycle. Virus replication is influenced by the initial steps of the virus entry pathway that occur at the host cell surface. Initial interactions between NV and sturgeon cells are probably mediated through sites on the virus capsid that bind to a specific cell surface receptor(s). NV, like other sNCLDVs, displays a tropism for epithelial cells suggesting that the target molecule may be present on these cells. If the primary lake sturgeon gill or gonad cell lines used in this study did not have the appropriate cell surface receptor(s), efficacy of virus binding (and possibly entry) and hence replication would be impaired. The nature of disease development in the host or cytopathic effect in cultured cells is complex involving host, viral and environmental factors. Our results indicate that NV can attach, penetrate and replicate in epithelial cells with some success in live lake sturgeon. The fitness of NV in culture may be improved once the cell biology and disease ecology of this group of viruses is better understood.

Considering the difficulty of growing NV on eukaryotic cell culture, the possibility of Namao virus and other sturgeon NCLDVs being propagated on cultured amoeba should be explored. Effective culturing of NV would facilitate the examination of disease dynamics. It would also provide a means to fulfill Koch's postulates, a set of criteria used to establish if a pathogen is the causative agent of a disease (Koch 1890, Hill 1965, Evan 1976). NCLDV

members including viruses in the *Mimiviridae* family (La Scola et al. 2003, 2008) and the Marseillevirus (Boyer et al. 2009) have been successfully grown in amoebae culture. Further metagenomics studies will provide insight into the relationship between host and pathogen and possible ways to culture the virus.

Taking into account the structural and pathological similarities shared among sturgeon NCLDVs, along with phylogenetic tree reconstructions, sturgeon NCLDVs most likely share a common lineage. Phylogenetic analysis was based on the orthologous MCP gene sequence and calculated using Bayesian inference analysis. The tree topology was consistent with other NCLDV trees reported in the literature regardless of whether they were based on the MCP or a different conserved NCLDV protein sequence (Yutin et al. 2009, Larson et al. 2008, Boyer et al. 2009, Raoult et al. 2004, Monier et al. 2008). Namao virus and WSIV grouped closest to each other and were partitioned into the *Mimiviridae* clade. The sturgeon viruses were separate from members of the *Iridoviridae* family. The current nomenclature identifying sturgeon NCLDVs as iridoviruses should be revaluated to incorporate our findings which are consistent with those of Kwak et al. (2006b).

According to phylogenetic tree reconstruction (Fig. 2.5), NV and WSIV have most likely evolved from a common ancestor. NV and WSIV may share a lineage with CroV and Mamavirus from the *Mimiviridae* family. As NCLDVs have a complex evolutionary history and acquire genes from viruses, bacteria, eukaryotes and virophages (Filee 2009), it is possible that the genomes of sturgeon NCLDVs are not as closely related to *Mimiviridae* or *Phycodnaviridae* as indicated by results of the present study, especially when taking to account genes other than the MCP. The NV and WSIV MCPs both have two conserved NCLDV capsid domains (Marchler-Bauer et al.

2010) separated by a non-conserved region of approximately 135 to 91 amino acids, respectively (Appendix 1 & 2). Further metagenomics studies (Chapter 3) will provide additional clarity into the taxonomy of these viruses. The genetic similarity between these viruses will provide the opportunity to develop sensitive diagnostic tests pan-specific for all sturgeon NCLDVs.

The conservation stocking program would greatly benefit from utilizing sensitive molecular tests to detect the presence of the NV. Infection may be inevitable, but understanding the infection dynamics and reducing the impact of risk factors will help mitigate hatchery mortalities. Diagnostic testing is important for identifying disease resistance in progeny, selection of broodstock, monitoring the infection status of sturgeon and screening fish upon release into the wild. Identification of Namao virus is the first step in managing these factors in order to help protect both reared and wild lake sturgeon populations from disease.

## 3.0 Development and Application of Molecular Tests used in the identification of Sturgeon Nucleo-Cytopasmic Large DNA Viruses in North America

### 3.1 Introduction

Nucleo-cytoplasmic large DNA viruses (NCLDV) have been associated with lethal epitheliotrophic infections in hatchery-reared and wild sturgeon throughout North America and northern Europe. Sturgeon NCLDVs include white sturgeon iridovirus (WSIV) identified in white sturgeon *Acipenser transmontanus* (Hedrick et al. 1990, Raverty et al. 2003), Missouri River sturgeon iridovirus (MRIV) recovered from pallid sturgeon *Scaphirhynchus albus* and shovelnose sturgeon *S. platorynchus* (Kurobe et al. 2010, 2011), shortnose sturgeon virus (SNSV) found in shortnose sturgeon *A. breviro* (LaPatra et al. 2014), Russian sturgeon iridovirus (RSIV) associated with infections in Russian sturgeon *A. gueldenstaedtii* (Adkison et al. 1998) and Namao virus found in lake sturgeon *A. fulvescens* (Clouthier et al. 2013). Sturgeon NCDVs belong to an unassigned genus and family (Clouthier et al. 2013) within the proposed order *Megavirales* (Colson et al. 2013).

Outbreaks of virus disease in hatchery populations are associated with stress factors such as high rearing density, handling, and changes in water temperature, levels and flow (LaPatra et al. 1994, 1996, Watson et al. 1998b, Georgiadis et al. 2000, 2001, Drennan et al. 2005, 2006). Sturgeon NCLDVs are more commonly found in the nasal, oral, gill, fin and abdominal epithelial cells of infected hosts (Elliot 2011, Hedrick et al. 1990, Watson et al. 1998a, Drennan et al. 2007, Kurobe et al. 2011). The damage to the osmoregulatory and chemosensory organs may lead to erratic swimming behavior and anorexia in infected sturgeon (Watson et al. 1998a). Histological analysis of H&E stained infected epithelial tissue reveal pathognomonic cellular changes such as hypertrophied amphophilic staining cells, eccentric nuclei and cytoplasmic

inclusion bodies. Transmission electron microscopy of the cytoplasmic inclusion bodies exposes icosahedral virus particles with two capsids ranging in diameter between 242 to 262 nm. The virions contain a condensed bar-shaped nucleoid core (Hedrick et al. 1990, 1992, Kurobe et al. 2011, Adkinson et al. 1998, Clouthier et al. 2013). Sturgeon NCLDVs have proven difficult to grow in cell culture, only some isolates of the WSIV have been successfully cultured and neither NV nor MRSIV has been isolated by cell culture (Hedrick et al. 1991, Watson et al. 1998a, Kurobe et al. 2011, Clouthier et al. 2013).

The analytical specificity of current PCR tests for detection of sturgeon viruses is isolate specific. MRSIV PCR tests are only capable of detecting MRSIV (Kurobe et al. 2010, Clouthier et al. 2013) and the WSIV PCR assay is specific to some sub-types of WSIV (Drennan et al. 2007). As sturgeon NCLDVs are found throughout different sturgeon species across North America, group-specific quantitative PCR tests were designed for their detection and analytically validated.

## 3.2 Materials and Methods

### 3.2.1 Viruses and Plasmids

The acronym and GenBank accession numbers for the major capsid protein sequence of each NCLDV member used in this study are shown in Table 3.1. Genomic viral DNA (Clouthier et al. 2013) and synthetic viral DNA were used for test development and optimization. Fin tissue samples from shortnose sturgeon infected with SNSV were provided by a commercial grower in Atlantic Canada and used to extract SNSV MCP DNA.

Table 3.1 Megavirales included in this study and analytical specificity of the conventional PCR test (C1), qPCR test optimized for NV DNA (Q1) and qPCR test pan-specific for sturgeon NCLDVs

(Q2). (+) = amplification. (-) = no amplification. (+/-) = inconsistent amplification. Blank = not done.

| Virus | Detection by test method | | | Accession # | |
|---|---|---|---|---|---|
| | C1 | Q2 | Q1 | DNA | Protein |
| ***Sturgeon NCLDVs*** | | | | | |
| Namao virus (NV) | + | + | + | JX155659 | AGH17555 |
| Missouri River sturgeon iridovirus (MRSIV) | + | + | + | JX155661 | AGH17557 |
| British Columbia white sturgeon virus (BCWSV) | + | + | + | JX155660 | AGH17556 |
| Shortnose sturgeon virus (SNSV) | + | + | + | KM606973 | |
| White sturgeon iridovirus (WSIV) | + | + | - | DQ897645 | ABK34555.1 |
| ***Mimiviridae*** | | | | | |
| ***Giant mimiviruses*** | | | | | |
| ***Clade I – Group A*** | | | | | |
| Acanthamoeba polyphage mimivirus (APMV-1) | - | - | - | NC014649 | YP003986929 |
| Acanthamoeba polyphage mimivirus (APMV-2) | - | - | - | JN036606 | AEJ34665 |
| Acanthamoeba castellanii mamavirus (ACMV) | - | - | - | JF801956 | AEQ60625 |
| ***Clade I – Group B*** | | | | | |
| Acanthamoeba polyphaga moumouvirus (APMM) | | | | NC020104 | YP007354349 |
| ***Clade I – Group C*** | | | | | |
| Megavirus chiliensis (MVChile) | + | - | - | NC016072 | YP004894515 |
| Megavirus courdo (MVCour) | - | - | - | JN885991 | AEX61606 |
| ***Clade II*** | | | | | |
| Cafeteria roenbergensis virus (CroV) | + | - | - | NC014637 | YP003969975 |
| ***Small mimiviruses*** | | | | | |
| Organic lake phycodnavirus 1 (OLPV-1) | | | | HQ704802 | ADX05938 |
| Organic lake phycodnavirus 2 (OLPV-2) | +/- | - | - | HQ704803 | ADX06358 |
| Phaeocystis pouchetii virus (PPV) | - | | | EU006631 | ABU23715 |
| Chrysochromulina ercina virus (CEV) | + | - | - | EU006628 | A7U6E7 |
| Pyramimonas orientalis virus (POV) | - | | | EU006630 | A7U6EP |
| Phaeocystis globosa virus (PGV) | | | | HQ634147 | AET73005 |
| ***Phycodnaviridae*** | | | | | |
| Heterosigma akashiwo virus (HAV) | + | - | - | AB198422 | BAE06835 |

| | | | | | |
|---|---|---|---|---|---|
| Acanthocystis turfacea chlorella virus (ATCV) | - | | | NC008724 | YP001426761 |
| Paramecium bursaria chlorella virus (PBCV) | - | | | NC009898 | YP001497813 |
| Micromonas pusilla virus (MPV) | - | | | HQ633072 | AET43572 |
| Ostreococcus tauri virus (OTV) | + | | | NC013288 | YP003495004 |
| Feldmannia species virus (FSV) | - | | | NC011183 | YP002154681 |
| Ectocarpus siliculosus virus (ESV) | - | | - | FN648730 | CBN80416 |
| ***Iridoviridae*** | | | | | |
| Chilo iridescent virus (CIV) | + | - | - | NC003038 | NP149737 |
| Frog virus 3 (FV3) | - | - | - | FJ459783 | ACP19256 |
| Invertebrate iridescent virus 3 (IIV3) | - | - | - | NC008187 | YP654586 |
| Infectious spleen & kidney virus (ISKNV) | - | - | - | AF371960 | AAL98730 |
| Lymphocystis disease virus (LDV) | - | - | - | EF103188 | ABL07488 |
| ***Marseilleviridae*** | | | | | |
| Marseillevirus (MV) | - | | | NC013756 | YP003407071 |
| ***Ascoviridae*** | | | | | |
| Diadromus pulchellus ascovirus 4a (DPAV) | - | | | AJ312705 | CAC84483 |
| ***Asfarviridae*** | | | | | |
| African swine fever virus (ASFV) | - | | | XM369245.2 | XP369245.2 |

WSIV and type 2 Acipenserid herpesviruses (AciHV-2) were grown on white sturgeon skin (WSSK-1) or white sturgeon spleen (WSS-2) primary cell lines (Hedrick et al. 1991). The cells were propagated at 16˚C in minimum essential medium with Hanks salts (MEM-H), 10% fetal bovine serum (FBS) and 2 mM L-glutamine (Life Technologies). Cell monolayers were inoculated with a suspension of WSIV or type 2 Acipenserid herpesviruses (AciHV-2) and after an absorption period of 1 h, MEM-H supplemented with 2% FBS (MEM-H2), 2 mM L-glutamine and 1 x antibiotic/antimycotic (Life Technologies) was added to each flask. Flasks were incubated at 16˚C and the monolayers were observed daily for evidence of cytopathic effects (CPE). At the

time of harvest, each monolayer was scraped from the bottom of the flask into the media and the cell suspension was frozen in a storage vial at -80˚C.

Genomic viral DNA was extracted from infected sturgeon tissue and used to infer part of the NV, MRSIV, BCWSV and SNSV MCP nucleotide and protein sequences (Clouthier et al. 2013). Synthetic constructs were designed to encode the sturgeon virus MCP DNA and inserted into cloning vectors pMA-RQ (Life Technologies) or pJ204 (DNA2.0). The constructs were named pNVmcp, pWSIVmcp, pMRSIVmcp, pBCWSVmcp or pSNSVmcp.

The MCP region of 25 of the 33 NCLDVs included in Table 3.1 were synthetically constructed and inserted into plasmids (DNA2.0). These synthetic constructs contained the target sequence for the conventional C1 test or qPCR tests Q1 or Q2 (Fig 3.1) inserted into the pJ204 cloning vector (DNA2.0). Three separate constructs were made for 14 of the viruses so they could be screened with each test. For example, the DNA sequence for APMV-1 was used to create plasmids pAPMV-1 C1, pAPMV-1 Q1 and pAPMV-1 Q2, each encompassing a different region of the MCP. The abbreviations C1, Q1 or Q2 refer to the test for which they were designed.

Artificial positive control (APC) plasmids were designed for the qPCR tests with the same approach as Snow et al. (2009). The APC constructs were designed with a region of the NV MCP inserted into a pJ204 vector back bone (DNA2.0) (Fig 3.2). These constructs also contain a non-viral segment of DNA which binds a distinct pAPC probe (Fig 3.2).

Q1

| | | sNCLDV F17 → | | sNCLDV P25 - - - → | | ← sNCLDV R15 | | Nucleotide mismatch | ASe |
|---|---|---|---|---|---|---|---|---|---|
| NV | 1 | ATAGGGTACAAGAGACATTCTC | $N_4$ | TGCCATGACACCAGTCGTT | $N_{15}$ | CTGGATTTTGGAAAGGAGGTCAACT | 85 | 0 | $10^{0.5}$ |
| WSIV | 1 | ..... | | .....C..T... | | .....A..T.... | 85 | 4 | – |
| BCWSV | 1 | ..... | | ..... | | ..... | 85 | 0 | $10^{0.7}$ |
| MRSIV | 1 | ..... | | ..... | | .....A.....A.... | 85 | 2 | $10^{0.7}$ |
| SNSV | 1 | ..... | | ..... | | ..... | 85 | 0 | $10^{0.7}$ |

Q2

| | | sNCLDV F21 → | | sNCLDV P19 - - - → | | ← sNCLDV R18 | | Nucleotide mismatch | ASe |
|---|---|---|---|---|---|---|---|---|---|
| NV | 1 | CCTGACGGTATCAACGTATATTCGTTT | $N_{18}$ | CACCAACCTTCTGGAAGTGCCAACTTTT | $N_5$ | TTTGAGTCGATTCGCATCGA | 98 | 0 | $10^{0.8}$ |
| WSIV | 1 | .....T..... | | .....C..A..... | | .....T..... | 98 | 4 | $10^{2.7}$ |
| BCWSV | 1 | ..... | | .....G..... | | ..... | 98 | 1 | $10^{0.7}$ |
| MRSIV | 1 | ..... | | .....T..... | | ..... | 98 | 1 | $10^{0.7}$ |
| SNSV | 1 | ..... | | .....C..... | | ..... | 98 | 1 | $10^{0.7}$ |

C1

| | | ginF → | | ← glaR | | Nucleotide mismatch | ASe |
|---|---|---|---|---|---|---|---|
| NV | 1 | GGTATCAACGTATATTCGTTTGC | $N_{175}$ | GGTCTAGCGGGTCTGGCATAC | 219 | 0 | $10^{1.7}$ |
| WSIV | 1 | ..... | | ..... | 219 | 0 | $10^{2.7}$ |
| BCWSV | 1 | ..... | | ..... | 219 | 0 | $10^{1.7}$ |
| MRSIV | 1 | ..... | | ..... | 219 | 0 | $10^{2.7}$ |
| SNSV | 1 | ..... | | ..... | 219 | 0 | $10^{1.7}$ |

Figure 3.1 QPCR (Q1 and Q2) and conventional PCR (C1) tests for the sturgeon viruses' major capsid protein gene. Namao virus (NV) is the referent sequence for nucleotide mismatches in primer/probe binding regions of Q1 (85 nucleotides), Q2 (98 nucleot nucleotides) amplicons. The symbols for primers (→, ←) and probes (– →) indicate polarity (5' to 3'). The sequence for sNCLDV R15, sNCLDV R18 and glaR are the reverse complement of the primer sequence shown above. The number of intervening nucleotides between primer and probe (Q1, Q2) or primers (C1) is $N_x$. Analytical sensitivity (ASe) of the tests is given for targets pNVmcp pWSIVmcp, pBCWSVmcp, pMRSIVmcp and pSNSVmcp. Virus acronyms are described in Table 1.

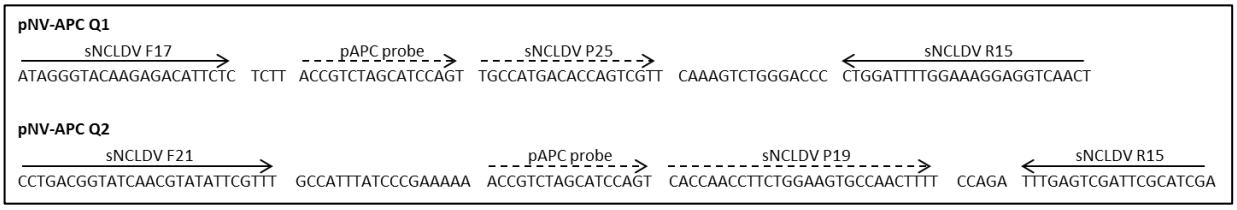



Figure 3.2 Artificial positive control (APC) plasmids pNV-APC Q1 and pNV-APC Q2. Namao virus (NV) is the referent for synthetic DNA sequences which also include a non-viral sequence (i.e. pAPC probe). Vector sequence (pJ204) is not shown. The symbols for primers (→, ←) and probes (– →) indicate polarity (5' to 3'). Primer sequences are provided in Table 1.

Plasmids were transformed into *Escherichia coli* DH5α or K12 (Life Technologies) and DNA was purified from the host using a QIAprep Spin Miniprep Kit (Qiagen).

### 3.2.2 Field Samples

Tissues samples were collected from lake sturgeon in the Nelson River and Winnipeg River drainage system and analysed with histology and/or qPCR (Fig 2.1). Broodstock collection sites in the Nelson River drainage include the Burntwood River, the mouth of the Landing River and Birthday Rapids. Broodstock collected on the Winnipeg River were taken from Pointe du Bois. The subsequent progeny were reared at either the Grand Rapids Hatchery or University of Manitoba Aquatic Animal Holding Facility. Tissue samples were collected from hatchery-reared fish originating from broodstock captured from Pointe du Bois Falls, Sturgeon Falls, Slave Falls, the Landing River, Birthday Rapids and Burntwood River (Fig 2.1).

Tissue samples (whole larvae, pectoral fins and gametes) for genomic DNA extraction or histological analysis were processed as described in Chapter 2. Gametes were placed in liquid nitrogen, fin clips were split in two and halves were placed in either RNA*later*® (Life Technologies) or Safe-Fix® (Fisher Scientific). Larvae were paired and also preserved in either RNA*later*® or Safe-Fix®.

### 3.2.3 Primer/probe Design

Sequence alignments of full or partial MCP nucleotide or protein sequence for NV, WSIV, BCWSV and MRSIV revealed conserved regions shared among viruses which formed the basis of primer targets. Sequence alignments were performed using T-Coffee (Notredame et al. 2000, Di Tommaso et al. 2011) (Fig. 3.1). Primer and probes for Q1 and Q2 assays were identified using Beacon Designer and Allele ID software (Premier Biosoft International).

### 3.2.4 Conventional PCR (cPCR) Test C1

The C1 cPCR primers were forward primer ginF 5'-GGT ATC AAC GTA TAT TCG TTT GC-3' and reverse primer glaR 5'-GCA AAC GAA TAT ACG TTG ATA CC-3' (Sigma-Aldrich; Clouthier et al. 2013). The C1 test was 25 µL of containing 100 nM ginF, 100 nM glaR, 3 mM magnesium chloride, 100 µM nucleotide triphosphates (dNTPs), 1.25 Units AmpliTaq Gold (Applied Biosystems) and 500 to 1500 ng of extracted DNA as a template. It was performed using an Applied Biosystems Veriti thermocycler. The thermocycling conditions were 2 min at 95°C, 40 cycles of 30 sec at 95°C, 30 sec at 54°C and 30 sec at 72°C followed by 1 cycle of 10 min at 72°C. Amplicons were separated by electrophoresis on 2% agarose gels (Life Technologies) containing 1x fluorescent nucleic acid stain GelRed (Biotium Inc). Gel images were captured using the Kodak Gel Logic 200 imaging system.

### 3.2.5 QPCR Tests Q1 and Q2

The primers for qPCR test Q1 were forward primer sNCLDV F17 5'-ATA GGG TAC AAG AGA CAT TCT C-3', reverse primer sNCLDV R15 5'-AGT TGA CCT CCT TTC CAA AAT CCA G-3' (Sigma-Aldrich), Q1 assay probe sNCLDV P25 5'-6FAM-TGC CAT GAC ACC AGT CGT T-MGBNFQ-3' and positive control pAPC probe 5'-VIC-ACC GTC TAG CAT CCA GT-MGBNFQ-3' (Life Technologies). The Q1 test was 25 µL containing 1000 nM sNCLDV F17, 800 nM sNCLDV R15, 250 nM sNCLDV P25, 250 nM pAPC probe, 1xTaqman Universal PCR Master Mix (Applied Biosystems) and 1500 ng genomic DNA.

The primers for qPCR test Q2 were forward primer sNCLDV F21 5'-CCT GAC GGT ATC AAC GTA TAT TCG TTT-3', reverse primer sNCLDV R18 5'-TCG ATG CGA ATC GAC TCA AA-3' (Sigma-Aldrich), Q2 assay probe sNCLDV P19 5'-6FAM-CAC CAA CCT TCT GGA AGT GCC AAC TTT T-

TAMRA-3' and positive control pAPC probe 5'-VIC-ACC GTC TAG CAT CCA GT-TAMRA-3' (Life Technologies). The Q2 test was 25 µl containing 400 nM sNCLDV F21, 800 nM sNCLDV R18, 200 nM sNCLDV P19, 250 nM pAPC probe, 1xTaqman Universal PCR Master Mix (Applied Biosystems) and 1500 ng genomic DNA.

Control samples of a known composition were included in each test run. The control material used during the DNA extraction included a negative extraction control containing no sturgeon tissue (N1), 50mg lake sturgeon tissue spiked with $10^{7.8}$ copies of pNV-APC Q1 or Q2 (P1), pNV-APC Q1 or Q2 diluted from $10^{7.7}$ to 5 copies per reaction (P2) and water (N2). The N1 and P1 samples were extracted and analyzed alongside the test samples while N2 and P2 were added at the qPCR step of the Q1 or Q2 tests.

Q1 and Q2 tests were run using the Stratagene Mx3000P system in a 96-well format. The thermocycling profile was 1 cycle of 2 min at 50°C, 1 cycle of 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Three to five replicates of each sample was tested. Data was analyzed using MxPro software (Stratagene) using the adaptive baseline and amplification-based threshold algorithm enhancements. The PCR cycle thresholds used for quantification in Q1 and Q2 will be referred to as the quantification cycle (Cq) (Bustin et al. 2009).

The copy number of Q1 or Q2 pNV-APC was used by the MxPro software to infer the copy number equivalent in test samples with the standard curve method. Q1 and Q2 constructs are 2,812 and 2,853 bp in length; correspond to $1.83 \times 10^6$ and $1.85 \times 10^6$ g mol-1 and the copy number per µg DNA was calculated as $3.29 \times 10^{11}$ and $3.26 \times 10^{11}$, respectively.

### 3.2.6 Conventional and qPCR Assays for WSIV MCP Gene or MRSIV Serpin Gene

The WSIV PCR analyses were performed as outlined by Kwak (2006b) and Kwak et al. (2006a). The MRSIV serpin qPCR assay was performed as described by Kurobe et al. (2010).

### 3.2.7 Primer/probe Screening and Optimization

Multiple qPCR primer pair combinations (N = 69) were analyzed with SYBR Green (Life Technologies) using 300 nM of each primer. The templates used for qPCR primer selection were DNA extracted from NV-infected lake sturgeon (1 µg) and plasmid DNA encoding a portion of the MCP gene from WSIV ($10^{6.7}$ copies). PCR products were evaluated using both dissociation curves and gel electrophoresis. Primer pair combinations that did not amplify both NV and WSIV DNA were eliminated from further testing.

Hydrolysis probe selection assays were performed using the DNA extracted from NV-infected lake sturgeon tissue or a plasmid carrying WSIV MCP DNA. The primer and probe combinations were 400 nM (eqimolar) and 80 nM, respectively. Primer/probe combinations that produced a detectable Cq value with NV template alone or with both NV and WSIV templates were used for further analysis.

Primer and probe concentrations and cycling conditions for the Q1 test were optimized for detection of NV in infected tissue while those for Q2 were optimized for detection of NV, WSIV, MRSIV and BCWSV. Templates were NV-infected lake sturgeon tissue or plasmid DNA encoding the full or partial MCP DNA sequence (i.e. pNVmcp, pWSIVmcp, pMRSIVmcp, pBCWSVmcp or pSNSVmcp). Primer and probe combinations were selected for their ability to amplify plasmid DNA from $10^{7.7}$ to $10^{0.7}$ copies per reaction. The concentration of primers was tested in increments of 100 nM, from 100 to 1000 nM while the hydrolysis probe concentration was 250

nM. Primer concentrations from each test associated with the lowest Cq value were selected for further analysis. Probe concentrations, in increments of 50 nM from 100 to 250 nM, were tested and those giving the highest final fluorescence value in the baseline-corrected, ROX-normalized view (i.e. dRn) were selected for further analysis.

The conventional PCR reaction was optimized with 100 to 800 nM of primers, 1 to 3 mM magnesium chloride and 0.1 to 0.3 mM deoxynucleotide triphosphates (dNTP). The reaction was optimized using the same templates as the qPCR tests. The primer concentrations and cycling conditions resulting in the higher relative fluorescence of amplified products with all four sturgeon NCLDVs were selected.

Different tissue types and quantities of template DNA were examined for the C1, Q1 and Q2 tests with 100, 500, 1000, 1500, 2000, 3000 and 4000 ng of gill, fin, snout and abdominal skin tissue.

### 3.2.8 Analytical Validation

The analytical sensitivity (ASe) of C1, Q1 and Q2 was evaluated using standard curves generated with three different kinds of template: i) plasmid DNA containing part (96%) of the MCP from NV, MRSIV, BCWSV, WSIV or SNSV, ii) APC plasmid DNA or iii) genomic DNA extracted from NV-infected sturgeon tissue. Standard curves of the plasmids encoding viral DNA were used to compare the qPCR reaction efficiencies across different virus isolates. Similarly, DNA derived from NV-infected tissue and plasmid DNA were compared to determine if plasmid DNA could be used as a proxy for absolute quantification of NV in infected tissue. Linear vs. circular plasmid DNA, the presence of host DNA (1500 ng) and/or the APC probe were investigated in terms of their effects on reaction efficiencies and ASe. The 50% and 100% limit

of detection was assessed using standard curves constructed from 2, 5 or 10-fold serial dilutions with each dilution tested in triplicate or replicates of five or six. The limit of detection was expressed as copies of plasmid DNA and was derived from measured concentrations of the last dilution with 50% or 100% positive detections. The 95% limit of detection was theoretically predicted using binominal logistic regression with the positive/negative sample status as the outcome and the concentration (log10) as the predictor. Linear regression was used to estimate the Cq value at the 95% probability of detection.

The analytical specificity (ASp) of each assay was evaluated and expressed as inclusivity or exclusivity (i.e. degree to which the assay detects all intended viruses and does not detect other viruses, respectively). Exclusivity was evaluated with synthetic constructs containing target sequences attributed to a selection of the NCLDVs listed in Table 3.1. Each construct was tested at a concentration of $10^{9.7}$, $10^{6.7}$ and $10^{3.7}$ plasmid copies. Exclusivity of the three tests was also evaluated with AciHV-1 and AciHV-2 isolates extracted from infected white sturgeon, shortnose sturgeon tissue or virus amplified in cell culture. Inclusivity of the molecular tests was determined using pNVmcp, pWSIVmcp, pMRSIVmcp, pBCWSVmcp and pSNSVmcp artificial constructs as well as well as DNA isolated from NV, MRSIV, BCWSV or SNSV-infected tissue and from WSIV amplified in cell culture. ASp evaluation involved using three replicates per sample and the previously described reaction conditions in section 1.1.5. The level of agreement between observed and expected results was quantified using two-way tables (Table 3.2). The 95% confidence intervals for these estimates were also calculated for each assay.

Table 3.2 The table used for determining analytical specificity of the C1, Q1 and Q2 tests. A = number of viruses expected to be detected and were. B = number of viruses expected to be detected but were not, C = number of viruses expected to be non-detectable and were not. D = number of viruses expected to be non-detectable and were. Inclusivity = A/(A + B); Exclusivity = D/(C + D)

| | | **Observed** | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| **Expected** | Positive | A | B | A + B |
| | Negative | C | D | C + D |
| | Total | A + C | B + D | A + B + C + D |

The analytical repeatability of Q1 and Q2 was tested with pNVmcp 10-fold serially diluted from $10^{7.7}$ to $10^{0.7}$ copies per reaction. The intra- and inter-assay repeatability was determined by comparing five replicates screened in five independent runs. Positive control samples P1 and P2 were used to evaluate the repeatability of Q1. Samples from three batches of P1 and eight batches of P2 were assessed in 88 independent runs performed by a single analyst over a nine month period. Q1 and Q2 were performed using reaction conditions outlined in section 1.1.5. The mean of the replicates was plotted against the standard deviation in a scatter-plot. Linear regression was used to determine if the inter-run or inter-batch variability was statistically significant (i.e. $p<0.05$). The coefficient of variation (CV) was calculated as (standard deviation (SD)/mean) x 100 using the qPCR Cq values as data. All statistical analyses were performed in StataI/C (12.1).

### 3.2.9 BLASTP, Sequence Alignment and Phylogenetic Analysis

BLASTP program searches revealed sequence similarity in the deduced amino acid sequence of the NV, WSIV, BCWSV, MRSIV or SNSV MCP (Altschul et al. 1990, 1997; Appendix 3). The protein sequence alignments were created by T-Coffee (Notredame et al. 2000, Di Tommaso et al. 2011) after sequences were trimmed to align with the first and last amino acids of the NV MCP fragment.

Bayesian inference analysis executed by MRBAYES v3.2.1 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) and maximum-likelihood by way of PHYML 3.69 (Guindon et al. 2010) were used to estimate phylogenetic tree reconstructions. The Bayesian trees were predicted using multiple independent runs of aamodelpr = mixed and default settings for 1,000,000 generations at which point the average deviation of the split frequencies was <0.002. Maximum-likelihood analysis estimated phylogenetic tree clade confidences by comparing 1000 bootstrap replicates. The output trees produced were visualized using FigTree v1.3.1 software (Rambaut 2008).

## 3.3 Results

### 3.3.1 Test Development

Phylogenetic tree reconstructions have placed sturgeon NCLDVs (NV, WSIV, MRSIV, BCWSV and SNSV) into a distinct group separate from the other NCLDVs (Fig. 2.3, Fig. 3.3). The sturgeon viruses belong to the proposed order *Megavirales* but are not associated with any known virus genera or family.

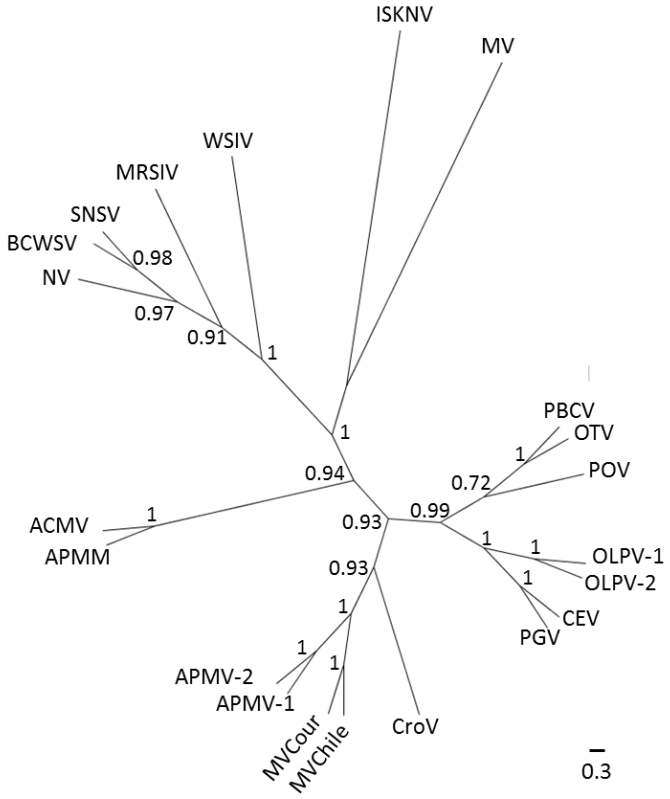



Figure 3.1 Bayesian inference of phylogeny for sturgeon viruses using the major capsid protein. Sequence from other nucleo-cytoplasmic large DNA viruses was included for comparison. Bayesian inference posterior probabilities are given at the nodes. Virus acronyms and accession numbers are provided in Appendix 1 and 2.

The evolutionary relationships revealed by phylogenetic analysis are reflected in the ASp and ASe of the C1, Q1 and Q2 tests. The C1 test has the broadest ASp and is able to detect the sturgeon NCLDVs along with members of the *Mimiviridae*, *Phycodnaviridae* or *Iridoviridae* families (Table 3.1). Q2 is more specific and recognizes the five sturgeon NCLDVs. Optimizing Q1 for detection of NV resulted in the most specific test that is able to detect NV, BCWSV, MRSIV and SNSV but not WSIV. Molecular assays designed to target the WSIV MCP or the serpin gene of MRSIV were unable to detect NV, BCWSV or SNSV.

Each molecular test targets a different region of the sturgeon virus MCP DNA sequence. The primers from the Q1 and Q2 qPCR tests bind to opposite ends of the MCP with Q1 targeting nucleotides 85 to 169 and Q2 targeting nucleotides 1363 to 1460, using the WSIV MCP sequence (DQ897645.1) as a reference. The C1 target region overlaps with that of the Q2 test as it targets the nucleotides 1369 to 1585 (Fig. 3.1)

The quantity of template DNA used in the cPCR test reactions ranged between 500 to 1500 ng, depending on the amount of DNA extracted. The qPCR test reactions used 1500 ng of template DNA regardless of the type of tissue. Testing the assays with different concentrations of template revealed that interference was evident if 2000 ng or more of DNA template was used.

Two positive controls, P1 and P2, were used to evaluate the efficiency of DNA extraction and monitor the qPCR reaction. These samples are also able to identify if cross-contamination is occurring. Each qPCR reaction is run with two probes which fluoresce on separate wavelengths and bind to different regions of the pNV-APC Q1 or Q2 (Fig. 3.1 & 3.2). The probes that are labelled with a FAM dye (P19 and P25) are able to bind to both the artificial plasmid control and sturgeon virus DNA. Probes labelled with a VIC dye can only bind the sequence of DNA encoded in the artificial plasmid controls pNV-APC Q1 or Q2 (Fig. 3.2). Therefore, if a qPCR test sample contains fluorescence with a VIC dye wavelength, cross contamination has occurred either in the DNA extraction or qPCR preparation steps. Including the pAPC probe and FAM labelled probe into qPCR reactions did not negatively impact the reaction efficiencies or sensitivities of the tests. Linearizing the plasmid DNA did not improve Q1 and Q2 test performance compared

to circular plasmid DNA; therefore, circular plasmid DNA was used as a template during qPCR test development and optimization.

### 3.3.2 Analytical Validation

The reaction efficiencies and detection limits of the FAM-based tests (qPCR reactions using P25 and P19) and the VIC-based test (qPCR involving the pAPC probe) were similar. The slopes of the qPCR standard curves for the Q1, Q2 and VIC-based test were very similar to one another and only varied by less than 0.1 (Fig 3.4). The standard curves remained linear over $10^{8.7}$ to $10^{0.7}$plasmid copies of target DNA with the cycle number and dilution factors showing strong correlation (Fig. 3.4). The reaction efficiencies of the Q1 and Q2 assays remained in the acceptable range when testing 5 or 10-fold serially dilutions of plasmid DNA or NV-infected tissue DNA (Fig 3.5). As the reaction efficiencies of the assays were similar regardless of the template used, plasmid could be used in lieu of NV-infected DNA to determine the sensitivity of the Q1 and Q2 tests.

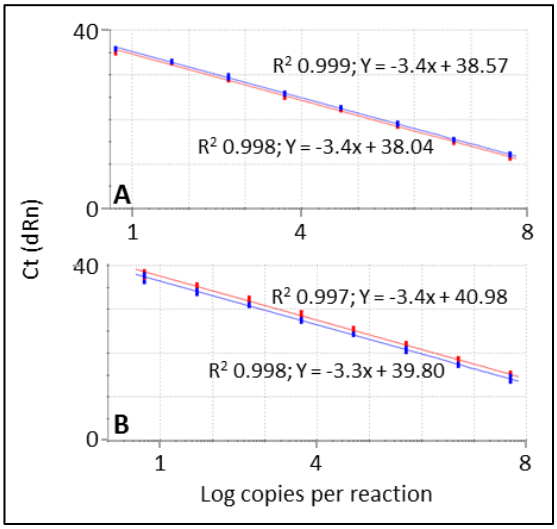



Figure 3.2 Reaction efficiency of Q1 and Q2 test. Ct values for VIC (red) or FAM-labelled (blue) probes were generated with 107.7 to 5 plasmid copies of pNV-APC Q1 (A) or pNV-APC Q2 (B). Standard curves were made using linear regression.

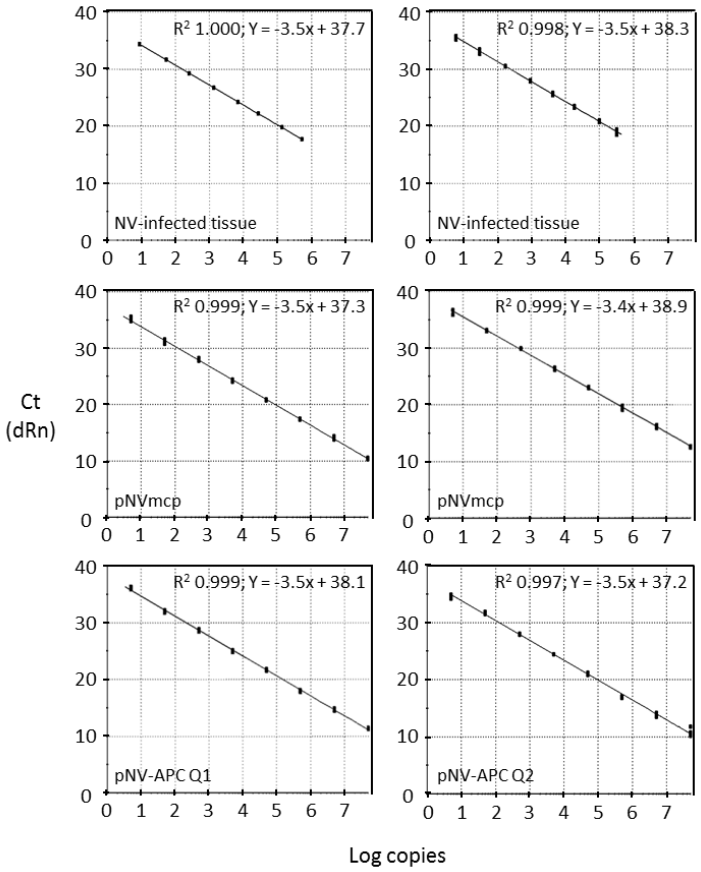



Figure 3.3 Reaction efficiency of Q1 and Q2 tests. Targets included DNA from Namao virus (NV)-infected tissue, plasmid encoding the NV major capsid protein (pNVmcp) or artificial positive control plasmids (pNV-APC Q1, Q2). Ct values for FAM-labelled probes were generated using infected tissue DNA (5-fold serially diluted) or plasmid DNA (10-fold serially diluted from $10^{7.7}$ to 5 copies). Standard curves were generated using linear regression.

The pNVmcp, pNV-APC Q1 and pNV-APC Q2 plasmids were used to determine the limit of detection for the three molecular tests. As the Q2 test has been optimized to be pan-specific for sturgeon NCLDVS, the pMRSIVmcp, pBCWSVmcp, pWSIVmcp, and pSNSVmcp plasmids were included in the Q2 evaluation. The assays were able to detect plasmid DNA across $10^{8.7}$ to $10^{0.7}$ copies or six to eight orders of magnitude (Fig. 3.6 and 3.7). For the Q2 test, amplification efficiencies shifted between -3.3 to -3.5 and the y-intercept values of the pMRSIVmcp,

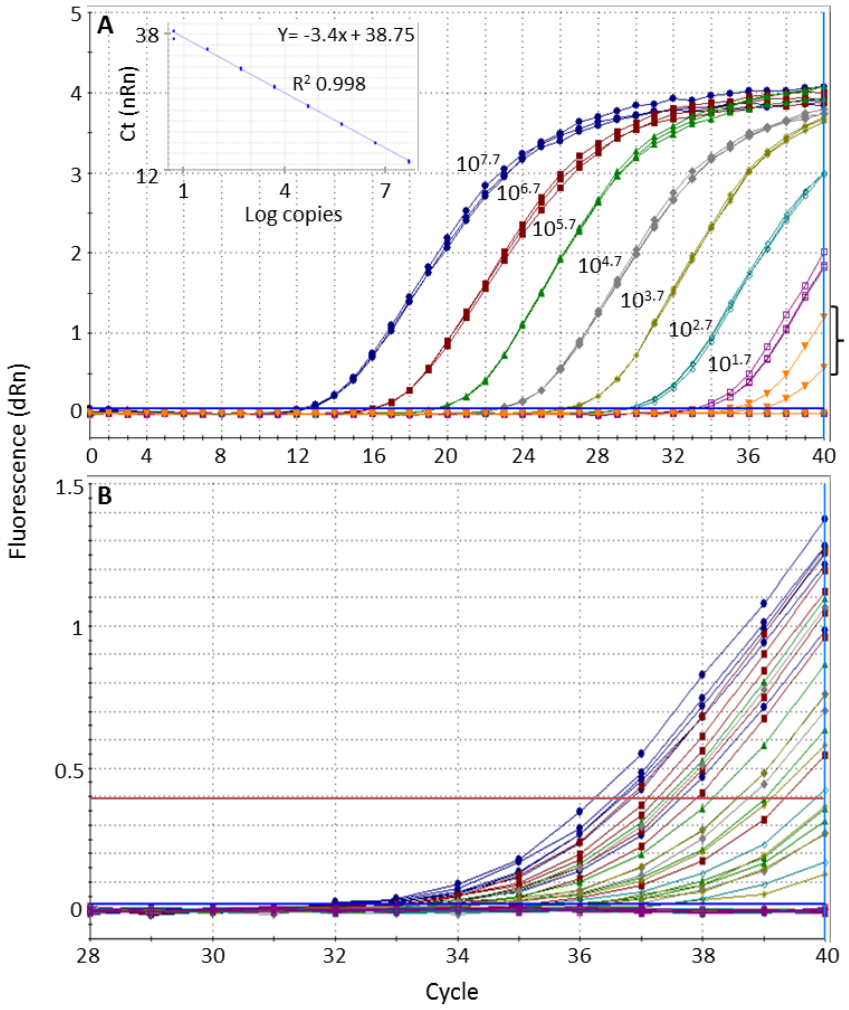



Figure 3.4 Analytical sensitivity of the Q1 test. Ct values for the FAM-labelled probe were generated using pNVmcp DNA diluted from $10^{7.7}$ to 5 copies (A) or from 50 to 0.75 copies (B). Linear regression for A is shown in the inset. The limit of detect detection is indicated by the horizontal red line in B.

pBCWSVmcp and pSNSVmcp standard curves varied from 39 to 41. The level of detection for the pNVmcp, pMRSIVmcp, pBCWSVmcp, and pSNSVmcp plasmids was similar using the Q2 test (Fig. 3.7) and the Q2 test had a lower sensitivity for pWSIVmcp which was reflected in the y-intercept value of 46 (Fig. 3.7E). For the Q1 test, the observed 50% limit of detection was three copies of pNVmcp (5 of 6 replicates tested positive; 35.84±2.11 Cq). The Q2 had a higher

sensitivity with an observed 50% limit of detection at 6.25 copies (4 of 6 replicates tested

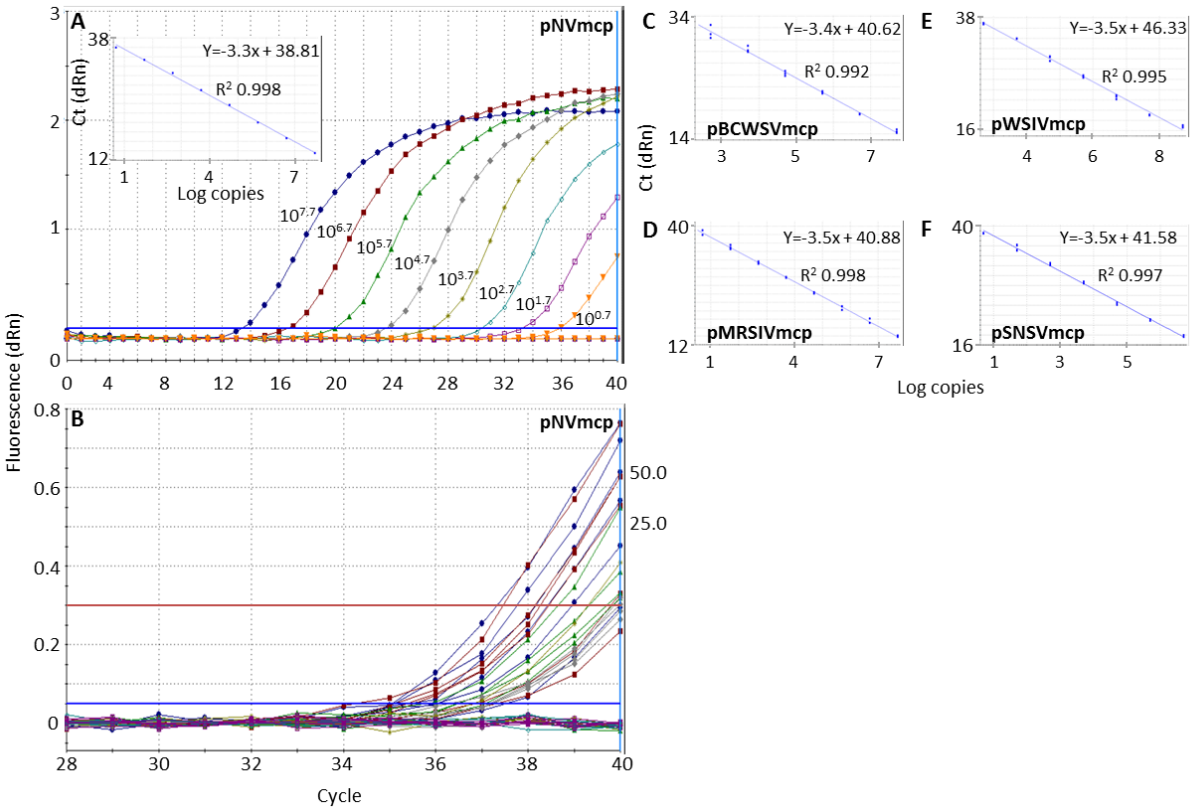



Figure 3.5 Analytical sensitivity of the Q2 test. Ct values for the FAM-labelled probe were generated with pNVmcp DNA diluted from $10^{7.7}$ to 5 copies (A) or from 50 to 0.75 copies (B). Linear regression for A is shown in the inset. The limit of detection is indicated by the horizontal red line in B. Linear regression is also shown for target DNA pBCWSVmcp (C), pMRSIVmcp (D), pWSIVmcp (E) and pSNSVmcp (F).

positive; 37.02±1.58 Cq) (Fig. 3.6B & 3.7B). The 100% limit of detection was 25 copies of pNVmcp for both the Q1 (33.66±0.75 Cq) and Q2 (35.78±1.14 Cq) tests. The QI and Q2 test sensitivity was not impacted by the presence of host tissue DNA and VIC-labelled pAPC probe. The theoretically determined 95% limit of detection estimates were very similar to those measured experimentally. The 95% limit of detection of the Q1 test is associated with 1.13 plasmid copies and a 39.39 Cq value. The Q2 test could detect 1.42 plasmid copies at 38.93 Cq value at the 95% limit of detection (Fig 3.8).

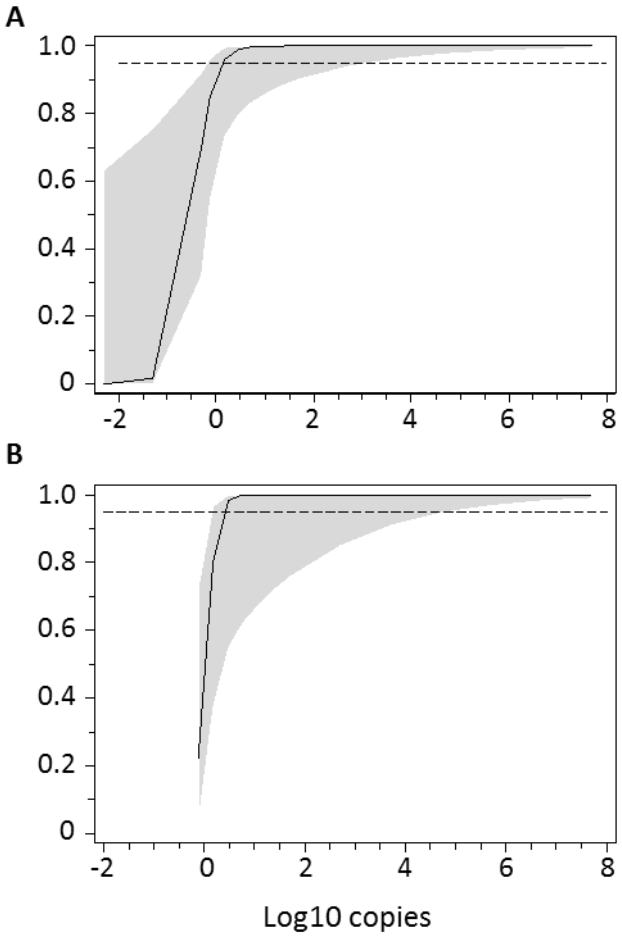
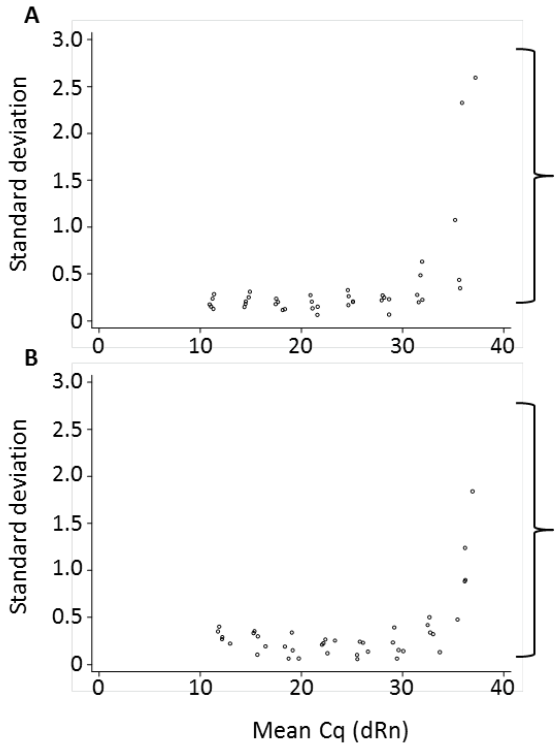



Figure 3.6 Analytical sensitivity predicted for Q1 (A) and Q2 (B) tests. The copy number of pNVmcp was estimated at the 95% probability of detection (Pr(detection)) for each test using logistic regression. The grey area depicts the 95% confidence interval and the dotted line marks the 95% probability of detection level.

The ASp of the Q1, Q2 and C1 tests was experimentally determined by screening the NCLDVs listed in Table 3.1. Herpesviruses AciHV-1 and -2 (Kelly et al. 2005, Kurobe et al. 2008) are not members of the *Megavirales* order but were included in specificity testing as they have been concurrently diagnosed with WSIV (Georgiadis et al. 2000) and unidentified sturgeon NCLDV-like infections (LaPatra et al. 2014). The herpesviruses could not be detected with the Q1, Q2 and C1 tests (data not shown). In order to quantify the ASp of each test, the level of agreement between the observed and expected status was ranked with the following formulas:

exclusivity (with 95% confidence intervals) is C1 (76% (57%-90%)) < Q1 (100% (81%-100%)) = Q2 (100% (81%-100%)); inclusivity is Q1 (80% (28%-99%)) < Q2 (100% (48%-100%)) = C1 (100% (48%-100%)). Neither the Q1 nor Q2 test was able to detect the MCP DNA from other viruses within *Megavirales* (Table 3.1). Both qPCR tests were able to detect the five sturgeon NCLDVS with the exception of Q1 which was not sensitive to WSIV DNA (Table 3.1). The C1 test has a broad specificity, not only could it detect the sturgeon NCLDVs, it could also amplify the MCP sequence of *Mimiviridae*, *Phycodnaviridae* or *Iridoviridae* family members (Table 3.1).

Five independent runs consisting of pNVmcp plasmid serially diluted across eight orders of




| Plasmid copies | Cq ± SD[a] | Coefficient of variation: Intra-assay[b] | Coefficient of variation: Inter-assay |
|---|---|---|---|
| $5x10^0$ | 35.92±0.76 | 3.74 | 2.12 |
| $5x10^1$ | 31.74±0.22 | 1.14 | 0.68 |
| $5x10^2$ | 28.30±0.35 | 0.73 | 1.23 |
| $5x10^3$ | 24.83±0.25 | 0.94 | 1.02 |
| $5x10^4$ | 21.25±0.32 | 0.78 | 1.51 |
| $5x10^5$ | 17.84±0.40 | 0.96 | 2.26 |
| $5x10^6$ | 14.62±0.23 | 1.49 | 1.58 |
| $5x10^7$ | 11.17±0.18 | 1.74 | 1.61 |

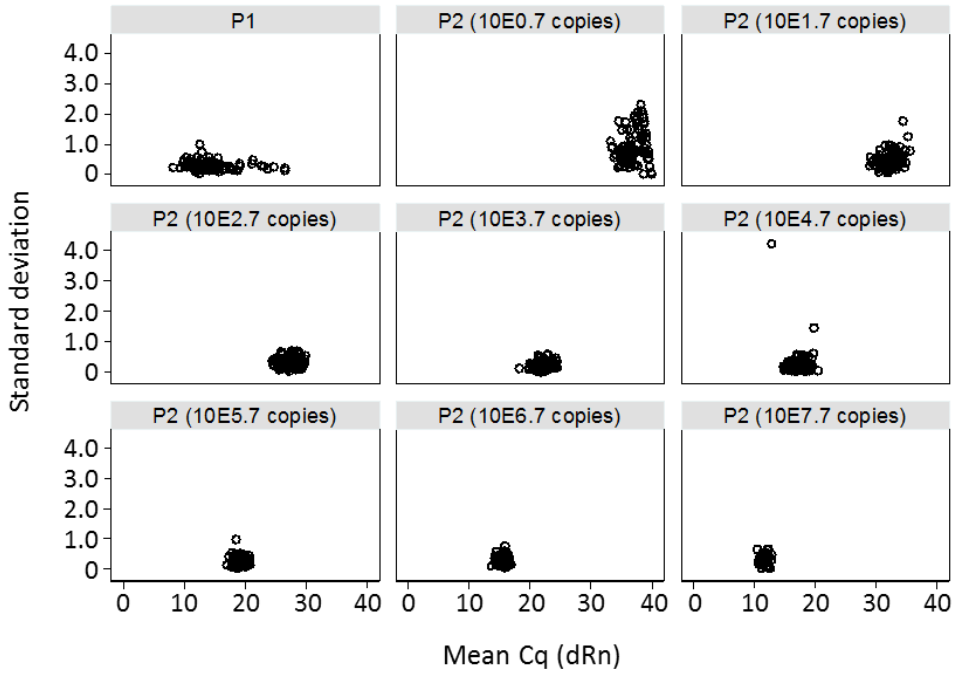
[a] Cq, mean of mean Cq/run; SD, mean of Cq standard deviation/run
[b] Average of within assay coefficient of variation for all runs

| Plasmid copies | Cq ± SD[b] | Coefficient of variation: Intra-assay[a] | Coefficient of variation: Inter-assay |
|---|---|---|---|
| $5x10^0$ | 36.19±0.52 | 2.93 | 1.45 |
| $5x10^1$ | 32.93±0.47 | 1.04 | 1.42 |
| $5x10^2$ | 29.48±0.40 | 0.67 | 1.34 |
| $5x10^3$ | 25.90±0.46 | 0.59 | 1.76 |
| $5x10^4$ | 22.50±0.51 | 0.95 | 2.28 |
| $5x10^5$ | 19.02±0.52 | 0.85 | 2.71 |
| $5x10^6$ | 15.69±0.47 | 1.64 | 2.97 |
| $5x10^7$ | 12.19±0.46 | 2.53 | 3.79 |

Figure 3.7 Analytical repeatability of the Q1 (A) and Q2 (B) tests. Cq values for the FAM-labelled probes were generated with pNVmcp DNA diluted from $10^{7.7}$ to 5 copies. Measurements were performed in replicates of five in five independent runs. Intra- and inter-assay coefficient of variation for Cq values as well as the standard deviation and mean Cq values for each of five runs are presented for each dilution in tables to the right of each graph

magnitude in five replicates was used to assess the repeatability of each qPCR assay. Plasmid DNA was diluted in a 10-fold dilution series from $10^{7.7}$ to 5 copies per reaction. The average intra- and inter- assay repeatability of Cq values were calculated using the coefficient of variation. The intra-assay CV for Cq values from the replicates ranged from 0.59 to 3.74 with the highest variation in dilutions with the lowest plasmid copy numbers (Fig 3.9). Comparing the five independent runs, the CV calculated from the Cq value of each dilution varied from 0.68 to 3.79 (Fig. 3.9). Higher variability was associated with the lowest and highest plasmid copy numbers.

The repeatability of the Q1 assay was evaluated using the P1 and P2 positive controls samples. Using the P1 positive control sample, the intra-assay CV for Cq values obtained was 1.4 and the inter-assay CV was 13.85 (Fig. 3.10). The P2 positive control sample was tested in a dilution series. The intra-assay CV, calculated from the Cq values from each dilution, ranged from 0.78 to 2.71 (Fig. 3.10). The higher CV values (2.46 and 2.71) were associated with the samples containing the lowest and highest plasmid copy numbers (5 and $10^{7.7}$). The variability between the P1 Cq values was statistically significant ($p<0.05$) between batches (+1.45 Cq) and runs (+0.034 Cq). The variability between the Cq values obtained from P1 were only statistically significant for samples with 5 plasmid copies between the batches (+0.18 Cq) and runs (0.01 Cq).




| Control sample | Number of Runs | Cq ± SD[a] | Coefficient of variation Intra-assay[b] | Inter-assay |
|---|---|---|---|---|
| P1 | 83 | 20.91±2.9 | 1.40 | 13.85 |
| P2 | | | | |
| $5x10^0$ | 88 | 37.87±1.33 | 2.46 | 3.50 |
| $5x10^1$ | 88 | 34.28±1.11 | 1.44 | 3.25 |
| $5x10^2$ | 88 | 30.60±1.02 | 1.10 | 3.32 |
| $5x10^3$ | 88 | 26.65±0.89 | 0.78 | 3.34 |
| $5x10^4$ | 88 | 23.16±1.03 | 1.28 | 4.44 |
| $5x10^5$ | 88 | 19.23±0.83 | 1.27 | 4.29 |
| $5x10^6$ | 88 | 15.51±0.63 | 1.66 | 4.07 |
| $5x10^7$ | 32 | 11.77±0.64 | 2.71 | 5.41 |

[a] Cq, mean of mean Cq/run; SD, mean of Cq standard deviation/run
[b] Average of within assay coefficient of variation for all runs

Figure 3.10 Analytical repeatability of the Q1 test with P1 and P2. Cq values for the FAM-labelled probe were generated with $10^{7.8}$ copies of pNV-APC Q1 DNA in 50 mg tissue (P1) or pNV-APC Q1 DNA diluted from $10^{7.7}$ to 5 copies (P2). Measurements were performed in replicates of three in 32 to 88 independent runs. Intra- and inter-assay coefficient of variation for Cq values as well as the standard deviation and mean Cq values are presented for P1 and each dilution of P2 in the table.

### 3.3.3 Application of Q1

The Q1 test has been used to screen endangered lake sturgeon populations in the Nelson River and Winnipeg River for the sturgeon viruses NV, MRSIV, BCWSV and SNSV. Fish testing positive have been traced back to both of the river drainage systems. Sturgeon gametes have not tested positive for virus DNA but larvae, juvenile, sub-adult and adult lake sturgeon somatic tissues have.

Samples collected from Pointe du Bois Falls (Winnipeg River) broodstock during the 2010 to 2012 spawning season (N = 35) all tested negative for virus however a wild adult sturgeon (1 of 12) collected at the same site during the 2013 spawning season tested positive. From years 2012 to 2014, broodstock tested positive (5 of 22) from the Nelson River drainage system. This includes the fish testing positive from Landing River (2 of 4; 2012), Burntwood River (1 of 4; 2013) and Birthday Rapids (2 of 4; 2014). The broodstock collected in 2011 and 2014 from the Landing River (N = 11) were all negative. The reproductive products collected from broodstock (N = 60) never tested positive with Q1 even if the donor fish tested positive for virus DNA. Larvae (N = 293), resulting from multiple gamete crosses, have not tested positive with the exception of a single larva that tested positive after being screened prior to transfer within the hatchery facility. That larva was the offspring of two positive testing broodstock.

Six wild broodstock tested positive by Q1 and carried 2 to 830 equivalent plasmid copies per µg of host tissue DNA. In comparison, the 130 juvenile sturgeon collected during the 2009 viral outbreak and tested with Q1 ranged from 136,033 to 388,089 equivalent plasmid copies per µg of host tissue DNA.

Histology was employed as a confirmatory diagnostic test when assessing the infection status of broodstock (N = 59), larvae (N = 109) and juvenile (N = 120) lake sturgeon collected from the Nelson and Winnipeg Rivers. Of the samples that tested negative by histology (N=256), nine samples also tested positive with the Q1 test. Visible pathognomonic changes consistent with sturgeon NCLDV infections were only observed in sturgeon tissues collected during a viral outbreak. All broodstock samples were collected from healthy-looking wild fish and tested negative by histology.

### 3.4 Discussion

Three new PCR assays were developed to detect and quantify sturgeon NCLDVs including NV, WSIV, MRSIV, SNSV and BCWSV in sturgeon tissue. Preliminary validation steps determined the tests had acceptable analytical sensitivity, specificity and repeatability as specified by the OIE Manual of Diagnostic tests for Aquatic Animals (OIE 2014). Phylogenetic analysis of sturgeon NCLDVs revealed they share a common evolutionary ancestor and may form a novel genus or family within the order *Megavirales*. The viruses appear to be endemic throughout North America, infecting members of the Acipenseridae family.

Five separate virus genotypes belonging to *Megavirales* have been uncovered in North America. These are epitheliotrophic viruses that are capable of causing acute and subclinical infections (Watson et al. 1998a, b, Raverty et al. 2003, Kurobe et al. 2010, 2011, Clouthier et al. 2013 LaPatra et al. 2014). The five genotypes were distributed across six river basins in North America. It is unknown if different genotypes can be found in the same water drainage systems. The geographic distribution of the genotypes does not correlate with the genetic similarity. For example, the SNSV was uncovered in shortnose sturgeon originating from the St. John River in

Atlantic Canada and the BCWSV was sourced from white sturgeon cultured in British Columbia, yet the MCP sequence is most similar between these two isolates. The individual genotypes may not be exclusively associated with a host species but the results from this study indicate that sturgeon NCLDV genotypes tend to be host family- or genus-specific.

It is possible to infer the evolutionary relationships among NCLDV members by comparing the shared MCP sequence. Phylogenetic tree reconstructions in this study resemble those reported in similar studies (Boyer et al. 2009, Fischer et al. 2010, Colson et al. 2011, 2012). Sturgeon NCLDVs fall outside the *Iridoviridae*, *Marseilleviridae* and *Mimiviridae* families and, therefore, may represent a new virus genus or family. The current practice of referring to WSIV and MRSIV as iridoviruses should be revised to eliminate confusion in the scientific literature.

As the MCP is a nucleocytoplasmic large DNA virus orthologous gene (Yutin et al. 2009), PCR tests were designed to target this region in order to develop group- specific tests for detection of all sturgeon NCLDVs. Specificity of the C1 assay was broader; it included all sturgeon NCLDVs as well as members of the *Phycoviridae, Mimiviridae*, or *Iridoviridae* families. The specificity of the Q1 and Q2 assays was limited to only the sturgeon NCLDVs. The Q1 test was unable to detect the WSIV but could detect the BCWSV, NV, SNSV and MRSIV. The Q2 test was pan-specific and capable of detecting all five sturgeon NCLDVs.

The three molecular tests developed in this study have been optimized for different applications. The sensitivity of the cPCR test was lower than the qPCR tests with a limit of detection of 50 to 500 plasmid copies. The C1 test could potentially be used sequentially with the Q1 or Q2 tests as a confirmatory diagnostic test. The cPCR assay could also be utilized as a tool to discover new sturgeon NCLDVs. Newly discovered sturgeon NCLDVs could potentially be

genotyped using the non-conserved DNA sequence found between two orthologous regions shared within all NCLDVs (Marchler-Bauer et al. 2015). If the genotype of a suspected sturgeon NCLDV is unknown, the Q2 test would be the most suitable screening assay as it is pan-specific for all sturgeon NCLDVs. The Q1 test has been optimized for NV detection and quantification but, if WSIV is not suspected, the two tests are interchangeable with the small exception of Q1 having a higher level of probe fluorescence.

The analytical performance characteristics of each PCR test were evaluated according to international standards (OIE 2014). These included the analytical sensitivity, specificity and repeatability. Efficiency of the qPCR reactions was 93% or higher for the Q1 and Q2 tests. The amplification products doubled consistently after each cycle when target DNA was present in quantities spanning over 8 orders of magnitude indicating the tests are reliable regardless of the viral load in the sample. Plasmid carrying NV MCP sequence was detectable in 50% of replicates when 3 plasmid copies were present and in 100% of replicates when 25 plasmid copies were tested for both the Q1 and Q2 tests. Other sturgeon NCLDV isolates were detected between 5 and 500 plasmid copies. Repeatability of the tests was high, the average CVs from Cq values ranged from 0.59 to 3.74 (intra-assay) and 0.68 to 3.79 (inter-assay). The highest variability was associated with samples carrying the lowest and highest plasmid copy numbers which is normal for a qPCR assays. The statistically significant variability in Cq values obtained for P1 controls were most likely a reflection of the control preparation rather than the test itself. The type lake sturgeon tissue varied between P1 controls and tissue type may influence qPCR sensitivity (Kwak et al. 2006a). The sensitivity and reliability of the tests indicate that they are appropriate to use in the screening and management of sturgeon viruses.

Namao virus is endemic in the Nelson River basin and lake sturgeon may act as carriers for the virus. Specifically, eleven fish, collected from the Winnipeg River and the Nelson Rivers, tested positive for NV. The last outbreak to occur in Manitoba was in 2009 in sturgeon originating from Winnipeg R. and Nelson R. broodstock. In the five years following, only one spawn sourced to broodstock collected at Birthday Rapids on the Nelson River in 2014, tested positive for NV. The offspring were tested again two months later and no virus was detected. It is possible the presence of NV within the river system is cyclical in nature, similar to observations of the WSIV in the Kootenai River (Drennan et al. 2005).

The Q1 test was used to measure the relative virus load present in infected sturgeon tissues. Virus was more abundant in tissues from dead or moribund fish collected during an outbreak compared to spawning wild adults. Kurobe et al. (2011, 2010) observed similar findings in wild pallid and shovelnose sturgeon broodstock. The relative virus abundance in moribund sturgeon was markedly higher compared to wild adult fish or fish recovering 8.5 months after initial exposure. Low virus levels are associated with the absence of clinical disease symptoms. Sturgeon NCLDV abundance is likely proportional to virus virulence or immunocompetence of the host.

The Namao virus has been maintained in lake sturgeon populations without being extinction-causing. It is likely that the virus and host are able to co-exist without the emergence of wide spread outbreaks. However, a shift in the host-pathogen-environment interplay can result in the expression of disease. For example, a dramatic shift in the environment, such as exposing a sturgeon to stressful hatchery conditions, could potentially a trigger manifestation of disease.

The C1, Q1 and Q2 tests developed as part of this study can be implemented by disease management programs to reduce the risk of virus transmission. Either the Q1 or Q2 test can be used to assess the infection status of potential broodstock thereby reducing the risk of virus exposure to cultured sturgeon populations. Screening lake sturgeon throughout their hatchery rearing and before release will ensure discharged sturgeon populations carry a lower prevalence of virus compared to populations in the wild. The molecular tests can be used to determine the prevalence and distribution of the virus by screening tissues collected from wild lake sturgeon. Screening inoculated live sturgeon with the assays will help establish the infection dynamics of the virus and the direct effect infection has on lake sturgeon mortality and health.

## 4.0 Transmission Dynamics of Namao Virus in Juvenile Lake Sturgeon *Acipenser fulvescens*

### 4.1 Introduction

Namao virus shares characteristics with a number of NCLDVs described in related sturgeon species. Clinical signs of disease associated with NV, WSIV and MRSIV infections include emaciation and petechial haemorrhaging around the pectoral fins, anal fins and mouth. Affected sturgeon may cease feeding, display unstable equilibrium, poor buoyancy and rapid opercular movement (Hedrick et al. 1992, Watson et al. 1998a, Kurobe et al. 2011, Clouthier et al 2013). Disease attributed to sturgeon NCLDV agents is age specific; it occurs in juvenile sturgeon less than a year old, and is influenced by stressors in the environment (Hedrick et al. 1992, Kurobe et al. 2011). Rearing conditions such as high densities of fish, low water inflow rates, poor water quality, handling, stressful transportation, temperature fluctuations as well as a genetic susceptibility to disease have been identified as factors that will encourage an outbreak (La Patra et al. 1994, Watson et al. 1998b, Georgiadis et al. 2001, Drennan et al. 2005).

The survivors of sturgeon NCLDV outbreaks act as asymptomatic carriers and may have the capacity to transmit virus vertically or horizontally (Watson et al. 1998, Raverty et al. 2003, Georgiadis et al 2001, Kurobe et al. 2011). Vertical transmission has been suspected among white sturgeon survivors (Georgiadis et al. 2001, Drennan et al. 2006). A study examining the effect of iodine disinfection of eggs post-fertilization was unable to establish whether WSIV could be transferred vertically either by intra-ovum or epibiotic pathways (Drennan et al. 2006). Experimental infection trials have successfully imparted WSIV and MRSIV horizontally to captive

sturgeon either through direct exposure to virus inoculant or by cohabitation with infected fish (Hedrick et al. 1990, Watson et al. 1998, Kwak et al 2006a, Drennan et al. 2007, Kurobe et al. 2010, 2011).

Koch's postulates, the classical standard of determining disease causality, cannot be strictly applied here as with most molecular and viral discoveries (Rivers 1937, Evan 1976). Rigid adherence to Koch's postulates requires isolating the pathogen from a diseased host, growing it in a pure culture and then using the agent to induce disease in a healthy host (Koch 1980). NV has thus far proven difficult to grow on cell culture (Clouthier et al. 2013). However, the overreaching principles of correlating the presence of a pathogen in diseased tissue with the newly caused illness can be realized with the help of molecular sequencing tools (Fredericks and Relman 1996). Like NV, MRSIV has not been isolated by culture on sturgeon cell lines. However, the presence of MRSIV was confirmed in dead or moribund fish collected during an infection trial and analyzed using both qPCR and histological methods. The results provided evidence that MRSIV was the etiological agent in susceptible sturgeon (Kurobe et al. 2011). The juvenile lake sturgeon were exposed to NV and the behavioral and physical characteristics of the fish were assessed. Histological and molecular testing was used to confirm if NV is present in sturgeon exhibiting signs of disease or increased mortality.

Namao virus was associated with disease in juvenile lake sturgeon reared as part of a conservation stocking program in Manitoba. In 2009 and 2010, juvenile lake sturgeon held at the Grand Rapids Hatchery (Grand Rapids, MB) and the University of Manitoba Aquatic Animal Holding Facility (Winnipeg, MB), experienced mortality. The disease outbreaks resulted in cumulative mortalities of 62 to 99.6% among progeny of wild Winnipeg River or Nelson River

lake sturgeon. Histological analysis of H&E stained gill and skin tissue collected from moribund lake sturgeon revealed intermittent hypertrophic cells containing inclusion bodies. Transmission electron microscopy of the inclusion bodies uncovered icosahedral-shaped virions in the cell cytoplasm. Presence of the virus has been observed in lake sturgeon using TEM, cPCR and qPCR; however, a direct link between NV and sturgeon morbidity and mortality has yet to be established (Clouthier et al. 2013). The goal of our study was to expose juvenile sturgeon to NV and apply Koch's postulates to determine the causality of infection.

## 4.2 Methods and Materials

### 4.2.1 Fish

Juvenile lake sturgeon sourced from multiple crosses of wild Nelson River broodstock were hatched in the spring of 2013 and reared at Grand Rapids Hatchery (Grand Rapids, MB). Fingerlings (3 to 10 g mean wet weight) from this population were transferred in October 2013 to the University of Manitoba Aquatic Animal Holding Facility (Winnipeg, MB) and were housed in two 600L tanks receiving de-chlorinated 16$^{o}$C municipal water at 1 to 2 L / min.

### 4.2.2 Virus

In February of 2009, a disease outbreak occurred in lake sturgeon reared at UMAAHF. Dead and diseased juvenile lake sturgeon, exhibiting signs of Namao virus infection, were collected during the mortality event and held at -20°C. Virus inoculant was prepared by homogenizing 123 of these archived lake sturgeon in Hank's minimal essential media (MEM) supplemented with 1 x antibiotic/antimycotic (Life Technologies). Five batches of homogenate were generated using an IKA Ultra Turrax Homogenizer. Each batch was tested with the Q1 sNCLDV qPCR test to establish virus load. They were also screened using virus-specific conventional PCR assays for

detection of acipenserid herpesviruses type 1 and 2 (AciHV-1, -2). The five batches of tissue homogenate were pooled to make 2 L of inoculate for the infection study.

### 4.2.3 Challenge

A subset of sturgeon was exposed to NV either through immersion in the virus inoculate or by cohabitation with NV-exposed sturgeon. Juvenile lake sturgeon were kept in six separate 75 L tanks throughout the study, two of the aquaria were identified as control tanks and the remaining four as immersion tanks (Fig 4.1).

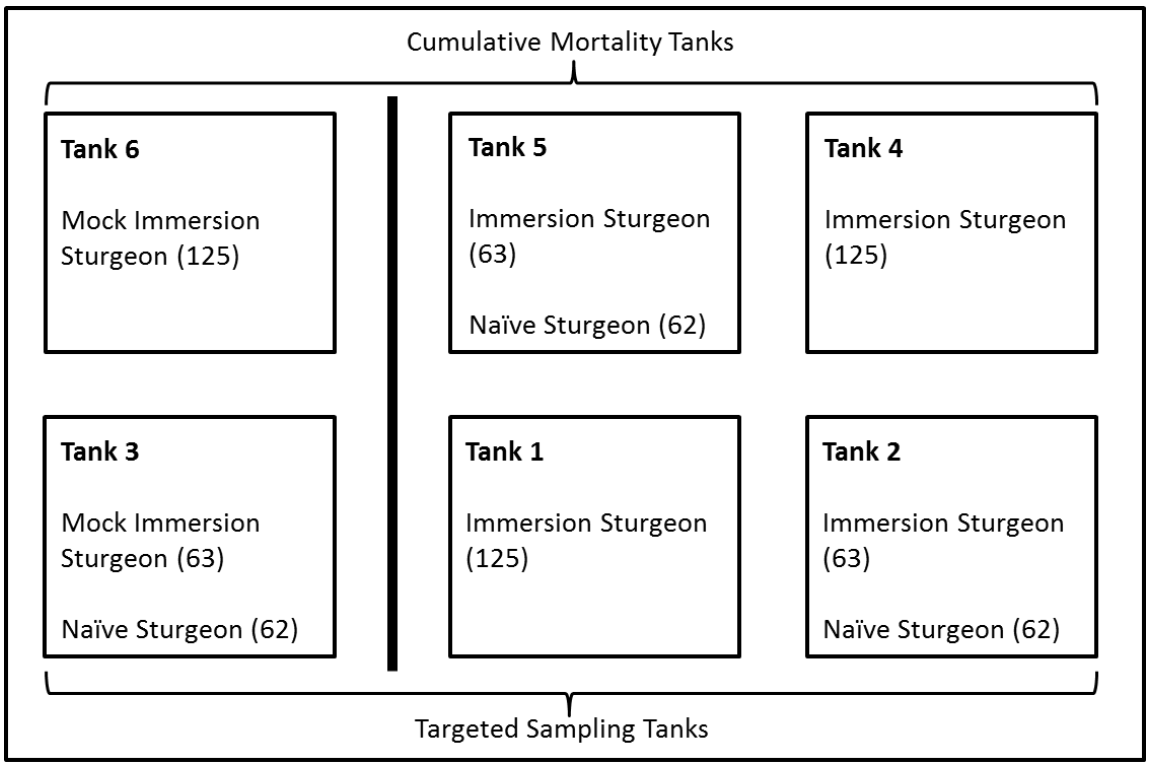



Figure 4.1 NV infection trial. Tank layout, treatment and number of sturgeon housed in each tank is presented. Number of fish per tank is in parenthesis. Only dead or moribund sturgeon were removed from Monitoring Tanks (i.e. tanks 4, 5, 6). Five fish per tank were removed weekly from the Targeted Sampling Tanks (i.e. tanks 1, 2, 3). Bolded line = plastic curtain barrier between mock immersion and virus challenge tanks, Mock Immersion = Immersed in MEM, Immersion = Immersed in NV-infected tissue homogenate, Naïve = cohabitant added 24 hours after treatment (anal fin clip).

The juvenile sturgeon were subjected to high-density rearing (15.5 g / L), as identified by Drennan et al. (2005), throughout the trial to elicit a manifestation of disease. The tanks were supplied with 15°C de-chlorinated city water at a flow rate of 0.4 L / min with dissolved oxygen levels at 80% or greater. Fish were fed brine shrimp and bloodworms (chironomids) at a rate of approximately 2% body weight/day throughout the trial. The water flow rate, water temperature, oxygen content and $NO_2$ /$NO_3$ levels were monitored daily. Fish were fed every morning and tanks were monitored three times daily for dead or moribund fish as well as overall fish behavior and health.

Two groups of 188 juvenile sturgeon were challenged with virus in two separate 40 L tanks by immersion in 1 L of NV-infected tissue homogenate mixed with 19 L of de-chlorinated city water. Fish were submerged in the static bath with aeration for 1 hour. An identical procedure was followed for control fish using 1 L of MEM containing 1x antibiotic/antimicotic for the mock immersion challenge. Following exposure, each treatment group was transferred into two 75 L tanks; one tank received 125 fish and the other 63 fish. Water flow rate was maintained at 0.4 L / min to clear any residual homogenate. Within 24 hours, 62 naïve juvenile lake sturgeon were placed into each of the tanks containing 63 fish (Figure 4.1). Immediately before addition, the naïve cohabitants were anesthetized with 100 mg / L sodium bicarbonate buffered tricaine methane sulfonate (TMS) (Syndel) and their right anal fin was clipped with sterile scissors. The clipped fin provided a means to distinguish naïve cohabitants from the virus-exposed sturgeon. Results of the NV challenge study have been presented as cumulative percent mortality.

### 4.2.4 Maintenance

Care was taken to eliminate cross contamination between the negative control and virus challenge tanks and between the virus challenge room and the rest of the animal care facility. The infection study was confined to a locked room within UMAAHF. Access to the facility was by keyed entry and access was restricted to authorized personnel. Within the virus challenge room, a footbath (Virkon) was used to disinfect footwear before entering the tank area. Tanks containing mock or virus immersion treatment groups were separated by a plastic curtain; footwear and coveralls were changed at the division. Instruments and equipment (nets, beakers, graduated cylinders, scrub brushes) exposed to tank water were disinfected in iodine solutions (Prepodyne) of 25:1 dilution (for a minimum of 20 minutes) and then 50:1 dilution (overnight). Each tank had its own net and equipment was not transferred between the mock and virus immersion treatment areas. Floors were mopped (Virkon) and shelving, curtains and equipment were cleaned daily (Peroxidase). The infection challenge and care for the lake sturgeon was carried out in accordance with the standards set forth by the Canadian Council on Animal Care (CCAC) under animal care protocol project title “Infection and transmission dynamics of a lake sturgeon virus” approved by the University of Manitoba animal care committee.

### 4.2.5 Sampling

At seven day intervals for the first 12 weeks, five live fish were collected from targeted sampling tanks. These tanks include one mock immersion tank (tank 3), and two immersion tanks (tanks 1 and 2) (Table 4.2, Figure 4.1). Each fish was euthanized with an overdose of TMS and placed individually into a labelled bag. Bags containing control or immersion fish were

always kept physically separated. Fish were dissected along the sagittal plane, one half of the sturgeon was preserved in Safe-Fix® (Fisher Scientific) for histological analysis and tissues were collected from the other half for qPCR analysis. The head skin, pectoral fin, anal fin, oropharynx, barbells, abdominal skin and kidney tissue (~50 mg each) were aseptically collected, transferred to individually labelled tubes and frozen immediately at -80°C. Dead or seriously moribund fish were removed from all tanks throughout the entire 17 weeks of the study and sampled in the same manner as fish collected during targeted sampling time points. After dissection, the remainder of the fish was stored at -80°C.

### 4.2.6 DNA Extraction

DNA was extracted from homogenates of frozen lake sturgeon tissue (50 mg) using the DNeasy Blood and Tissue Kit (Qiagen) and the QIAcube HT instrument (Qiagen) as outlined by the manufacturer. The tissue was homogenized with a 5 mm steel bead (Qiagen) and TissueLyser (Qiagen) in lysis buffer (Qiagen buffer ATL) for 2 min at 30 Hz, twice. The extracted DNA was quantified on the Take-3 Micro-Volume Plate using the Synergy HT spectrophotometer (Biotek).

### 4.2.7 Histology

For histological analysis, tissues were embedded in paraffin, sectioned at 5 µm, deparaffinised, hematoxylin and eosin (H&E) stained and analyzed using light microscopy.

### 4.2.8 Conventional PCR (cPCR) test for Acipenserid herpesviruses type 1 and 2 (AciHV-1, -2)

Conventional PCR tests for detection of AciHV-1 or AciHV-2 were kindly provided by Dr. Hedrick (UC Davis, unpublished). The tests targeted the AciHV-1 or AciHV-2 terminase genes

(197 bp and 141 bp, respectively) and were used to test virus challenge tissue homogenate. The cPCR targeting AciHV-1 used forward PCR primer 292 AciHv1F1 and reverse primer 294 AciHV1R1. The AciHV-2 test used forward primer 291 AciHV2F1 and reverse primer 293 AciHV2R1. Both PCR tests were 25 µL containing 100 µM forward primer, 100 µM reverse primer, 10X PCR Buffer (Applied Biosystems), 1.5 mM magnesium chloride, 200 µM nucleotide triphosphates (dNTPs), 2.5 units of AmpliTaq Gold (Applied Biosystems) and 200 ng of genomic DNA. Cycling conditions were 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 5 min, and 1 remaining cycle at 72°C for 5 min. The cPCR tests were run in an Applied Biosystems Veriti thermocycler. A 2% agarose gel (Life Technologies) containing 1x fluorescent nucleic acid stain GelRed (Biotium Inc.) was used to electrophoretically separate the amplicons. Data was recorded with a Kodak Gel Logic 200 imaging system.

### 4.2.9 qPCR and Postitive Control Material

The qPCR test Q1 was performed as described in Chapter 3. A forward qPCR primer sNCLDV F17 (5'-ATA GGG TAC AAG AGA CAT TCT C-3'), reverse primer sNCLDV R15 (5'-AGT TGA CCT CCT TTC CAA AAT CCA G-3'), assay probe sNCLDV P25 (5'-6FAM-TGC CAT GAC ACC AGT CGT T-MGBNFQ-3') and the positive control probe sNCLDV pAPC (5'-VIC-ACC GTC TAG CAT CCA GT-MGBNFQ-3' ) (Life Technologies) were designed based on the alignment of major capsid protein (MCP) regions of NV, WSIV, BCWSIV and MRSIV. The Q1 test was run using the Stratagene MX 3000P platform in a 96 well format. Tests were run in three replicates of each sample. The qPCR cocktail was 25 µL containing 1000 nM of sNCLDV F17, 800 nM sNCLDV R15, 250 nM of sNCLDV P25 and 250 nM sNCLDV pAPC, Taqman PCR Master Mix (Applied Biosystems) and 1500 ng of genomic DNA. The cycling conditions were 1 cycle of 2 m at 50°C, 1 cycle of 10 m at 95°C

followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. An artificial positive control (APC) plasmid (pNV–APC Q1) was used to generate positive control samples that were included in the nucleic acid extraction step (P1) and qPCR step (P2).

Viral copy numbers were calculated using the standard curve method. The positive control plasmid pNV-APC Q1 was diluted from $10^{7.7}$ to 5 copies / reaction in every qPCR run. The construct is 2,812 bp in length, $1.83 \times 10^{6}$ g / mol, and therefore, $3.29 \times 10^{11}$ copy numbers / µg DNA. This information was used to infer the copy number equivalent in test samples via a method provided by the MxPro software.

## 4.3 Results

### 4.3.1 NV Infection Trial Cumulative Percent Mortalities

In this study, the cumulative percent mortality in the groups of sturgeon exposed to NV by immersion was similar to the mortality in the groups of mock challenged sturgeon (Table 4.1). The cumulative mortality in fish housed in tanks sampled weekly (i.e. targeted sampling tanks) ranged from 9.52 to 16% (Control; mock immersion = 11.11%, naïve cohabitant = 14.52%). The cumulative mortality in fish housed in tanks where only dead or moribund sturgeon were removed (i.e. monitoring tanks) was between 40.32 and 52.8% (Control; mock immersion = 50.79%, naïve cohabitant = 40.32%). Sturgeon in the mock immersion and virus immersion groups displayed poor buoyancy, erratic swimming behaviour and visible wasting before death, throughout the study.

Table 4.1 Cumulative percent mortality of juvenile lake sturgeon in the NV infection study.

| | Tank # | Treatment | No. of Fish / tank | No. of Mortalities | Cumulative % Mortality |
|---|---|---|---|---|---|
| Targeted sampling tanks | 1 | NV immersion challenge | 125 | 20 | 16.00% |
| | 2 | NV immersion challenge | 63 | 6 | 9.52% |
| | | Naïve cohabitant | 62 | 13 | 20.97% |
| | 3 | Mock immersion challenge | 63 | 7 | 11.11% |
| | | Naïve cohabitant | 62 | 9 | 14.52% |
| | | | | | |
| Monitoring tanks | 4 | NV immersion challenge | 125 | 66 | 52.80% |
| | 5 | NV immersion challenge | 63 | 28 | 44.44% |
| | | Naïve cohabitant | 62 | 26 | 41.94% |
| | 6 | Mock immersion challenge | 63 | 32 | 50.79% |
| | | Naïve cohabitant | 62 | 25 | 40.32% |

### 4.3.2 NV-infected Tissue Homogenate Used as the challenge Virus

The quantity of NV present in the five batches of tissue homogenate was determined using the Q1 test. The virus load in each batch ranged from $2.41 \times 10^5$ to $4.52 \times 10^5$ equivalent plasmid copies / µg of DNA. Final virus challenge dose was 9.5 log10 plasmid copies/L of water. Homogenates tested negative for AciHV-1 and AciHV-2 using Dr. Hedrick's cPCR test methods.

### 4.3.3 Virus Transmission

A total of 3152 tissue samples from 469 fish were collected during the 17 week study. Of those, 1421 tissues were tested with Q1 from the 216 dead or moribund fish collected

throughout the trial. The head skin, gill, pectoral fin, anal fin, oropharynx, barbels, abdominal skin and kidney tissue collected from each dead or moribund fish were screened with Q1 with two exceptions. Only three types of tissue (i.e. head skin, pectoral fin and abdominal skin) were tested from dead fish collected from tanks 1, 2 and 5 between days 14 and 85 post-infection. Dead fish collected from tank 3 were not analysed (Figure 4.2).

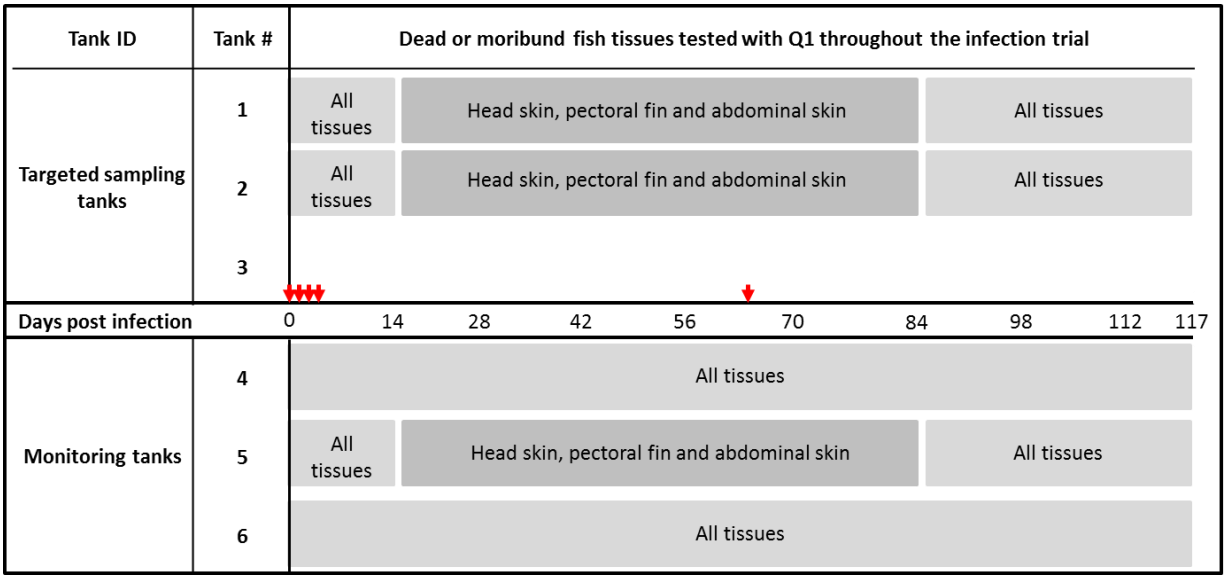



Figure 4.2 Q1 testing of tissues from dead or moribund lake sturgeon. An overview of tissue type and sampling timepoints for dead or moribund lake sturgeon tested with Q1. All tissues = head skin, pectoral fin, anal fin, gill, oropharynx, abdominal skin, barbels and kidney. Red arrows (↓) indicate sampling timepoints at which Q1-positive fish were collected (0, 1, 2, 3, 62 days post-infection).

None of the fish (0/152) collected as part of the weekly sampling regimen tested positive for Namao virus using the Q1 test (Table 4.2). In total, 7% (11/159) of NV-exposed fish that died during the study tested positive by the Q1 test (Table 4.2). There was no evidence of cross contamination from the qPCR positive control samples in any of the tissues testing positive by Q1. Fish collected within the first 3 days of the trial provided 95% of the positive tissues; the

remaining 5% were collected on day 62 (Table 4.3). An originally naïve cohabitant tested positive for NV DNA on the second day post-challenge (Table 4.3).

Table 4.2 Q1 diagnostic test results for the NV challenge study. The number of lake sturgeon testing positive with Q1 is presented for the dead or moribund fish and targeted sampling fish relative to each treatment group.

| Sample Group | Tank # | Treatment | No. positive fish / no. fish screened with Q1 |
|---|---|---|---|
| Dead or Moribund Fish | 1 | NV immersion challenge | 1/23 (4.3%) |
| | 2 | Cohabitant immersion challenge | 3/18 (16.7%) |
| | 3 | Mock cohabitation immersion challenge | Not tested |
| | 4 | NV immersion challenge | 5/66 (7.6%) |
| | 5 | Cohabitant immersion challenge | 2/52 (3.8%) |
| | 6 | Mock immersion challenge | 0/57 (0%) |
| | | | |
| Targeted Sampling Fish | 1 | NV immersion challenge | 0/65 (0%) |
| | 2 | Cohabitant immersion challenge | 0/87 (0%) |
| | 3 | Mock cohabitation immersion challenge | Not tested |

Table 4.3 Q1 diagnostic test results for each day a NV-positive fish was collected. The results are presented as a ratio of the number of Q1 positive fish to the total number of dead fish tested per treatment group.

| Days Post Challenge | Treatment | |
|---|---|---|
| | NV Immersion Challenge | Naïve Cohabitant |
| 0 | 2/2 | - |
| 1 | 2/5 | 0/1 |
| 2 | 3/3 | 1/3 |
| 3 | 2/8 | 0/4 |
| 62 | 1/6 | 0/2 |

### 4.3.4 Histological Results

Histological examination was performed with tissue collected from dead or moribund fish from tanks 4 (virus immersion challenge) and 6 (mock immersion challenge) as well as from all sturgeon that tested positive with Q1. Pathognomonic signs consistent with the presence of Namao virus were not evident. *Costia ichthyobode,* a protozoan flagellate parasite, was reported in 67% (38/57) of sturgeon from the tank 6 mock exposed group and not in any histological samples from other tanks.

### 4.3.5 NV Tissue Tropism

Positive test results with Q1 were obtained with all eight tissues that were sampled throughout the study (Table 4.3). NV was detected most frequently in gill (82%), abdominal skin (27%) and kidney tissue (27%) (Table 4.4).

Table 4.4 Q1 diagnostic test results for each lake sturgeon tissue type. Results are presented as a ratio of the number of positive samples to the total number of samples tested by Q1 for each treatment group.

| Treatment Group | Head Skin | Gill | Pectoral Fin | Pelvic Fin | Oropharynx | Abdominal Skin | Kidney | Barbel |
|---|---|---|---|---|---|---|---|---|
| Immersion | 2/122 | 8/122 | 1/85 | 1/85 | 2/85 | 3/122 | 3/85 | 1/85 |
| Naïve | 0/37 | 1/37 | 0/17 | 0/17 | 0/17 | 0/37 | 0/17 | 0/17 |
| Immersion (Control) | 0/32 | 0/32 | 0/32 | 0/32 | 0/32 | 0/32 | 0/32 | 0/32 |
| Naïve (Control) | 0/25 | 0/25 | 0/25 | 0/25 | 0/25 | 0/25 | 0/25 | 0/25 |

### 4.3.6 Virus Load of Lake Sturgeon Tissue

The quantity of equivalent plasmid copies/µg of host DNA was established for each of the tissues testing positive using the Q1 test. The greatest concentrations of virus DNA was found in

kidney and gill tissue (357.48 and 135.22 equivalent plasmid copies / µg of DNA, respectively) (Table 4.5). The mean NV DNA concentration of the positive tissues was 41.0 equivalent plasmid copies / µg of DNA.

Table 4.5 The relative quantity of namao virus in lake sturgeon tissues testing positive by Q1. The virus copy number is expressed as equivalent plasmid copies/µg of DNA. "-" indicates no NV DNA was detected.

| Fish no. | Head Skin | Gill | Pectoral | Pelvic | Oropharynx | Abdominal Skin | Kidney | Barbel |
|---|---|---|---|---|---|---|---|---|
| 1 | 2.99 | 135.22 | 2.43 | - | 5.27 | 8.25 | 7.57 | 3.32 |
| 2 | - | 120.91 | - | - | - | - | - | - |
| 3 | - | 1.31 | - | - | - | - | - | - |
| 4 | - | 48.45 | - | - | - | - | 357.48 | - |
| 5 | - | 4.25 | - | - | - | - | - | - |
| 6 | - | 18.29 | - | - | - | - | - | - |
| 7 | - | 28.49 | - | 17.96 | 18.94 | 71.86 | - | - |
| 8 | - | 1.44 | - | - | - | - | - | - |
| 9 | - | 6.00 | - | - | - | - | - | - |
| 10 | - | - | - | - | - | 1.80 | 8.29 | - |
| 11 | 31.42 | - | - | - | - | - | - | - |

## 4.4 Discussion

Our study provided evidence that NV could be transmitted to juvenile lake sturgeon. The transmission frequency was lower than expected given that mortality as high as 93% has been reported for WSIV in white sturgeon *Acipenser transmontanus* (Drennan et al 2006) and 85% for MRSIV in pallid sturgeon *Scaphirhynchus albus* (Kurobe et al 2011) following exposure to the virus by experimental challenge. Juvenile lake sturgeon exposed to NV exhibited mortality and some fish displayed clinical signs of disease characteristic of this group of viruses (i.e.

emaciation, erratic swimming behavior, unstable equilibrium). Pathognomonic changes typical of sturgeon NCLDV infection were not identified following histological examination and NV DNA was present in only 3.5% of the dead fish tested. This information indicated that a causal relationship between NV and disease had not been established and that further studies would be required to fulfill Koch's postulates.

A low virus challenge dose may have been a critical determinant in the outcome of our study. The pathogen used in this study was sourced from lake sturgeon that had died during a disease outbreak in 2009. These sturgeon tested positive for NV nucleic acid in various tissues examined by cPCR and qPCR; however, virus viability testing was not possible as NV could not be amplified by cell culture (Clouthier et al, 2013). The viability of NV used in this study may have been reduced through storage of the source fish for almost five years at -20°C. The largemouth bass *Micropterus salmoides* iridovirus is viable in frozen fish for 5 months; however, viability is dependent on the freshness of the fish when frozen (Plumb and Zilberg 1999). The process of partially thawing the sturgeon tissue to generate a tissue homogenate and then refreezing it may have further decreased the infectivity of NV. Infectious haematopoietic necrosis virus, which causes necrosis in salmonid fish, loses infectivity when freeze-thawed in some circumstances (Pietsch et al. 1977).

Genetic susceptibility or resistance of sturgeon stocks has been identified as one of the most important determinants for disease outbreaks attributable to this group of viruses. For example, epidemiological analysis of WSIV outbreaks in commercial sturgeon farms performed by Georgiadis et al. (2000) revealed progeny from different spawning broodstock pairs experienced dissimilar mortality rates even though they were exposed to the same fish

management procedures. The sturgeon used in our infection trial were progeny of only two parental mating crosses, one or both of which may have had a genetic resistance to infection with NV. The latter may explain the low rate of NV transmission and mortality observed in our study.

High density rearing of juvenile white sturgeon and water temperature are other risk factors that have been shown to contribute to the onset and severity of sturgeon NCLDV disease outbreaks (LaPatra et al. 1994, Georgiadis et al. 2001, Drennan et al. 2006). Lake sturgeon in this study were held at a stocking density of 15.5 g / L in water maintained at 15°C in an effort to induce manifestation of disease upon challenge with NV. These environmental conditions have been shown to induce a higher daily mortality rate in white sturgeon exposed to WSIV (Drennan et al 2005, Watson et al. 1998b). The cumulative percent mortality rate in this study did not reach the relatively high levels associated with an acute sturgeon NCLDV outbreak and could not be attributed to the NV.

NV displayed a tropism for the integument of the body and gill tissue. Virus DNA was more commonly found in lake sturgeon gill tissue and, to a lesser extent, in abdominal skin tissue shortly following NV challenge. Similarly, WSIV is more likely to localize to gill tissue in white sturgeon after exposure to a high dose of virus (Kwak et al 2006a). White sturgeon gill is one of the first tissues to display epithelial hyperplasia and dysplasia upon virus exposure compared to the skin, esophagus, kidney, liver and spleen (Watson et al. 1998). Though gill tissue tested positive initially, Namao virus exhibited the ability to persist in sturgeon head skin for up to 62 days. WSIV has been identified in fish snout tissues up to nine months post exposure (Kwak et al. 2006a) and MRSIV has been found in fin tissue 8.5 months following

challenge (Kurobe et al 2011). Gill tissue appears to be the initial site of a NV infection but the virus may localize to the epithelial cells of the integument during persistent subclinical and chronic infections.

Histological analysis has consistently proven to be less sensitive than qPCR testing and has a reduced capacity to detect light viral loads (Kwak et al 2006a, Drennan et al. 2007, Kurobe et al. 2010, 2011). The relative quantity of virus detected in tissues in this study was relatively light with equivalent plasmid copies per µg of host DNA ranging from 1.31 to 357.48. Comparatively, the viral loads of sturgeon presenting pathological signs during a 2009 mortality event reached 136,033 to 388,089 equivalent plasmid copies per µg of DNA. The light viral loads account for the lack of histopathological signs of a NV infection in sturgeon testing positive via qPCR.

We were unable to establish that Namao virus was the cause of lake sturgeon mortalities; however we demonstrated that NV can be transmitted horizontally and is able to persist in sturgeon tissue. As NV can be transmitted horizontally, it is important to screen lake sturgeon skin tissue with Q1 before exposing sturgeon to new cohabitants. Sturgeon not displaying signs of infection may be carriers of NV. The similarities shared between NV and other sturgeon NCLDVs indicate that it would be prudent to eliminate the risk factors that contribute to outbreaks when culturing lake sturgeon. High density rearing, water temperature fluctuations and unnecessary handling may negatively impact sturgeon health and facilitate NV outbreaks. Future research examining the genetic susceptibility of lake sturgeon will be an important step in understanding NV transmission dynamics.

## 5.0 Synthesis

Namao virus was associated with mortality events in juvenile sturgeon reared as part of a conservation stocking program meant to supplement endangered lake sturgeon populations in Manitoba. Namao virus was discovered and identified as part on this Master's thesis. I developed three molecular tests capable of detecting and diagnosing NV along with multiple related sturgeon viruses. Each of the assays were optimized and the analytical performance characteristics evaluated. The molecular tests were used in conjunction with a live infection trial in order to better understand the transmission dynamics of NV. The results from this thesis indicate gills act as a primary entry site for NV and the virus has the ability to persist in cranial epithelial tissue.

The structure of NV is consistent with a growing number of agents described in several Acipenseridae species (Hedrick et al. 1990, Adkison et al. 1998, Kurobe et al. 2011, LaPatra et al. 2014). NV, WSIV, BCWSIV, MRSIV, SNSV and RSIV have been associated with epitheliotrophic infections targeting sturgeon skin, gills and olfactory organs including the oropharynx and barbels (Hedrick et al. 1990, Adkison et al. 1998, Watson et al. 1998a, Drennan et al. 2007, Kurobe et al. 2011). Hypertrophied amphophilic to eosinophilic-staining cells with irregular nuclei are visible in diseased sturgeon tissue sections (Hedrick et al 1990, 1992 Adkison et al 1998, Watson et al. 1998a, Kurobe et al. 2011,). The impairment of osmoregulation, respiration, navigation and sensory organs alters the swimming and feeding behavior of sturgeon. This may account for the erratic swimming and chronic wasting observed during a sturgeon NCLDV infection.

The causal relationship between Namao virus infection and mortalities in lake sturgeon could not be established in this thesis; however, the link can be inferred by comparison to similar sturgeon NCLDV infections. The cumulative mortalities caused by WSIV in white sturgeon and MRSIV in pallid and shovelnose sturgeon can reach close to 100% (Hedrick et al 1990, 1992, Kurobe et al. 2011). Similarly, cumulative mortalities in NV-associated outbreaks ranged from 62 to 99%. Conventional PCR analysis revealed that 93.6% of lake sturgeon tissues tested from 2008 and 2009 outbreaks contained NV DNA. WSIV die-offs often follow stressful events that occur 10 – 40 days prior to appearance of symptoms (Watson et al 1998b, Georgiadis et al. 2001, Drennan et al. 2007). he kinetics of NV outbreaks varied across tanks and facilities and may have corresponded to individual stressful events. NV has a similar size, structure and pathology as other sturgeon NCLDVs; therefore, it is not unreasonable to infer that a NV infection can develop into an acute lethal disease.

Phylogenetic analyses of NV, WSIV, BCWSIV, MRSIV and SNSV MCP sequences place sturgeon NCLDVs into a new virus genus within the order *Megavirales.* These five genotypes of virus exist in multiple river basins across North America and may be endemic in the wild sturgeon populations they infect. Molecular tests targeting sturgeon NCLDVs with and without isolate-specific analytical specificity are important for the detection and identification of various sturgeon NCLDVs in the environment. The Q1 qPCR test developed in this study has been optimized to detect Namao virus. It is applicable for screening broodstock, gametes and progeny collected in Manitoba where NV is endemic. It is also suitable for testing lake sturgeon exposed to NV in experimental conditions. The second molecular test developed in this study, Q2, is pan-specific for all sturgeon viruses and can be utilized if a sturgeon NCDLV is suspected

but the genotype unknown. The cPCR test was instrumental in the discovery of NV and the development of tests Q1 and Q2. The C1 test is suitable for discovering new sturgeon NCLDVs or confirmatory diagnostic testing.

Sturgeon NCLDVs can coexist with their hosts as persistent subclinical localized infections possibly for the lifetime of the fish (LaPatra et al 1994, Kwak et al. 2006a, Kurobe et al. 2011). Subclinical carriers are thought to occasionally shed virus-laden epithelial cells into the water column (Hedrick et al. 1990, Watson et al. 1998a). Stressful conditions may compromise the immunity of captive sturgeon and result in the replication of virus particles and manifestation of disease. Spawning is considered to be a stressful event (Bruch and Binkowski 2002) and wild sturgeon engaged in spawning may be more likely to amplify, shed and transmit virus. The practise of sampling broodstock during the spawning season is ideal for detecting subclinical carriers. Sturgeon may have a higher concentration of virus during this period, making it more likely to be detected by diagnostic testing regimens.

Studies examining the transmission dynamics of WSIV suggest the virus may be passed vertically from broodstock to progeny (Geogiadis et al. 2001). Namao virus might share a similar dynamic as a single hatchery-reared larva, generated from a cross of virus-positive broodstock, tested positive for Namao virus DNA. However, gametes from the infected broodstock did not test positive nor did the vast majority of larvae screened throughout this study (N = 239). This thesis is unable to provide conclusive evidence on vertical transmission. Larvae may have been exposed to virus horizontally, through contact or exposure to a waterborne agent sometime after hatch. River water has been the suspected source of multiple sturgeon NCLDV outbreaks (LaPatra et al. 1994, Drennan et al 2006) and may have contributed to the 2008 and 2009 die-offs of lake

sturgeon in Manitoba. Water from the Winnipeg, Saskatchewan and Nelson drainage systems may be the source of infection and disinfection procedures should be considered when supplying rearing facilities with fresh water.

Research addressing current gaps in knowledge, regarding these emerging pathogens, will enhance our ability to manage and protect endangered lake sturgeon populations. Similar to MRSIV and some isolates of WSIV, NV has not been successfully cultured on sturgeon cell lines. The possible propagation of NV on cultured amoeba would provide a source of viable NV as well as a means to fulfill Koch's postulates. The genetic susceptibility of lake sturgeon to NV is not fully understood and further research in this field may influence the future selection of broodstock and progeny. Phylogenetic analysis using additional sequences or whole genome analysis of NV may provide more insight into the evolutionary history and provide a clearer taxonomic picture for sturgeon NCLDVs. The current practice of the conservation stocking program in Manitoba is to release hatchery-reared sturgeon into their river of origin only if the prevalence of NV is equal to or below that of the wild population. As a result, continued research into the distribution and prevalence of NV in these populations is warranted. Continued study of NV transmission dynamics and the role of stressors in the manifestation of disease may influence the culturing and rearing practices of lake sturgeon. The findings of such research would provide considerable insight into NV and aid in the recovery and maintenance of healthy lake sturgeon populations in Manitoba.

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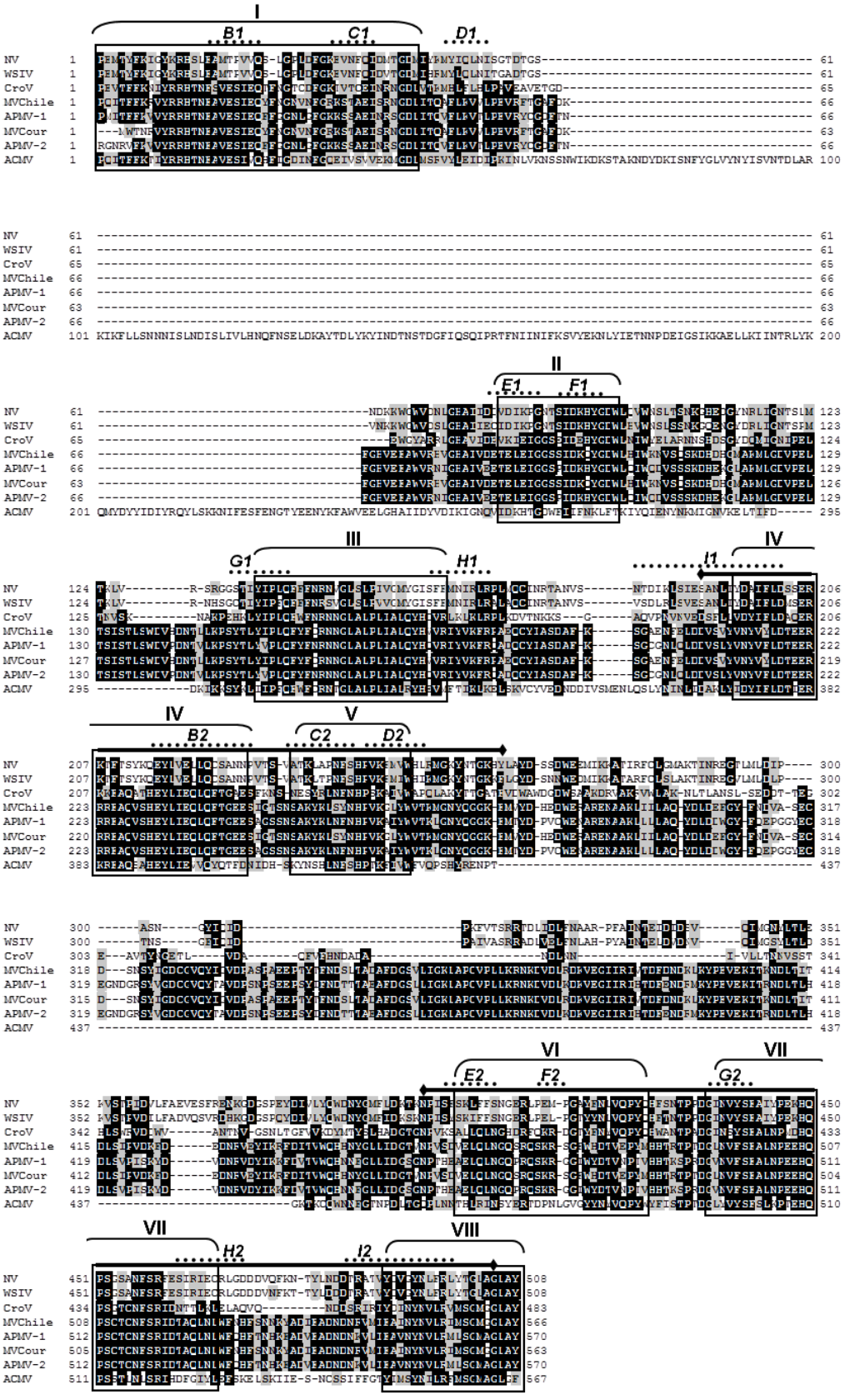
## Appendices

Appendix 1. Major capsid protein of representative members of the nucleo-cytoplasmic large DNA virus (NCLDV) superfamily

| Classification & Virus | Acronym | Gene (bp) | Protein (aa) | # NCLDV domains[a] | Accession # (DNA/protein) | Reference |
|---|---|---|---|---|---|---|
| Unclassified Mimivirus-like | | | | | | |
| Namao virus | NV | 1524 partial | 508 partial | 2 | JX155659 | This study |
| White sturgeon iridovirus | WSIV | 1596 | 531 | 2 | DQ897645 ABK34555.1 | Direct submission |
| **Mimiviridae** | | | | | | |
| Acanthamoeba polyphage mimivirus (Bradford isolate) | APMV-1 | 1782 | 593 | 2 | NC 014649.1 YP003986929 | Legendre et al. (2011) |
| Acanthamoeba polyphage mimivirus (M4 isolate) | APMV-2 | 1776 | 591 | 2 | JN036606.1 AEJ34665.1 | Boyer et al. (2011) |
| Cafeteria roenbergensis virus | CroV | 1521 | 506 | 2 | NC 014637.1 YP003969975.1 | Fischer et al. (2010) |
| Megavirus chiliensis | MVChile | 1770 | 589 | 2 | NC 016072.1 YP004894515.1 | Arsian et al. (2011) |
| Megavirus courdo | MVCour | 1698 | 565 | 2 | JN885991.1 AEX61606 | Direct submission |
| Acanthamoeba castellanii mamavirus | ACMV | 1776 | 591 | 1 | JF801956.1 AEQ60611.1 | Colson et al. (2011b) |
| **Phycodnaviridae** | | | | | | |
| Organic lake phycodnavirus 2 | OLPV-2 | 1611 | 536 | 1 | HQ704803.1 ADX06358 | Yau et al. (2011) |
| Phaeocystis pouchetii virus | PPV | 1563 | 520 | 1 | EU006631.1 ABU23715.1 | Larsen et al. (2008) |
| Chrysochromulina ercina virus | CEV | 1755 | 584 | 1 | EU006628.1 ABU23712.1 | Larsen et al. (2008) |
| Pyramimonas orientalis virus | POV | 1191 | 396 | 1 | EU006630.1 ABU23714 | Larsen et al. (2008) |
| Heterosigma akashiwo virus | HAV | 1323 | 440 | 1 | AB198422.1 BAE06835 | Nagasaki et al. (2005) |
| Acanthocystis turfacea chlorella virus | ATCV | 1317 | 438 | 1 | NC 008724.1 YP001426761.1 | Direct submission |
| Paramecium bursaria chlorella | PBCV | 1314 | 437 | 1 | NC 009898.1 YP001497813.1 | Fitzgerald et al. (2007) |

| | | | | | | |
|---|---|---|---|---|---|---|
| virus | | | | | | |
| Micromonas pusilla virus | MPV | 1257 | 418 | 1 | HQ633072.1 AET43572 | Direct submission |
| Ostreococcus tauri virus | OTV | 1269 | 422 | 1 | NC013288.1 YP003495004.1 | Weynberg et al. (2009) |
| Feldmannia species virus | FSV | 1308 | 435 | 1 | NC011183.1 YP002154681.1 | Schroeder et al. (2009) |
| Ectocarpus siliculosus virus | ESV | 1428 | 475 | 1 | FN648730.1 CBN80416.1 | Cock et al. (2010) |
| **Iridoviridae** | | | | | | |
| Chilo iridescent virus | CIV | 1404 | 467 | 1 | NC003038.1 NP149737 | Jakob et al. (2001) |
| Invertebrate iridescent virus 3 | IIV3 | 1401 | 466 | 1 | NC008187.1 YP654586 | Delhon et al. (2006) |
| Frog virus 3 | FV3 | 1392 | 463 | 1 | FJ459783.1 ACP19256.1 | Holopainen et al. (2009) |
| Infectious spleen & kidney virus | ISKNV | 1362 | 453 | 1 | AF371960.1 AAL98730.1 | He et al. (2001) |
| Lymphocystis disease virus | LDV | 1380 | 459 | 1 | EF103188.1 ABL07488.1 | Direct submission |
| **Unclassified** | | | | | | |
| Marseillevirus | MV | 1434 | 477 | 1 | NC013756.1 YP003407071.1 | Boyer et al. (2009) |
| **Ascoviridae** | | | | | | |
| Diadromus pulchellus ascovirus 4a | DPAV | 1305 | 434 | 1 | AJ312705.1 CAC84483.1 | Stasiak et al. (2003) |
| **Asfarviridae** | | | | | | |
| African swine fever virus | ASFV | 1941 | 646 | 1 | XM369245.2 XP369245.2 | Direct submission |

[a] Capsid NCLDV domain identified by the Conserved Domain Database (Materials and Methods)


I
B1
C1
D1
II
E1
F1
III
G1
H1
I1
IV
IV
B2
V
C2
D2
VI
E2
F2
VII
G2
VII
H2
I2
VIII

Appendix 2 Alignment of major capsid protein (MCP) sequences from 8 viruses found to cluster together in the phylogenetic tree presented in Figure 2.5. The MCP sequences were trimmed to the first and last amino acids aligning with the N and C terminal ends, respectively, of the namao virus (NV) MCP fragment targeted by primers from the present study. Identical amino acid residues shared by two or more sequences are shaded black whereas similar residues are shaded grey. The numbers preceding and following the alignments indicate the positions of the first and last residues of the aligned regions in the corresponding protein sequences. Bars above the sequence delineate the conserved nucleo-cytoplasmic large DNA virus domains identified in the NV MCP through the Conserved Domain Database and National Center for Biotechnology Information's Conserved Domain Search service. The beta strands predicted in the first and second jelly-roll motifs are designated B1 to I1 and B2 to I2, respectively. The 8 conserved capsid domains reported by Larsen et al. (2008) for viruses classified as members of family Phycodnaviridae are boxed and labelled I to VIII. Virus abbreviations are defined in Appendix 1