CHAPTER 6.3

Alloherpesviruses of fish

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Abstract

The family *Alloherpesviridae* includes herpesviruses of fish and amphibians. This group of viruses are phylogenetically distant from the better characterized herpesviruses found in birds and mammals. However, many biological and structural properties are conserved in the order *Herpesvirales*. The known alloherpesviruses typically exhibit host specificity which is a biological feature shared by nearly all herpesviruses. Of the alloherpesviruses of fish at least 11 cause significant economic losses to aquaculture. These include: *Ictalurid herpesvirus 1* and *2* (IcHV1, 2) impacting catfish aquaculture; *Cyprinid herpesvirus 1, 2* and *3* (CyHV1-3) impacting carp and goldfish aquaculture; *Anguillid herpesvirus 1* (AngHV1) impacting eel aquaculture; *Acipenserid herpesvirus 1* and *2* (AciHV1, 2) impacting sturgeon aquaculture; and *Salmonid herpesvirus 2, 3* and *4* (SalHV2-4) impacting salmon and trout aquaculture. The most notable impact has been koi herpesvirus disease caused by *Cyprinid herpesvirus 3*. This virus has caused devastating losses worldwide in all strains of common carp and is an OIE notifiable pathogen.

Key words

Ictalurid herpesvirus, Cyprinid herpesvirus, Anguillid herpesvirus, Acipenserid herpesvirus, Salmonid herpesvirus

Chapter starts here

6.3.1 Introduction

Herpesviruses are a diverse group of viruses with large DNA genomes. Genetic analysis has shown that there are 3 distinct groups (families) of herpesviruses: *Herpesviridae*- that infect the reptiles, birds and mammals; *Malacoherpesviridae*- that infect mollusks; and *Alloherpesviridae* that infect fish and amphibians (Davison *et al.,* 2009; McGeoch *et al.,* 2006; Waltzek *et al.* 2009). The possible exception to these general trends is tilapia larvae encephalitis virus which showed more sequence similarity in a small fragment of the polymerase gene to *Herpesviridae* than *Alloherpesviridae* (Shlapobersky *et al.,* 2010). Sequence similarity between

herpesvirus families is almost non-existent. In fact in large scale comparisons between the deduced amino acid sequences of the genes of the DNA viruses the *Herpesvirales* did not form a monophyletic group (Wu *et al.,* 2009). The only gene with clear homology is the ATPase subunit of the terminase, a protein involved in packaging genome into the capsid during virion assembly (Davison *et al.,* 2009; Davison, 2002; Davison *et al.,* 2005). The encoding gene also appears to be related to a similar gene in T4 like bacteriophages. Within *Alloherpesviridae* there are 12 genes that are consistently conserved (van Beurden *et al.,* 2010). The more highly conserved regions with these genes can be used to design degenerate primers for PCR based targeted gene sequence amplification and for sequence comparisons (Hanson *et al.,* 2006; Kelley *et al.,* 2005; Waltzek *et al.,* 2009). This method was used by Waltzek *et al.* (2009) to amplify and sequence a portion of the DNA polymerase gene, and ATPase subunit of the terminase gene and allow phylogenic assessment of 13 fish and amphibian herpesviruses. This study showed that there were 2 monophyletic clades within *Alloherpesviridae* with AngHV1, CyHV1, CyHV2, and CyHV3 in Clade 1 and IcHV1, IcHV2, AciHV1, AciHV2, SalHV1, SalHV2, SalHV3, *Ranid herpesvirus 1* and *Ranid herpesvirus 2* in Clade 2 (Table 6.3-1) (Waltzek *et al.,* 2009). A later study highlighted the uniqueness of the ranid herpesviruses from the aforementioned clade 2 and suggested three subfamilies should be considered within the *Alloherpesviridae* (Doszpoly et al. 2011). Currently, the ICTV recognizes the following genera and associated species: *Batrachovirus* containing *Ranid herpesvirus 1* and *2*; *Cyprinivirus* containing *Cyprinid herpesvirus 1*, *2*, and *3* and *Anguillid herpesvirus 1*; *Ictalurivirus* containing *Ictalurid herpesvirus 1* and *2* and *Acipenserid herpesvirus 2*; and *Salmonivirus* containing *Salmonid herpesvirus 1*, *2* and *3* (ICTV, 2014).

Aquaculture	Virus name (abbreviation)	Common name (abbreviation)	Host(s)	Disease	Ref.
Eels	Anguillid HV 1 (AngHV1)	HV anguillae (HVA)	Japanese eel *Anguilla japonica* and European eel *A. Anguilla*	Hemorrhages of skin, fins, gills, liver	(Sano et al., 1990; van Beurden et al., 2010)
Cyprinids	Cyprinid HV 1 (CyHV1)	HV cyprini, carp pox HV, carp HV(CHV)	Common carp *Cyprinus carpio*	High losses in fry- exopthalmia hemorrhages, survivors have papillomas	(Sano et al., 1985; Sano et al., 1991)
	Cyprinid HV 2 (CyHV2)	Goldfish hematopoietic necrosis virus (GFHNV)	Goldfish *Carassius auratus* Prussian carp *Carassius gibelio*	High mortality all ages. Necrosis of hematopoietic tissue, spleen, pancreas, intestine	(Goodwin et al., 2006; Jung and Miyazaki, 1995) (Doszpoly et al., 2011)
	Cyprinid HV 3 (CyHV3)	Koi HV (KHV), carp nephritis and gill necrosis virus (CNGV)	Common carp	gill inflammation, hyperplasia, and necrosis, hematopoietic tissue necrosis, high mortality,18-26 °C, all ages	(Hedrick et al., 2000)
Catfish	Ictalurid HV 1 (IcHV1)	channel catfish virus (CCV), Channel catfish herpesvirus	Channel catfish *Ictalurus punctatus*	Kidney, liver and intestinal necrosis, hemorrhages, high mortality in young fish at above 27 °C	(Fijan et al., 1970; Wolf and Darlington, 1971)
	Ictalurid HV 2 (IcHV2)	Ictalurus melas HV (IcmHV)	Black bullhead *Ameiurus melas*	Kidney necrosis, hemorrhages, high mortality all ages	(Alborali et al., 1996; Hedrick et al., 2003)
Sturgeon	Acipenserid HV 1 (AciHV1)	White sturgeon HV 1	White sturgeon *Acipenser transmontanus*	diffuse dermatitis, high losses in juveniles	(Hedrick et al., 1991)
	Acipenserid HV 2 (AciHV2)	White sturgeon HV 2	White sturgeon Shortnose sturgeon	Epithelial hyperplasia, hemorrhages, paleness of internal organs, high mortality rate	(Watson et al., 1995) (LaPatra et al., 2014)

			Acipenser brevirostrum Lake sturgeon *Acipenser fulvescens* Siberian sturgeon *Acipenser baeri*		(Doszpoly & Waltzek, unpublished) (Shchelkunov et al., 2009) (Doszpoly & Shchelkunov, 2010)
Salmonids	Salmonid HV 1(SalHV1)	HV salmonis (HPV) Steelhead herpesvirus (SHV)	Rainbow trout *Oncorhynchus mykiss*	Mild disease low losses at 10 C. Adults- Virus shedding in ovarian fluid. No signs of disease.	(Wolf et al., 1978)
	Salmonid HV 2(SalHV2)	Oncorhynchus masou virus (OMV)	Cherry salmon *O. masou,*, coho salmon *O. kisutch*, sockeye salmon *O. nerka*, coho salmon *O. keta,* rainbow trout,	Viremia, external hemorrhages exophthalmia, hepatic necrosis with high losses in young. Survivors- oral papillomas, virus shed in ovarian fluid	(Kimura et al., 1981; Sano, 1976)
	Salmonid HV 3 (SalHV3)	Epizootic epitheliotropic disease virus (EEDV)	Lake trout *Salvelinus namaycush,* lake trout X brook trout *S. fontinalis* hybrids	Epithelial hyperplasia, hypertrophy, hemorrhages on eye and jaw. High losses in juveniles at 6-15 C	(Bradley et al., 1989; McAllister and Herman, 1989)
Cod	*Gadid herpesvirus* 1 (GaHV1)	Atlantic cod herpesvirus (ACHV)	Atlantic cod *Gadus morhua*	Hypertrophy of cells in gills. High losses in adults.	(Marcos-Lopez et al., 2012)
Pilchards (wild)	Pilchard HV		Australian pilchard *Sardinops sagax*	Acute losses with gill inflammation, epithelial hyperplasia and hypertrophy	(Crockford et al., 2005; Doszpoly et al., 2011; Hyatt et al., 1997; Whittington et al., 1997)
Tilapia	tilapia HV	Tilapia larvae encephalitis virus (TLEV)	Blue tilapia (*Oreochromis aureus*)	Encephalitis and high loses in larvae	(Shlapobersky et al., 2010)

Table 6.3-1. Alloherpesviruses and associated diseases that impact aquaculture and wild fisheries

Even though there is almost no sequence conservation between the families, there is structural and biological conservation. Structurally a herpesvirus virion contains a linear, double-stranded DNA genome packaged within an icosahedral nucleocapsid (capsid diameter ~100 nm) that is surrounded by a proteinaceous tegument layer and finally a host-derived envelope (Minson *et al.,* 2000). The linear genome is packed in an electron dense staining core in transmission electron microscopy. Cryoelectron microscopy and computer based image reconstruction demonstrates that the physical structure of the capsids of *Human herpesvirus 1* (*Herpesviridae*), *Ictalurid herpesvirus 1* (*Alloherpesviridae*) and *Ostreid herpesvirus 1* (*Malacoherpesviridae*) areremarkably conserved. All three are composed of 162 capsomers (150 hexons, 12 pentons) and each capsomer has a chimney like protrusion with an axial channel in the middle (Booy *et al.,* 1996; Davison *et al.,* 2005).

The replicative cycles of these viruses also appear to be conserved and have been best characterized in the *Herpesviridae* (Mettenleiter *et al.,* 2009). The glycoproteins on the envelope of virions attach to the cellular receptors, and envelope-membrane fusion releases the nucleocapsid and tegument into the cytoplasm. The nucleocapsid is transported to a nuclear pore

and the linear genome is released into the nucleus. The genome is then circularized in the nucleus and genes are expressed using the cell's transcription and translation machinery. Replication using the viral DNA polymerase generates branching concatamers. Empty capsids assemble in the nucleus then DNA is loaded into them and cleaved at specific sites using a portal complex. These newly formed nucleocapsids then bud out of the nucleus and are released into the cytoplasm where they acquire the tegument and then bud into the vesicles of the trans-Golgi network, acquiring the glycoprotein studded envelope, after which they are exocytosed as infectious virions.

The genomes contain over 70 genes. The smallest characterized alloherpesvirus, *Ictalurid herpesvirus 1*, is 134 kb and contains 76 deduced genes (Davison, 1992). The largest, *Cyprinid herpesvirus 3*, is 295 kb and contains 155 genes (Aoki *et al.,* 2007; Davison *et al.,* 2013). The gene structures are by in large simple with only one out of ten genes in the genome containing introns. However transciptome analysis demonstrates more frequent splicing and alternate splicing than what was predicted from genomic data (van Beurden at el., 2012). Also overlapping transcription is relatively rare and transcription occurs from both strands. These include genes that encode proteins involved in basic structural components of the virion, virus replicative functions, and a variety of genes that encode proteins which intricately interact with the host's physiology. A common characteristic of herpesviruses is they establish a latent state in the host after an initial replicative infection. During this latent state, no infectious virus is produced and very little viral protein is expressed. The triggers that regulate latency and productive replication are intricately tied to the physiology of the host. Most often these viruses will not infect or will establish a limited infection in the non-natural host, but occasionally herpesviruses can cause an extremely lethal disease in the non-natural host. For example, herpes B virus, *Cercopithecine herpesvirus 1*, is a common pathogen causing mild disease in macaque monkeys but is highly lethal to humans, causing over 70% mortality if untreated.

Even though herpesviruses have large complex genomes, the viral DNA is infectious and can be manipulated allowing for intricate reverse genetics experiments and recombinant vaccine development. One of the most promising developments is the establishment of infectious clones in bacterial artificial chromosomes (BAC) because genetic manipulation can be done using highly efficient bacterial systems. Recombinant BAC technology has been applied to IcHV1 and CyHV3 (Costes *et al.,* 2008; Kunec *et al.,* 2008; Kunec *et al.,* 2009).

The diseases produced by herpesviruses vary substantially and latent or recrudescent virus can cause different disease signs and outcomes than the primary infection. They undergo productive replication during primary infection. This often involves replication in a variety of cell types and may result in viremia and acute disease. Disease outcome from a primary infection is often controlled by the dose of the virus and the immune status of the fish. In the natural host, primary infection often results in mild disease and dissemination within a population but under optimal conditions that often occur in high density aquaculture several alloherpesviruses can cause severe epizootics with high morbidity and mortality. Typical lesions seen in primary herpesvirus infections include epithelial proliferation of the skin and gills (Figure 6.3-1.jpg C, D), hemorrhages (Figure 6.3-1.jpg E), ascites (Figure 6.3-1.jpg F), and kidney and liver necrosis. The most common manifestation of disease associated with recrudescent virus expression is dermal or epidermal cell proliferation (Figure 6.3-1.jpg A). These proliferative lesions are often unsightly but are rarely fatal. Cellular changes that are often seen with alloherpesviruses include hypertrophy and karyomegaly, margination of the chromatin with eosinophilic or light staining intranuclear inclusions (Figure 6.3-2.jpg A, C, D).

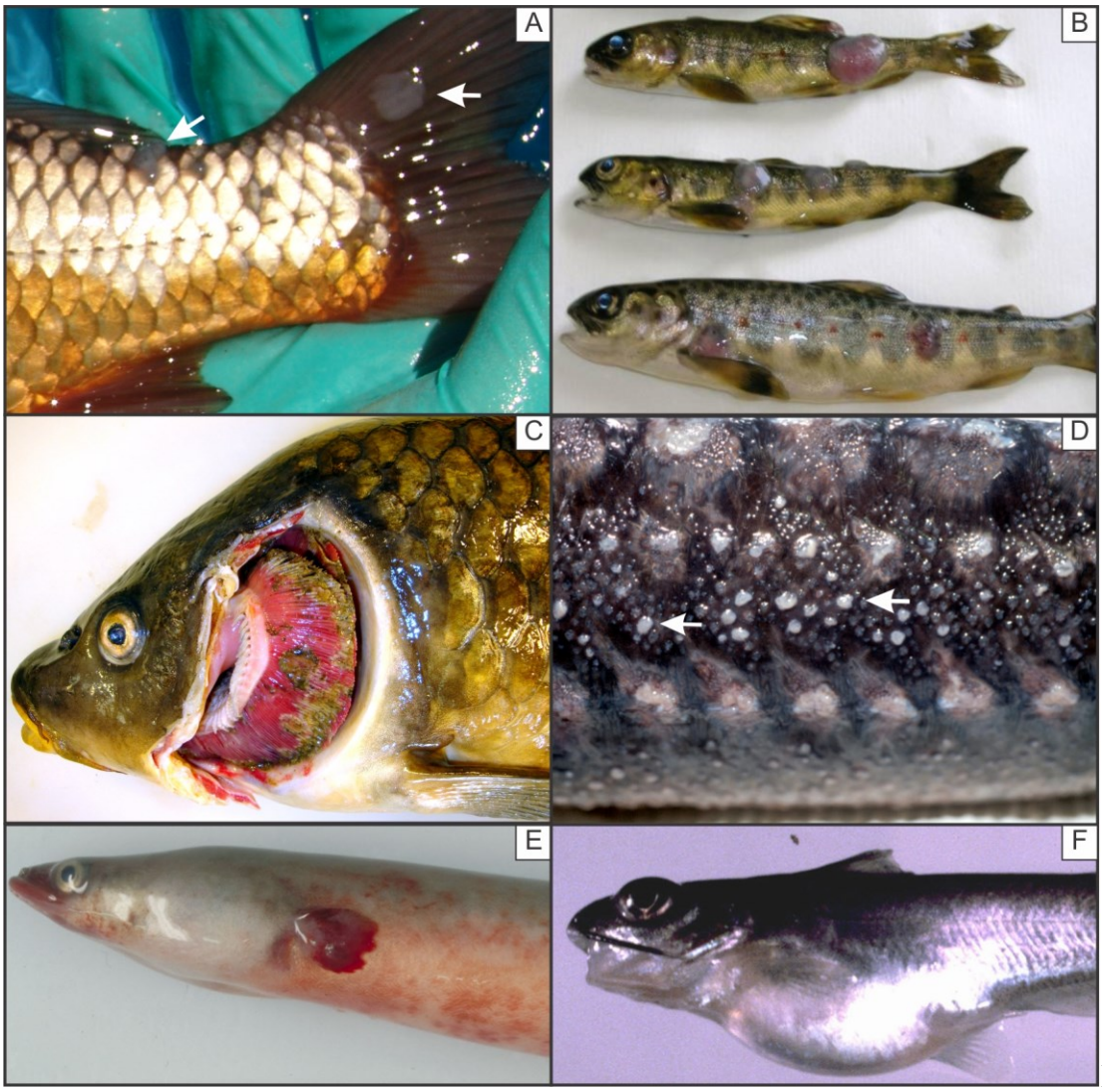

FIGURE 6.3-1.jpg: Images displaying external gross pathology induced by several alloherpesviruses that negatively impact aquaculture. (A) A common carp infected with *Cyprinid herpesvirus 1*displaying foci of epidermal hyperplasia (arrows). (B) Atlantic salmon infected with *Salmonid herpesvirus 4* displaying Atlantic salmon papillomatosis lesions. (C) Common carp infected with *Cyprinid herpesvirus 3* displaying necrotic gill lesions associated with koi herpesvirus disease. (D) Skin of white sturgeon infected with *Acipenserid herpesvirus 2* displaying white multifocal epithelial hyperplasia on skin (arrows). (E) European eel infected *with Anguillid herpesvirus 1* displaying petechial hemorrhages on the skin. (F) Channel catfish fingerling infected with *Ictalurid herpesvirus 1* displaying exophthalmia and abdominal distension associated with channel catfish virus disease. Image B from Doszpoly *et al.* (2013) by permission from Disease of Aquatic Organisms. Image C provided by Mark Okihiro. Image E from Haenen *et al.* (2002); CVI-NL no. 519348.

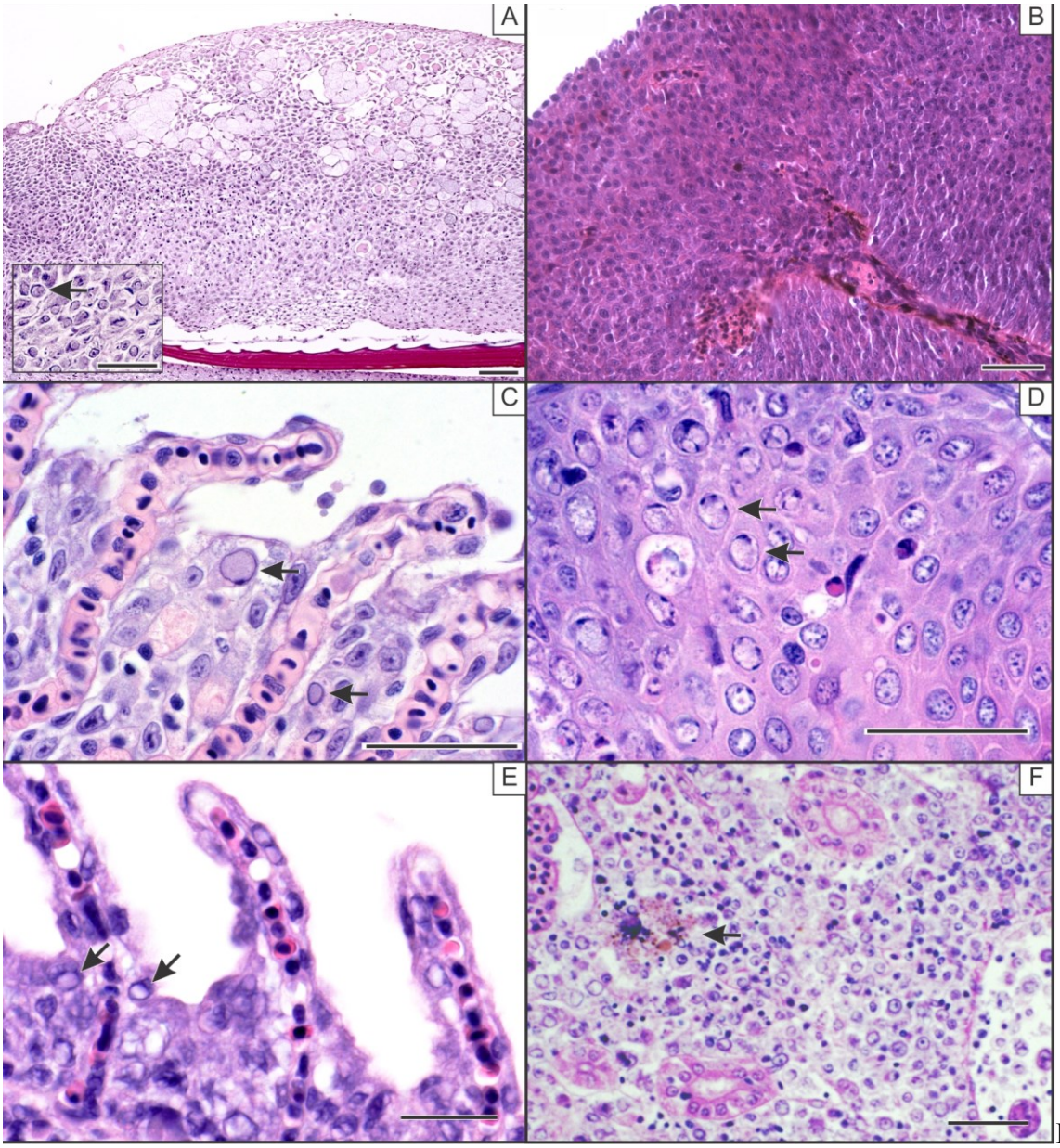

FIGURE 6.3-2.jpg: Histopathologic findings of alloherpesvirus infections in cultivated finfishes (H&E stain). (A) Koi infected with *Cyprinid herpesvirus 1* displaying epidermal hyperplasia of the integument. Inset shows higher magnification of the keratinocytes revealing karyomegaly, margination of chromatin, and pale eosinophilic nuclear inclusions (black arrow). (B) Skin papillomatosis in young Atlantic salmon, caused by SalHV4. (C) Koi infected with *Cyprinid herpesvirus 3* displaying severe branchitis including hyperplasia and fusion of the secondary lamellae. Gill epithelial cells displaying karyomegaly, margination of chromatin, and pale eosinophilic nuclear inclusions (black arrow). (D) Skin of white sturgeon, with keratinocytes displaying karyomegaly, marginated chromatin, and hypochromatic nuclei (black arrows), caused by AciHV2. (E) Gill of eel (*Anguilla anguilla*) infected with AngHV1, showing severe hyperplasia of the secondary lamellae and pale eosinophilic nuclear inclusions (black arrows) (F) Kidney of catfish with IcHV2, showing severe necrosis (black arrows) and lymphocytic

inflammation. Scale bars = (A) 50 μm, (B-F) 20 μm. Images C, D and F provided by Ronald Hedrick. Images B (Doszpoly *et al.* 2013) and E (Hangalapura et al. 2007) by permission from Disease of Aquatic Organisms.

Alloherpesviruses cause economically important diseases in catfish, carps, sturgeon, eel and salmonid aquaculture industries (Table 6.3-1). Additionally alloherpesviruses cause disease in amphibians, *Ranid herpesvirus* 1 and 2 (Davison *et al.,* 2006), and are likely the cause of disease in at least 13 other species of fish (the pathogens appear to be herpesviruses by EM but have not been genetically characterized) (Waltzek *et al.,* 2009).

6.3.2- Catfish Alloherpesviruses

There are two characterized alloherpesviruses that cause substantial losses in catfish aquaculture, *Ictalurid herpesvirus 1* (IcHV1, channel catfish virus) and *Ictalurid herpesvirus 2* (IcHV2, black bullhead virus). In a diagnostic setting, distinguishing between the two viruses is important because IcHV2 has not been detected in North America and it may be more virulent to channel catfish than IcHV1 (Hedrick *et al.,* 2003). The two virus species are phylogenetically close and are within the genus *Ictalurivius*with IcHV1 being the types species.

6.3.2.1. *Ictalurid herpesvirus 1*

Ictalurid herpesvirus 1 was first isolated in 1968 from populations of juvenile channel catfish in Alabama, Arkansas and Kentucky suffering massive mortalities due to a severe hemorrhagic disease termed channel catfish virus disease (CCVD) (Fijan *et al.,* 1970). This was the first intensively studied of the alloherpesviruses. It was first characterized in 1971 (Wolf and Darlington 1971).

Ictalurid herpesvirus 2 was first identified in aquacultured adult black bullheads in Italy in 1994 (Alborali *et al.,* 1996). This virus caused severe disease outbreaks that had a part in devastating the small black bullhead aquaculture industry in Italy (Roncarati *et al.,* 2014).

CCVD can cause high mortality, reduced growth and a predisposition to bacterial diseases (Plumb, 1978). Severe outbreaks of CCVD are often associated with environmental stress and crowding. Almost all CCVD cases occur in heavily stocked channel catfish fingerling ponds during warm summer months. The optimum temperature for disease progression is 27 °C or higher (Plumb, 1973a). Although older fish can be affected (Hedrick *et al.,* 1987), natural outbreaks almost exclusively occur in catfish that are less than one year of age. In experimental challenge trials very young fish are more resistant to the virus and become more sensitive as they age to 60 day post hatch. Furthermore the resistance was correlated to the maternal parent's CCV neutralizing antibody titer suggesting that maternal transfer of antibodies may influence early susceptibility (Hanson *et al.,* 2004). In the catfish producing region of United States, the optimum temperatures for CCVD occur from July through September when fingerlings are less than 4 months of age. Under these conditions over 90% of the population may die in less than 2 weeks from the first signs of disease, yet most IcHV1 endemic populations will experience no obvious CCVD. Losses due to CCVD on fingerling production operations can vary substantially from year-to-year without obvious environmental cues or changes in management.

Once catfish become infected with IcHV1, either through vertical or horizontal transmission, they become lifelong carriers. The first evidence of vertical transmission was the

finding that CCVD affected fry in one of the first documented outbreaks were offspring from brood fish that had high IcHV1 neutralizing titers (Plumb, 1973b). Detection and/or experimental induction of IcHV1 recrudescence has been difficult. In one experiment IcHV1 antigen (but no culturable virus) was detected in ovarian tissue of immunosuppressed adult fish immediately after spawning *(Plumb et al.,* 1981). Later IcHV1 were cultured from adult catfish during the winter when temperatures were below 8 ºC and this isolation was enhanced if the fish were immunosupressed with dexamethazone and the leukocytes co-cultured with CCO cells (Bowser *et al.,* 1985). However, conditions needed for recrudescence appear to be specific, as several researchers have tried to induce IcHV1 production in carrier fish using dexamethazone with little success (Arnizaut and Hanson 2011). Because there are no infectious virus produced during latency, the detection of carrier fish requires indirect evaluation (antibody detection) (Amend and McDowell 1984; Crawford *et al.,* 1999) or the detection of latent viral DNA. The detection of IcHV1 DNA in asymptomatic adult channel catfish was first done using Southern blot analysis (Wise *et al.,* 1985). This method was used to demonstrate that offspring from IcHV1 carriers were positive for the IcHV1 genome (Wise *et al.,* 1988). Subsequently several IcHV1 specific PCR assays were developed and used to evaluate carrier fish (Baek and Boyle 1996; Boyle and Blackwell 1991; Gray *et al.,* 1999).

Vertical transmission allows IcHV1 to be effectively maintained in a population. IcHV1 specific PCR on recently hatched fry from 5 representative fingerling operations demonstrated latent carrier status in 10–20 percent in each population of the sampled fish (Thompson *et al.,* 2005). These farms represented approximately 20% of the commercial catfish fingerling production. Therefore, it can be assumed that IcHV1 is endemic in most aquaculture populations of channel catfish in the Southeastern United States. Furthermore, by evaluating incidence of latent IcHV1 in endemic fingerling populations over time showed an increase even though no CCVD was detected. This suggests that horizontal transmission occurs in the populations without overt disease (Thompson *et al.,* 2005). When evaluating the use of PCR to detect and eliminate carrier brooders we found that numbers of carrier offspring were reduced but it did not eliminate vertical transmission to the subsequent generation (unpublished data). This suggests that the PCR detection of carrier brood stock was not sensitive enough for effective culling.

Channel catfish with acute CCVD may swim disoriented or hang at the surface or pond bank. These fish often display mild hemorrhaging of the fin bases, exophthalmia, and distended abdomen (Figure 6.3-1.jpg F). Internally there is clear or yellow ascites and a swollen posterior kidney. The posterior kidney is the primary target tissue during acute CCVD. Early in infection this organ displays extensive edema, inflammation and necrosis of hematopoietic tissue and tubules. This is followed by focal necrosis, hemorrhage and edema of the liver and gastrointestinal tract and necrosis of pancreatic tissue, congested spleen and focal areas of hemorrhage in musculature (Plumb *et al.,* 1974; Wolf *et al.,* 1972). Occasionally margination of the chromatin and eosinophilic intra-nuclear inclusion may be seen (Figure 6.3-2.jpg B) but these cellular changes are not as common as with other herpesviruses.

The innate immunity of channel catfish to IcHV1 likely plays and important role in CCVD resistance. Exposure of catfish to poly I:C or catfish reovirus as inducers of the innate immune system make channel catfish resistant to experimental CCVD (Chinchar *et al.,* 1998; Plant *et al.,* 2005). Also natural killer like cells from naïve channel catfish have been shown to kill IcHV1 infected cells (Hogan *et al.,* 1996).

The adaptive immune response of channel catfish to a productive IcHV1 infection is protective and long-lasting. Once a population of channel catfish recovers from CCVD or an

IcHV1 positive population has been through the first summer, they become resistant to experimental infection (Arnizaut and Hanson 2011; Plumb, 1973b). The protective components of this immunity have not been clearly defined but the antibody response is important. Passive immunity can be conferred to channel catfish if serum from a CCVD survivor is transferred to a naïve recipient (Hedrick and McDowell 1987). IcHV1 antibodies can persist for over 2 years after exposure (Bowser and Munson 1986; Hedrick *et al.,* 1987). The IcHV1 specific antibodies in the serum of older fish increase during summer months (Bowser and Munson 1986), and may be boosted by periodic virus recrudescence (Arnizaut and Hanson 2011). Furthermore, early-life-stage resistance was correlated to neutralizing antibodies in the maternal parent suggesting that maternal transfer of antibodies may play a role in preventing disease outbreaks (Hanson *et al.,* 2004).The immunity and control of IcHV1 latency in fingerlings that are carriers due to vertical transmission of the virus is not understood. We have found the carrier rate within from positive spawns are 40-75% of the fish (Hanson *et al.,* 2004) and these carrier fry are susceptible to CCVD when exposed to exogenous IcHV1 (Hanson *et al.,* 2004; Kancharla and Hanson 1996). However, these fish do not express culturable virus under experimental conditions even when exposed to high temperatures and/or injected with dexamethasone (Arnizaut and Hanson 2011).

6.3.2.2. *Ictalurid herpesvirus 2*

Much less has been reported about the biology of IcHV2. Natural outbreaks of acute herpesvirus disease occurred in adult black bullheads in Italy at above 20°C. The disease was similar to CCVD in causing disoriented swimming scattered hemorrhages and extensive necrosis of the kidney tissue (Alborali *et al.,* 1996). This virus was shown to be antigenically and genetically distinct from IcHV2 and it has been experimentally shown to be capable of causing high losses in channel catfish fry and juveniles at relatively lower temperatures (24 °C) (Hedrick *et al.,* 2003). Partial genomic sequencing reveals that IcHV2 is closely related to IcHV1 and they are grouped in genus *Ictalurivirus* (Doszpoly *et al.,* 2008, 2011; Waltzek *et al.,* 2009).

The most common method for diagnosing CCVD is a combination of clinical signs, pathology and cell culture. IcHV1 produces rapidly developing cytopathic effect (CPE) on cultures of channel catfish ovary or brown bullhead cell lines. The CPE consists of syncytium formation and subsequent contraction forming cellular with radiating spindles to attachments on the plate or neighboring cells. For cell cultures, the use of whole fry (up to 2 cm), viscera from small fingerlings (2-5 cm) or posterior kidney from larger juveniles is suggested. When a 0.5 ml inoculum of a 1:10 dilution of tissue from a CCVD affected fish is inoculated into a 12 cm^2 flask of cells, CPE is usually seen within 2 days at 30°C.

Similar clinical signs, pathology and CPE are seen with IcHV2. However, IcHV2 has a broader cell host range; it will produce CPE on bluegill fry (BF2) cell and the EPC (fathead minnow) cell line; whereas, IcHV1 will not (Alborali *et al.,* 1996).

Molecular means are suggested for confirmation and are required for detecting carriers. Several traditional PCR assays are available to detect IcHV1 (Baek and Boyle 1996; Boyle and Blackwell 1991; Gray *et al.,* 1999; Thompson *et al.,* 2005). These methods are highly sensitive and effective in detecting latent virus. Their specificity has been tested against other alloherpesviruses but not between IcHV1 and IcHV2. Recently IcHV2 specific hydrolysis probe (TaqMan) PCR was developed and tested against multiple isolates of IcHV1 with no false positives (Goodwin and Marecaux 2010). Hydrolysis probe assays that are specific for two

genotypes of IcHV1 can also be used to distinguish these two species (Hanson, *in press*).Because IcHV1 is widespread in the catfish industry, there is little effort to contain or eliminate the virus. Instead catfish fingerling producers primarily use management to prevent disease outbreaks. Primarily this involves minimizing handling and crowding of fish during warm summer months. If CCVD occurs in the hatchery, the fry are generally depopulated and facility sterilized before using again. Once CCVD does break every effort should be made to contain the infection because in can spread to other ponds. Experimental vaccines include subunit vaccines (Awad *et al.,* 1989), DNA vaccines (Nusbaum *et al.,* 2002) and live attenuated virus vaccines (Noga and Hartmann 1981; Vanderheijden *et al.,* 2001; Zhang and Hanson 1995).

The black bullhead aquaculture industry was devastated by IcHV2 and has since retreated to facilities that could rear the fish at below 24 °C (Roncarati *et al.,* 2014).

6.3.3- Carp Alloherpesviruses

There are three characterized alloherpesviruses of carp that cause important diseases in aquaculture and the ornamental fish trade. These are *Cyprinid herpesvirus 1* (CyHV1, carp pox herpesvirus), *Cyprinid herpesvirus 2* (CyHV2, goldfish hematopoietic necrosis virus), and *Cyprinid herpesvirus 3* (CyHV3, koi herpesvirus). All three are closely related in sequence and genome structure and are considered within the genus *Cyprinivirus* (Davison *et al.,* 2013; Waltzek *et al.,* 2009; Waltzek *et al.,* 2005). The genomes of all three have been sequenced; all are over 290 kb in length with CyHV3 being the largest known herpesvirus genome at 295 kb (Aoki *et al.,* 2007; Davison *et al.,* 2013). These pathogens are restricted in host range, CyHV1 and CyHV3 are only known cause disease in common carp strains and CyHV2 is only known cause disease in goldfish (*Carassius auratus*) and the closely related Prussian carp, *Carassius gibelio* (Danek *et al.,* 2012; Doszpoly *et al.,* 2011b). Also, all three are relatively recalcitrant to cell culture and when they do replicate CPE develops slowly.

6.3.3.1. *Cyprinid herpesvirus 1*

The cause of a disease known for centuries as carp pox, characterized as a benign papilloma of common carp, was first cultured and shown to be a herpesvirus by Sano *et al.* (1985a,b). CyHV1 can cause systemic disease with high mortality in fry (Sano *et al.,* 1991). These fish displayed aberrant swimming behavior, distended abdomens, exophthalmia, darkened skin pigmentation, and hemorrhages on the operculum and abdomen. Histological changes seen in moribund fry included necrosis of the liver, kidney and intestines with some cells displaying Cowdry type A nuclear inclusion bodies. Grass carp, crucian carp and willow shiners were refractive to infection. Tracing viral antigen expression using indirect fluorescent antibody (IFA) staining demonstrated high expression in the viscera early during the primary infection and a progression to the epidermis late in infection (Sano *et al.,* 1991). There was an obvious effect of temperature on virus replication and pathogenicity. Evaluating virus production at 10, 15, 20, 25 and 30 °C in cell culture showed the virus replication was highest at 15 and 20 °C, moderate at 10 and 25 °C and undetectable at 30 °C. In challenge studies highest losses occurred at 15 °C, moderate losses occurred at 20 °C and no mortality occurred at 25 °C. The survivors of the immersion challenge and adults injected with CyHV1 developed a high incidence of papillomas (Figure 6.3-1.jpg A). Histologically many of the affected cells display marginated chromatin and pale eosinophilic intranuclear inclusions (Figure 6.3-2.jpg A). These epidermal proliferations developed months after primary exposure and expressed culturable virus and were positive for

viral antigens by IFA staining (Sano *et al.,* 1991). In experimentally challenged fish the papillomas developed and persisted at 15 °C, and developed and began to regress at 20 °C and 25°C. When the papillomas were allowed to develop at lower temperatures and then the temperature raised to 30 °C, they rapidly regressed (Sano *et al.,* 1993). Often the papillomas would reoccur several months after regression (Sano *et al.,* 1991). *In situ* hybridization studies demonstrated the presence of latent virus in cranial and spinal nerves and subcutaneous tissue when not viral antigens or infectious virus were present (Sano *et al.,* 1993). Whole genome sequencing revealed CyHV1 encodes the oncogene JUNB and this may be partially responsible for tumor development (Davison *et al.,* 2013). The disease has a worldwide distribution and its impact on commercial aquaculture is difficult to assess. The tumors are unsightly and affect marketability of ornamental fish and fish sold live or whole for food but high mortality due to primary infection has not been reported as a major concern in aquaculture systems. Presumptive diagnosis of CyHV1 infection can be done by observation of papillomas on common carp that regress at elevated temperatures. The diagnosis can be confirmed by testing infected tissues by IFA (Sano *et al.*, 1991) or by PCR (Viadanna et al. in submission). Also, virus isolation can be attempted on EPC or Fat head minnow (FHM) cell lines at 20 °C which develop CPE at about 5 days post inoculation consisting of vacuolation and rounding cells.

6.3.3.2. *Cyprinid herpesvirus 2*

A severe disease of cultured goldfish designated as herpesviral hematopoietic necrosis (HVHN) was first described from cases in Japan in 1992 and 1993 by Jung and Miyazaki (1995). The outbreaks occurred in the spring and fall in goldfish of all ages with high mortality. Affected fish were lethargic but showed no external lesions other than occasional pale patches in the gills. Internally the swollen and pale kidney and splenomegaly were the most notable signs. Histopathology consisted primarily of necrosis of the hematopoietic tissues of the anterior and posterior kidney and the splenic pulp. Other lesions included focal necrosis and inflammation of the lamina propria and submucosa of the intestine and occasional pancreatic necrosis (Jung and Miyazaki 1995). Filtrates from spleen and kidney produced CPE on FHM and EPC cells at 20 °C consisting of vacuolation and cell rounding but the virus disappeared after 3-4 passages. Other outbreaks were reported in the USA (Goodwin *et al.,* 2006; Groff *et al.,* 1998; Neiger *et al.*, 2015), Australia (Stephens *et al.,* 2004), the UK (Jeffery *et al.,* 2007) Hungary (Doszpoly *et al.,* 2011) and China. These studies found similar pathology with the additional observation of diffuse branchial hyperplasia, leukocyte infiltration and necrosis. Also others were unsuccessful in culturing the virus except Jeffry *et al.* (2007) was able to demonstrate CPE on the Koi Fin (KF1) cell line at 20 °C. A goldfish cell line (Li and Fukuda 2003) has been used to propagate CyHV2 allowing for additional challenge studies (Hedrick *et al.,* 2006) and the ability to sequence the genome (Davison *et al.,* 2013).

The disease appears restricted to goldfish and closely related species such as Prussian carp. Common carp (Hedrick *et al.,* 2006; Jung and Miyazaki 1995) and koi x goldfish hybrids are resistant to HVHN (Hedrick *et al.,* 2006). Prussian carp are very sensitive with several outbreaks occurring in aquacultured and wild populations (Danek *et al.,* 2012; Doszpoly *et al.,* 2011b; Luo *et al.,* 2013).

Given the importance of HVHN to goldfish production and the ornamental fish trade and the recalcitrant nature to CyHV2 to cell culture, two PCR assays have been established and validated. These include conventional PCR (Waltzek *et al.,* 2009) and hydrolysis probe (TaqMan) quantitative PCR (Goodwin and Marecaux 2010; Goodwin *et al.,* 2006). The sensitive

qPCR was used to demonstrate viral genome presence in health adult goldfish suggesting latency (Goodwin *et al.,* 2006).

The options for HVHN disease prevention is limited to avoidance, and possibly the use of resistant fish. The qPCR assay detected latent CyHV2 in non-diseased fish (Goodwin *et al.,* 2006) so the use of this method in screening populations during broodfish selection would likely reduce the potential for HVHN. Also in a problematic environment the use of resistant fish may be warranted such as the Koi x goldfish hybrid (Hedrick *et al.,* 2006). However, whether fish become carriers and shed virus after CyHV2 exposure has not been evaluated. Ito & Maeno (2015) developed a formalin inactivated vaccine that was effective against CyHV2 in goldfish; but, future research is needed to determine the length of protection.

6.3.3.3. *Cyprinid herpesvirus 3*

Since 1997, disease causing extremely high mortality in all ages of common carp (many of the first cases occurred in koi carp) has been identified in USA, Europe and Asia (Bretzinger *et al.,* 1999; Hedrick *et al.,* 1999). The disease was recognized by severe gill hyperplasia and necrosis. Hedrick *et al.* (2000) were able to isolate the causative agent in cell culture and reproduce the disease experimentally. Based on TEM of the isolated virus, infected tissues and infected cell cultures the virus was classified as a herpesvirus and designated as koi herpesvirus (KHV) with the disease designated Koi herpesvirus disease KHVD (Hedrick *et al.,* 2000). Based on partial genome sequence data it was apparent that KHV was closely related to CyHV1 and CyHV2, and therefore was renamed *Cyprinid herpesvirus 3* (Waltzek *et al.,* 2005). Genome sequencing of CyHV3 and later CyHV2 and CyHV1 confirmed that these viruses were very closely related supporting the designation of the three in the *Cyprinivirus* genus (Aoki *et al.,* 2007; Davison *et al.,* 2013).

The disease is highly transmissible and causes high mortality in all strains of common carp both in the wild and in aquaculture, when the temperature is between 18 and 28 °C, with the optimum being 22-26 °C. Affected fish are lethargic, and display endophthamia, and white patches on the skin and gills due to epithelial hyperplasia and necrosis (Figure 6.3-1.jpg C). The gills filaments often will be visibly swollen, with necrosis and sloughing occurring at the tips. Secondary bacterial infection such as *Flavobacterium columnare* is common in affected fish. Internally the fish commonly have swollen kidneys and splenomegaly. Histopathology demonstrates epithelial hyperplasia, hypertrophy and necrosis in the affected gill and epidermal tissue and occasionally epithelial cells within the affected areas will have enlarged nuclei with marginated chromatin surrounded a lighter staining central region (Figure 6.3-2.jpg C). There is also necrosis and inflammation in the hematopoietic tissues of the kidneys, the spleen, liver and lamina propria of the intestine (Hedrick *et al.,* 2000).

The disease is restricted to *Cyprinus carpio* and Koi x crucian carp (*Carasius carasius*) hybrids with goldfish x common carp hybrids being somewhat susceptible (Bergmann *et al.,* 2010; Hedrick *et al.,* 2006). However other species may become infected and shed KHV and thus, may function as unapparent carriers on aquaculture facilities (El-Matbouli *et al.,* 2007; El-Matbouli and Soliman 2011; Fabian *et al.,* 2013; Kempter and Bergmann 2007; Sadler *et al.,* 2008).

Although CyHV3 can grow on several common carp cell lines and produce vacuolating CPE after 4-6 days, this is not considered reliable. Instead it is recommended that a presumptive diagnosis of KHVD be made based on clinical signs and pathology with confirmation done using CyHV-3 PCR (OIE, 2015). There are several PCR methods published; the OIE manual suggests

the use of the assay developed by Bercovier *et al.* (2005) or that of Yuasa *et al.* (2005) or the hydrolysis probe based qPCR developed by Gilad *et al.* (2004). Bergmann *et al.* (2010) compared several molecular assays including nested and semi nested PCRs and found that of the OIE suggested traditional assays the Bercovier assay detected the lowest levels, and the Gilad qPCR assay was by far much more sensitive than either. Also, they found that sequence variation could result in some false negatives in case samples. Engelsma et al. (2013) developed a nested PCR that detects all CyHV1-3 including newly discovered divergent strains of CyHV3.

Current KHVD management is largely based on either avoidance or the use of vaccines. If possible avoidance is generally the best option but this requires vigilant screening of fish stocks, thorough disinfection of infected facilities and the use of a non-infected source of water. Also, non-carp sources of the virus including residual virus present in invertebrates should be considered (Fabian *et al.,* 2013; Kempter and Bergmann 2007; Kielpinski *et al.*, 2010). If KHVD does occur elevating the temperature will reduce losses but survivors will carry the virus and subsequent shedding will likely result in new KHVD outbreaks in any naïve fish that are added to the facility. The use of vaccines are also promising, one vaccine (KoVax) is a natural attenuated live vaccine that is commercially produced (Perelberg *et al.,* 2005; Ronen *et al.,* 2003; Weber *et al.,* 2014). Another is a designed recombinant CyHV3 vaccine developed using BAC technology (Boutier *et al.,* 2015).

6.3.4 Eel Alloherpesvirus- *Anguillid herpesvirus 1*

In 1985, Japanese eels (*Anguilla japonica*) and European eels *(Anguilla anguilla*) farmed in recirculation systems in Japan suffered from increased mortality rates (Sano *et al.,* 1990). Using a cell line derived from the kidneys of an eel (EK-1) (Chen *et al.,* 1982), a virus was isolated from the affected eels (Sano *et al.,* 1990). By TEM typical herpesvirus-like virions were observed, and the virus was tentatively named herpesvirus anguillae. At the same time, a herpesvirus was also reported in eels in Europe (Békési *et al.,* 1986), but isolation was only successful after the importation of the susceptible EK-1 cell line more than a decade later (Davidse *et al.,* 1999). Serologically and genetically, herpesvirus isolates from eels from European and Asia are highly similar, and are regarded as a single virus species *Anguillid herpesvirus 1* (AngHV1), that belongs to the genus *Cyprinivirus* (Chang *et al.,* 2002; Rijsewijk *et al.,* 2005; Waltzek *et al.,* 2009;ICTV, 2014; van Beurden *et al.,* 2010).

Since its first discovery in the mid-1980s, AngHV1 has been reported repeatedly in Japanese and European eels in warm water culture systems in East Asia, and in cultured and wild European eels in Europe (reviewed in Van Beurden *et al.,* 2012). As such, AngHV1 has been suggested to play a contributory role in the decline of the endangered wild European eel stocks (Haenen *et al.,* 2012). In addition, AngHV1 has been detected by PCR in another freshwater eel species, the namely American eel (*Anguilla rostrata*) cultured in Taiwan and imported from China to Poland (Shih, 2004;Kempter *et al.,* 2014). Upon experimental infection with eel herpesvirus in Formosa, Ueno *et al.* (1992) reported high mortality of common carp, but this observation has not been confirmed by others. So far, the host range of AngHV1 seems to be restricted to freshwater eels of the genus *Anguillidae*, in particular the Japanese and European eel, and putatively the American eel. Eels of all ages are susceptible, although for most experimental infections smaller eels were used.

In high-density intensive recirculation production systems, AngHV1 outbreaks have been associated with stress-factors, such as poor water quality or eel sorting (Haenen *et al.,* 2002; van Beurden *et al.,* 2012). Progress of the disease shows a clear temperature dependency in culture

systems, with clinical symptoms being most severe at 25-26 °C. Depending on the circumstances, morbidity can be high resulting in decreased growth rates, whereas mortality rates are usually no more than 10% (Davidse *et al.,* 1999; Haenen *et al.,* 2002; Sano *et al.,* 1990). In case of complicating factors, such as secondary infections, mortality rates can be considerably higher (Chang *et al.,* 2002). Clinical symptoms may vary among and within outbreaks, and include apathy, varying degrees of fin and skin hemorrhages (particularly in the head, beak, operculum and caudal abdominal surface) (Figure 6.3-1f), and congestion of the gills (van Beurden *et al.,* 2012). Gross pathological findings may range from clinically normal to a pale spleen, a pale and hemorrhagic liver, a distended gall bladder, swollen kidneys, and ascites (Chang *et al.,* 2002; Davidse *et al.,* 1999; Haenen *et al.,* 2002; Lee *et al.,* 1999; Sano *et al.,* 1990; Ueno *et al.,* 1992). In general, experimental infections demonstrated comparable symptoms, although to a more limited degree and generally without mortality.

Diagnosis of AngHV1 should not be solely based on clinical symptoms and gross pathology, as skin and fin hemorrhages are rather non-specific in eels (Haenen *et al.,* 2010), and two pathogenic RNA viruses, namely Eel virus European (EVE) and Eel virus European X (EVEX), may cause a similar hemorrhagic disease (van Beurden *et al.,* 2012). Descriptions of histopathological changes induced by AngHV1 infection are limited, but include skin necrosis, gill hyperplasia, inflammation, necrosis and lamellar fusion, and liver inflammation and necrosis (Armitage *et al.,* 2014; Hangalapura *et al.,* 2007; Lee *et al.,* 1999; Sano *et al.,* 1990). An immunohistochemistry assay and an *in situ* hybridization assay have been developed for the detection of AngHV1 in histological slides *(Chang et al., 2002; Hangalapura et al., 2007; Shih et al.,* 2003). Virus isolation by inoculation of suspensions of the gills and a pool of internal organs (including liver, kidney and spleen) on a susceptible cell line remains the golden standard. AngHV1 can successfully be isolated and propagated in several cell lines of eel origin, but the most widely used is the EK-1 cell line (Chen *et al.,* 1982). The optimal virus growth temperature for AngHV1 in EK-1 cells is around 25 °C (Sano *et al.,* 1990). Upon the observation of cytopathic effect, characterized by giant cell formation, immunological assays can be used to identify the causative virus as AngHV1 (Davidse *et al.,* 1999; Varvarigos *et al.,* 2011). More recently, molecular assays have been developed to demonstrate the presence of AngHV1 genetic material in positive cell cultures or directly in organs of affected eels. These include three conventional PCR assays targeting the DNA polymerase gene (Armitage *et al.,* 2014; Rijsewijk *et al.,* 2005; Shih, 2004), and a qPCR targeting a conserved capsid protein gene (van Beurden *et al.,* 2015).

No vaccines or antivirals against AngHV1 are commercially available. In addition, experimental AngHV1 infection studies have suggested the possibility of stress-dependent reactivation of latent virus (van Nieuwstadt *et al.,* 2001). Losses due to AngHV1 infection in eel farms can therefore only be reduced by management practices, i.e. quarantine measures, stress reduction and water temperature adjustment (Haenen *et al.,* 2002). AngHV1 infection proceeds horizontally, hence hygienic measures between infected and non-infected culture units should be taken in order to avoid cross-contamination. Stress factors play an important role in the development of AngHV1 associated disease, and should be minimalized in case of an outbreak. These include avoidance of handling infected eels, optimization of water quality, and treatment of secondary parasitic or bacterial infections. Finally, at eel farms with a regulated water temperature, lowering the water temperature to below 22 °C may reduce the mortality rates and clinical symptoms due to AngHV1 infection (Haenen *et al.,* 2002).

6.3.5. Sturgeon Alloherpesviruses

Two alloherpesviruses, *Acipenserid herpesvirus 1* (AciHV1) and *Acipenserid herpesvirus 2* (AciHV2), have been characterized from wild and farm-reared sturgeon in North America and Europe. Although AciHV1 has occasionally been associated with disease in farm-reared sturgeon, AciHV2 has repeatedly caused significant epizootics in both North American and European sturgeon farms (Doszpoly and Shchelkunov 2010; Hedrick *et al.,* 1991; Kelley *et al.,* 2005; Kurobe *et al.,* 2008; Watson *et al.,* 1995). Serologic and phylogenetic evidence suggests that these sturgeon alloherpesviruses are only distantly related (Kelley *et al.,* 2005; Kurobe *et al.,* 2008; Waltzek*et al.,* 2009; Watson *et al.,* 1995). Phylogenomic analyses have revealed that AciHV2 is most closely related to IcHV1 and IcHV2 in the genus *Ictalurivirus* (Doszpoly *et al.,* 2008, 2011; Kelley *et al.,* 2005; Waltzek *et al.,* 2009).

6.3.5.1. *Acipenserid herpesvirus 1*

Acipenserid herpesvirus 1 was isolated from juvenile white sturgeon (*Acipenser transmontanus*) experiencing increased mortality on a farm in California, USA (Hedrick *et al.,* 1991). Years later AciHV1 was again isolated from farmed white sturgeon in California and for the first and only time in Europe on an Italian farm (Kelley *et al.,* 2005; Kurobe *et al.,* 2008). Experimental infection trials resulted in 35% cumulative mortality among juvenile white sturgeon (Hedrick *et al.,* 1991). Few gross external or internal signs were observed; however, microscopic examination of hematoxylin and eosin stained tissues revealed a diffuse hyperplastic dermatitis. Affected keratinocytes appeared swollen with slightly enlarged nuclei displaying marginated chromatin and occasional nuclear inclusions (Hedrick *et al.,* 1991).

Diagnosis of AciHV1 is best accomplished through cell culture followed by ancillary testing. Viral replication occurs on white sturgeon skin cells (WSSK-1) at temperatures between 10-20°C with the optimum being 15°C (Hedrick *et al.,* 1991). Cytopathic effect includes the formation of syncytia 2-4 days post inoculation at 15°C. Analysis of infected tissues or WSSK-1 cells by TEM reveals naked hexagonal nucleocapsids (110 nm in diameter) in the nucleus and enveloped particles surrounded by a coarse electron-dense tegument layer (230 nm in diameter) within cytoplasmic vacuoles (Hedrick *et al.,* 1991). Although AciHV1 specific molecular diagnostic tests have not been developed, PCR amplification and sequencing of the viral DNA polymerase or terminase genes has been used to confirm the presence of AciHV1 (Kelley *et al.,* 2005; Kurobe *et al.,* 2008; Waltzek *et al.,* 2009). To date, no vaccines or antiviral drugs have been tested against AciHV1.

6.3.5.2. *Acipenserid herpesvirus 2*

Acipenserid herpesvirus 2 was first detected in adult white sturgeon in California, USA, during routine sampling. The virus was isolated on the white sturgeon spleen (WSS-2) cell line from the ovarian fluid of a healthy adult sturgeon (Watson *et al.,* 1995). Shortly thereafter, the virus was again isolated from the skin of a subadult white sturgeon from a farm-reared population experiencing erosive skin lesions associated with elevated morbidity and mortality. Other North American detections of AciHV2 include wild white sturgeon from Idaho and Oregon (Kelley *et al.,* 2005; Kurobe *et al.,* 2008) farm-reared shortnose sturgeon (*Acipenser brevirostrum*) from north-eastern Canada (Kelley *et al.,* 2005; Kurobe *et al.,* 2008; LaPatra *et al.,* 2014) and managed lake sturgeon (*Acipenser fulvescens*) from Wisconsin (Waltzek and Doszpoly, unpublished). Experimental bath infection trials resulted in up to 80% cumulative mortality among juvenile white sturgeon after 6 weeks (Watson *et al.,* 1995). Affected juveniles

displayed erratic swimming behaviour, lethargy, raised spherical mucoid skin lesions on the head and pectoral fins (Figure 6.3-1d), and hyperemia of the ventral scutes, mouth, and anus (Watson *et al.,* 1995). Lower dosage challenge studies failed to induce clinical signs and mortality; however, subclinical infections were detected 70 days post infection (Watson *et al.,* 1995). Microscopic examination of hematoxylin and eosin stained tissues from moribund juveniles revealed diffuse hyperplastic dermatitis with marked secondary erosions of the integument, oropharyngeal mucosa, and respiratory mucosa. Affected keratinocytes displayed karyomegaly, marginated chromatin, and hypochromatic nuclei (Watson *et al.,* 1995).

The first detection of AciHV2 in Europe was a severe epizootic that occurred on a Russian farm rearing Siberian sturgeon (*Acipenser baeri*) fingerlings (Shchelkunov *et al.,* 2009). The disease predominantly affected Siberian sturgeon but was also isolated from Bester (*Acipenser ruthenus x Huso huso* hybrid) juveniles. Similar to AciHV2 epizootics in North American sturgeon (LaPatra *et al.,* 2014; Watson *et al.,* 1995), the Siberian sturgeon herpesvirus displayed a clear tropism for the integument resulting in focal hyperplastic and hemorrhagic lesions. Siberian sturgeon fingerlings experienced 100% cumulative mortality by 14 days following bath exposure. Two year old Siberian sturgeon experienced a less severe (36% cumulative mortality) and protracted disease course (40 post bath exposure).

Diagnosis of AciHV2 is best accomplished through cell culture followed by ancillary testing. Strains of AciHV2 have been isolated on cell lines derived from white sturgeon (skin; WSSK-1, spleen; WSS-2, liver; WSLV, gonad; WSGO) and Siberian sturgeon (organ, SSO-2; fin; SSF-2) cell lines at 15°C (Shchelkunov *et al.,* 2009; Watson *et al.,* 1995). Cytopathic effect involves the focal destruction of the monolayer with grape-like clusters of cells surrounding infective foci (day 3-7) and progresses to the complete destruction of the monolayer by 2 weeks at 15°C (Shchelkunov *et al.,* 2009; Watson *et al.,* 1995). Analysis of infected tissues or WSSK-1 cells by TEM reveals naked hexagonal nucleocapsids (95-110 nm in diameter) in the nucleus and enveloped particles surrounded by a coarse electron-dense tegument layer (176-250 nm in diameter) within the cytoplasm (LaPatra *et al.,* 2014; Shchelkunov *et al.,* 2009; Watson *et al.,* 1995). Although AciHV2 specific molecular diagnostic tests have not been developed, PCR amplification and sequencing of the viral DNA polymerase or terminase genes has been used to confirm the presence of AciHV2 (Doszpoly *et al.,* 2008, 2011; Doszpoly and Shchelkunov 2010; Kelley *et al.,* 2005; Kurobe *et al.,* 2008; Waltzek *et al.,* 2009). To date, no vaccines or antiviral drugs have been tested against AciHV2.

6.3.6. Salmonid Alloherpesviruses

There are four characterized alloherpesviruses in trout and salmon; all appear phylogenetically related and have been designated *Salmonid herpesvirus 1*, *2*, *3* and 4 (SalHV1-4) in the genus *Salmonivirus*. Three of these viruses are associated with significant losses in aquaculture: SalHV2 causes *Oncorhynchus masou* virus disease (OMVD) in pacific salmon species in Japan; SalHV3 causes epizootic epitheliotropic disease (EED) in lake trout (*Salvelinus namaycush*) in the Great Lakes region in North America and SalHV4 causes Atlantic salmon papillomatosis in Atlantic salmon (*Salmo salar*) in northern Europe.

6.3.6.1. *Salmonid herpesvirus 1*

Salmonid herpesvirus 1 was first detected in spawning rainbow trout (*Oncorhynchus mykiss*) in 1971-1975 in Winthrop fish hatchery in Washington State (Wolf and Taylor 1975). The virus was characterized and designated as a *Herpesvirus salmonis* (Wolf *et al.,* 1978). The

virus was again isolated in California ten years later in steelhead trout (Hedrick *et al.,* 1986). This isolate designated Steelhead herpesvirus was shown to be a strain of SalHV1 by serology and DNA hybridization (Eaton *et al.,* 1991; Hedrick *et al.,* 1987). This virus was the first salmonid herpesvirus characterized at the genomic level (Davison 1998) and later analysis showed genetic grouping of salmonid herpesviruses (Waltzek et al 2009) and SalHV1 was designated the type species for genus *Salmonivirus* (ICTV 2014)

SalHV-1 has only been associated with mild disease in natural outbreaks and thus is not of great concern to aquaculture. The clinical signs include general edema of the visceral organs and hyperemia of the liver and adipose tissue (Eaton and Hedrick 1994). In experimental infections of rainbow trout fry by parenteral administrations of the virus, the fish developed darkened pigmentation, exophthalmia, abdominal distension and shed fecal casts (Wolf and Smith 1981). The fish also displayed hemorrhages in the fins and pale gills. Internal gross pathology included yellow and blood tinged ascites. Histology revealed edema of cardiac and skeletal tissue, extensive congestion and necrosis in the liver and kidneys and syncytia was seen in pancreatic tissue. Highest virus concentrations were detected in the kidney tissue. A study using the Steelhead isolate in challenges showed much more limited pathology but did report on the syncytia of hepatocytes (Eaton *et al.,* 1989).

SalHV1 is distantly related to SalHV2 which is much more pathogenic. SalHV-1 and SalHV2 have similar characteristics in cell culture and must be distinguished from each other using serology or molecular methods. The presumptive diagnosis of SalHV1 can be done using traditional cell culture. It replicated on the common salmonid cells lines Chinook salmon embryo (CHSE-214) and rainbow trout gonad (RTG-2) at 10 °C. The virus produces a disseminating syncytium in the cells but it may take 10 day to 2 weeks to develop and blind passage may be needed to detect low level infection. Poor replication occurs at higher temperatures and this helps distinguish it from SalHV2 which replicates at 10-20 °C. Confirmation requires the use of neutralizing antibodies (Eaton and Hedrick 1994) or PCR. The PCR method of Aso *et al.* (2001) produces a ~800 bp band for SalHV1 and a 439 bp band for SalHV2.

There has been minimal research done on controlling SalHV1 but avoidance appears to be effective. Carrier brood fish shed virus in ovarian and seminal fluid during spawning and these can be used to detect carriers. In the original cases the virus was isolated from the ovarian fluid of post spawning adults that had higher than expected losses. In this hatchery the brood fish were replaced and the virus was no longer found.

6.3.6.2. *Salmonid herpesvirus 2*

Salmonid herpesvirus 2 is an important pathogen to farmed pacific salmon and rainbow trout in japan. This pathogen has several common names including *Oncorhynchus masou* virus, nerka virus Towada Lake, Akita Perfecture, and yamame tumor virus. After serological analysis demonstrated serum cross reaction with these isolates and they were serologically distinct from SalHV1 and molecular analysis confirmed, these isolates were designated as a single species (Eaton *et al.,* 1991; Hayashi *et al.,* 1993; Hedrick *et al.,* 1987; Waltzek *et al.,* 2009; Yoshimizu *et al.,* 1995). This virus causes disease in masu salmon (*Oncorhynchus masou*), coho salmon (*O. kisutch*) and kokanee salmon (*O. nerka*), rainbow trout (O. mykiss), yearling rainbow trout and Chum salmon (O. keta).

There are two recognized forms of disease caused by SalHV2: an acute form caused by primary virus infection and a tumor form caused by recrudescent infection. The acute form is a

disseminated viremia and can cause mortality that exceeds 80%. In this form affected fish appear darkened, may have skin ulcers, and lethargic with a low appetite. Internally the liver may have white patches and the intestine is often hemorrhagic. Some fish may display focal areas of epithelial proliferation on the mouth and body (Yoshimizu, 2012). The tumor form of infection is a papilloma primarily of the mouth region. This develops months after the primary infection. In an experimental challenge of juvenile chum salmon, tumors began to develop 4 months post infection and persisted for over 1 year. Tumor occurrence after experimental challenge was 40-60 % in chum salmon, 12% for rainbow trout and almost 100% for masu salmon (Yoshimizu *et al.,* 1987).

Presumptive diagnosis of SalHV2 in diseased fish can be done using clinical signs and cell culture on RTG-2 or CHSE 215 cells at 15 °C. For diagnosis of the acute infection liver, kidney and spleen are processed for cell culture. In the tumor form of the disease it is best to co-cultivate tumor tissue with RTG-2 cells (Yoshimizu, 2012). Carrier fish can be detected by screening ovarian fluid and tissues from post spawning adults. Confirmation can be done using serum neutralization assays using SalHV2 specific antisera or by SalHV-2 specific PCR (Aso *et al.,* 2001).

Management to control SalHV-2 is a combination of sanitation of facilities and equipment, disinfection of incoming water and prevention of vertical transmission using surveillance of broodstock, and iodine disinfection of eggs. The use of injectable formalin killed vaccine in broodstock also helps reduce the vertical transmission of the virus (Yoshimizu, 2009; Yoshimizu and Kasai 2011).

6.3.6.3. *Salmonid herpesvirus 3*

Salmonid herpesvirus 3 was characterized by partial sequencing and shown to be a member of the genus *Salmonivirus* (Waltzek *et al.,* 2009). This virus is recalcitrant to cell culture but was identified as the cause of epidermal proliferative disease in lake trout fingerlings designated epizootic epitheliotropic disease (EED) or lake trout epidermal hyperplasia. This disease has caused high losses of lake trout in state and federal hatcheries in the Great Lakes region of North America since 1983. The herpesvirus cause of EED was determined by observation of herpesvirus particles in infected tissues, isolation of the virions using differential centrifugation and experimentally reproducing the disease in lake trout using purified virus or tissue filtrates (Bradley *et al.,* 1989; McAllister and Herman 1989). After detailed structural analysis the designation *Salmonid herpesvirus 3* was proposed (Bradley *et al.,* 1989). This virus causes disease in lake trout and lake trout hybrids. Cohabitation and immersion challenge trials using filtrates from infected lake trout show that rainbow trout, brown trout, brook trout and Atlantic salmon are resistant to disease (Bradley *et al.,* 1989; McAllister and Herman 1989).

Outbreaks of EED have only been documented in aquaculture facilities and usually occur in the spring when temperatures are near 8-10 °C and can often be correlated with a predisposing stressing event such as fin clipping. The mortality pattern can be protracted often lasting more several months and cumulative losses may approach 100% (McAllister, 1993). Fish with EED are lethargic, swim near the surface and may display periods of hyperexcitability with disoriented or spiral swimming patterns. Lower portion of the eyes mouth and fish may display hemorrhages and gray to white mucoid patches are seen in the skin and fins. Fins may also be frayed and secondary *Saprolegnia* infections are common. Internally the fish appear normal except for enlarged spleens and swollen kidneys. Histopathology reveals patches of hypertrophy, hyperplasia, and necrosis of keratinocytes with an associated lymphocytic

infiltration. Some affected epithelial cells display eosinophilic intranuclear inclusions. Other lesions include edema in the gills, and the presence of macrophages laden with cellular debris in kidneys and liver (Bradley et al., 1989; McAllister and Herman, 1989).

Diagnosis of EED has traditionally relied on clinical signs and histopathology with the use visualization of herpesvirus particles in TEM for confirmation. However, genomic sequence data obtained from infected tissues (Waltzek *et al.,* 2009), provided the information needed for highly sensitive PCR based diagnostics (Kurobe *et al.,* 2009) and highly sensitive qPCR (Glenney *et al. in press*). These methods have been used effectively to diagnose EED and SalHV3 carriers.

Management to reduce EED has focused on the reducing stress, reducing fish density and on minimizing recirculation of hatchery water. No vaccines have been developed or studied for this virus. Some hatcheries that experience high losses during the initial outbreaks depopulated and disinfected their facilities then obtained eggs from Wyoming (an area with no history of disease) (Kurobe *et al.,* 2009). The SalHV3 specific PCR can be used to detect carrier brood fish and to allow culling of eggs and fry that may have received the infections by vertical transmission (Kurobe *et al.,* 2009; Glenney *et al., in press*).

6.3.6.4. Salmonid herpesvirus 4

Salmonid herpesvirus 4 is an alloherpesvirus that causes Atlantic salmon papillomatosis (ASP). As the name implies ASP is characterized by dermal proliferation in Atlantic salmon. This disease has been recognized as a problem in salmon culture facilities since the 1950s and is found in Scotland, the Scandinavian countries and Northwestern Russia. In early studies a herpesvirus was shown to be associated with the proliferative lesions using TEM but all attempts to culture the virus were unsuccessful (Carlisle, 1977; Shchelkunov *et al.,* 1992). DNA analysis of the tumor tissue using degenerate PCR targeted to conserved alloherpesvirus sequences demonstrated the presence of a unique alloherpesvirus most closely related to SalHV3 (Doszpoly *et al.,* 2013). With this sequence information they proposed the virus be designated Salmonid herpesvirus 4 and its placement in the *Salmonivirus* genus.

Outbreaks of ASP occur primarily in older Atlantic salmon juveniles (over 2 years of age) during summer months when water temperatures are 10 - 16°C, especially during times of smoltification (Bylund *et al.,* 1980; Doszpoly *et al.,* 2013). When temperatures drop below 10 °C the papillomas degenerate and ASP associated mortality peaks due to secondary infections. High incidences of ASP (sometimes exceeding 85% of some stocks of fish) are problematic in certain aquaculture facilities and losses exceeding 50% due to secondary infections have been documented (Bylund *et al.,* 1980). The disease is seen primarily in aquaculture facilities but has been documented in wild, natural spawned stocks of juvenile Atlantic salmon in Finland (Bylund *et al.,* 1980). The clinical signs are well demarcated elevated epidermal proliferative growths that may exceed 1 cm in diameter and 5 mm in height (Figure 6.3-1b). These lesions can occur anywhere on the epidermis but predominantly on the caudal peduncle. Histologically these lesions display epithelial cell hyperplasia and karyomegaly of affected cells. These proliferative lesions also display a loss of goblet cells, loss of the basement membrane, and deformation and/or loss of the scales (Doszpoly *et al.,* 2013).

Diagnosis of ASP has relied on histopathology but with the availability of genomic sequence data molecular detection of the presence of SalHV4 is replacing this. Recent work by Glenney *et al.* (*in press*) with SalHV3 qPCR assays based on the terminase gene had demonstrated cross reactivity with SalHV4 and the presence of another closely related

herpesvirus in lake trout (tentatively designated Salmonid herpesvirus 5 SalHV5, Glenney, *et al., in press* b) . They subsequently developed SalHV3, SalHV4 and SalHV5 specific qPCR assays. Management by avoidance will rely on the development and use of sensitive molecular methods. Prevalence appears to be related to parental source which suggests possible vertical transmission (Carlisle and Roberts 1977; Doszpoly *et al.,* 2013). Reported management methods include the use of prophylactic salt and formalin treatments on alternating weeks, minimizing stress and treating secondary infections when the papilloma regression and sloughing occurs (Bylund *et al.,* 1980). Because this is a disease that occurs predominantly in older juveniles, hatcheries in the Kola Peninsula of Russia have shortened the rearing period to 1 year before fish are released into the rivers (Doszpoly *et al.,* 2013).

6.3.7. Conclusions

Alloherpesviruses of fish are responsible for many important diseases in aquaculture. They persist in affected populations as inapparent latent infections and can spread to naïve hosts by recrudescence and horizontal transmission or to offspring by vertical transmission. Diseases caused by these viruses are often closely tied to specific temperatures and stress. Management of these diseases generally relies on avoiding carrier sources of fish, minimizing stress and minimizing crowding during the most susceptible ages and temperatures. The development of efficient methods of producing recombinant attenuated viruses using BACs will facilitated vaccine development and the use of affordable live vaccines may become more common management tools in the future. The latent form is difficult to detect and many alloherpesviruses are recalcitrant to cell culture isolation making detecting them a challenge for diagnostic laboratories. The identification of conserved genes and the use of degenerate PCR followed by sequencing has allowed the molecular characterization and development of diagnostic tools for uncultivable members of this group.

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