CHAPTER THREE

Cyprinid Herpesvirus 3: An Archetype of Fish Alloherpesviruses

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Abstract

The order *Herpesvirales* encompasses viruses that share structural, genetic, and biological properties. However, members of this order infect hosts ranging from molluscs to humans. It is currently divided into three phylogenetically related families. The *Alloherpesviridae* family contains viruses infecting fish and amphibians. There are 12 alloherpesviruses described to date, 10 of which infect fish. Over the last decade, cyprinid herpesvirus 3 (CyHV-3) infecting common and koi carp has emerged as the archetype of fish alloherpesviruses. Since its first description in the late 1990s, this virus has induced important economic losses in common and koi carp worldwide. It has also

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had negative environmental implications by affecting wild carp populations. These negative impacts and the importance of the host species have stimulated studies aimed at developing diagnostic and prophylactic tools. Unexpectedly, the data generated by these applied studies have stimulated interest in CyHV-3 as a model for fundamental research. This review intends to provide a complete overview of the knowledge currently available on CyHV-3.

1. INTRODUCTION

The order *Herpesvirales* contains a large number of viruses that share structural, genetic, and biological properties. It is divided into three phylogenetically related families infecting a wide range of hosts (Pellett et al., 2011b). The *Herpesviridae* family encompasses viruses infecting mammals, birds, or reptiles. It is by far the most important, both in terms of the number of its members and the volume of studies that have been devoted to them. The *Malacoherpesviridae* family comprises viruses infecting molluscs. Finally, the *Alloherpesviridae* family encompasses viruses infecting fish and amphibians. Twelve alloherpesviruses have been described to date, ten of them infecting fish (Hanson, Dishon, & Kotler, 2011; Waltzek et al., 2009).

Over the last decade, an increasing number of studies have been devoted to alloherpesviruses that infect fish. Scientific interest in a specific virus tends to originate from its impact on wildlife, the economic losses it causes to the aquaculture industry, or its importance as a fundamental research object. On rare occasions, all three of these reasons apply. This is the case for cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), which has emerged as the archetype of fish alloherpesviruses (Adamek, Steinhagen, et al., 2014; Rakus et al., 2013).

Since its emergence in the late 1990s, CyHV-3 has had an ecological impact and induced severe economic losses in the common and koi carp industries (Bondad-Reantaso et al., 2005; Perelberg et al., 2003; Rakus et al., 2013). The common carp (*Cyprinus carpio*) is one of the oldest cultivated freshwater fish species (Balon, 1995) and is now one of the most economically valuable species in aquaculture. It is widely cultivated for human consumption, with a worldwide production of 3.8 million tons in 2012 representing US$5.2 billion (FAO, 2012). Furthermore, its colorful ornamental varieties (koi carp), grown for personal pleasure and competitive exhibitions, represent one of the most expensive markets for individual freshwater fish. The economic importance of CyHV-3 has rapidly

stimulated research efforts aimed at building essential knowledge for the development of diagnostic and prophylactic tools (Ilouze, Dishon, & Kotler, 2006; Rakus et al., 2013). In addition, these studies have stimulated interest in CyHV-3 as an object of fundamental research. As a result, CyHV-3 can be considered today as the archetype of fish alloherpesviruses and is the subject of an increasing number of studies. Most of the present review is devoted to this virus.

This review consists of two sections. In the first part, we describe an up-to-date phylogenetic analysis of the family *Alloherpesviridae* as a component of the order *Herpesvirales*. We also summarize the main properties of herpesviruses and the specific properties of fish alloherpesviruses. In the second and main part, we provide a full overview of the knowledge currently available on CyHV-3.

2. THE ORDER *HERPESVIRALES*

2.1 Phylogeny

2.1.1 Phylogeny of the Order Herpesvirales

In historical terms, recognition of an agent as a herpesvirus has rested on morphology: a linear, double-stranded DNA genome packed into a $T=16$ icosahedral capsid, embedded in a complex protein layer known as the tegument, wrapped in a glycoprotein-containing lipid membrane, yielding a spherical virion. However, extensive understanding of the genetic structure of herpesviruses, especially in relation to conserved genes, now allows these features to be inferred rather than demonstrated directly. As a result, classification of an entity as a herpesvirus and determination of its detailed taxonomy depend principally on the interpretation of primary sequence data.

For many years, the International Committee on Taxonomy of Viruses (ICTV) counted several fish pathogens as being likely members of the family *Herpesviridae* based on morphology. In 1998, the first species of fish herpesvirus was founded in the family, namely *Ictalurid herpesvirus 1* (ictalurid herpesvirus 1 [IcHV-1], also known as channel catfish virus). The genus in which this species was placed adopted the name *Ictalurivirus*. However, it had been clear for some years that this virus was only very distantly related to mammalian herpesviruses (Davison, 1992). In 2008, this, as well as other considerations, led to the adoption of the order *Herpesvirales* (Davison et al., 2009; Pellett et al., 2011b). This order was established to contain three

families: the already existing family *Herpesviridae*, which now contains herpesviruses of mammals, birds, and reptiles (Pellett et al., 2011c), the new families *Alloherpesviridae*, encompassing herpesviruses of amphibians and fish (Pellett et al., 2011a), and *Malacoherpesviridae*, containing herpesviruses of invertebrates (Pellett et al., 2011d). The assignment of herpesviruses of certain hosts to these families is descriptive rather than prescriptive.

The ICTV (http://www.ictvonline.org) currently lists 87 species in the family *Herpesviridae* distributed among the three subfamilies, *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*, plus one unassigned species. The subfamilies contain five, four, and four genera, respectively. Establishment of this taxonomical structure has been fostered by an extensively researched phylogeny (McGeoch, Dolan, & Ralph, 2000; McGeoch & Gatherer, 2005; McGeoch, Rixon, & Davison, 2006). A phylogenetic description of 65 viruses classified in this family, based on the complete sequence of the highly conserved viral gene encoding DNA polymerase, is shown in Fig. 1A. The overall genetic coherence of the family is apparent from the fact that 43 genes are conserved among members of the family. These genes are presumed to have been present in the last common ancestor, which has been inferred to have existed 400 million years ago (McGeoch et al., 2006).

A description of the phylogeny of the family *Alloherpesviridae*, to which CyHV-3 belongs, is given in Section 2.1.2. The third family, *Malacoherpesviridae*, consists of two genera, *Aurivirus*, which contains the species *Haliotid herpesvirus 1* (haliotid herpesvirus 1 or abalone herpesvirus), and *Ostreavirus*, which includes the species *Ostreid herpesvirus 1* (ostreid herpesvirus 1 or oyster herpesvirus).

Since herpesviruses continue to be identified, it seems likely that more members of the order *Herpesvirales* remain to be discovered. Although the coherence of the order is apparent from structural conservation of the virion, particularly the capsid, among the three families (Booy, Trus, Davison, & Steven, 1996; Davison et al., 2005), detectable genetic similarities are very few. The most convincingly conserved gene is that encoding DNA packaging terminase subunit 1, a subunit of an enzyme complex responsible for incorporating genomes into preformed capsids. Conservation of the predicted amino-acid sequence of this protein in herpesviruses and tailed bacteriophages (Davison, 1992), as well as the existence of conserved structural elements in other proteins (Rixon & Schmid, 2014), points to an origin of all herpesviruses from ancient precursors having existed in bacteria.

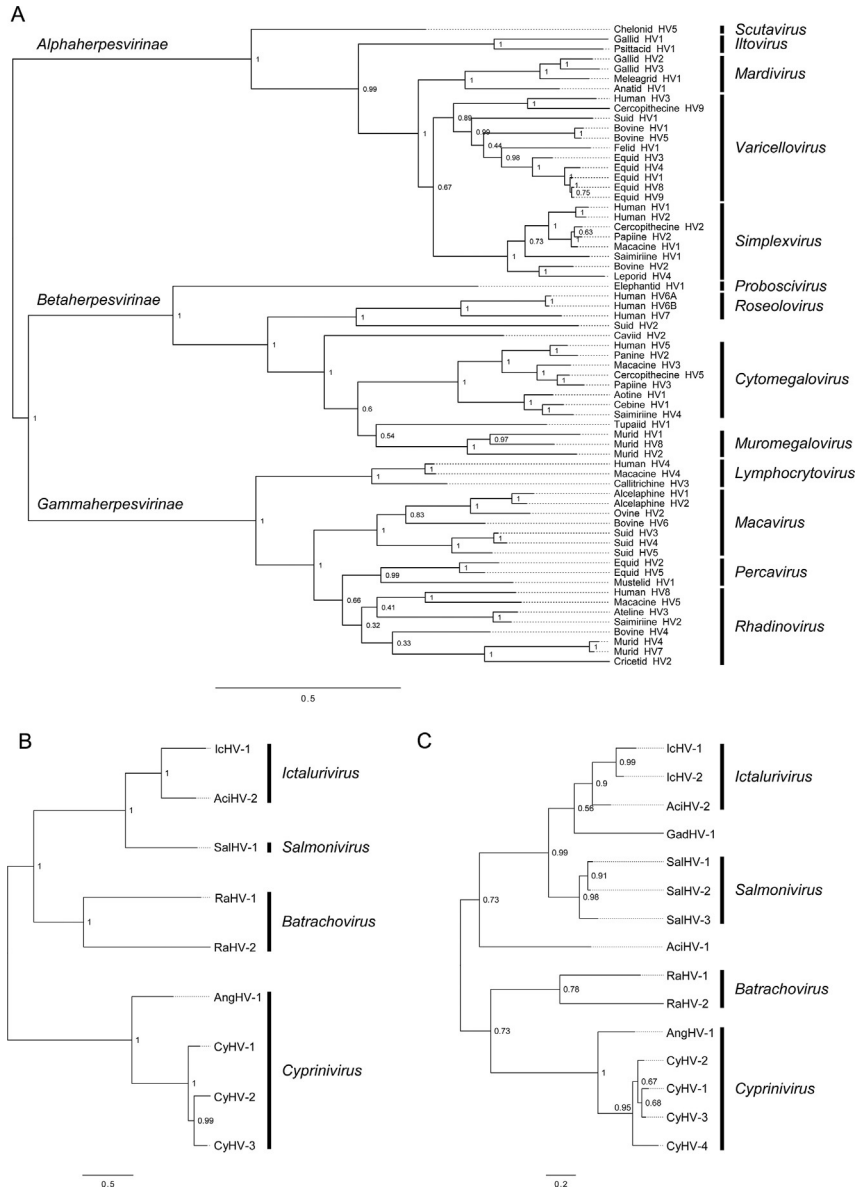

Figure 1 Phylogenetic analysis of the *Herpesviridae* and *Alloherpesviridae* families. Unrooted phylogenetic tree based on (A) the full-length DNA polymerases of members of the family *Herpesviridae*, (B) the full-length DNA polymerases of members of the family *Alloherpesviridae*, and (C) partial DNA polymerases of members or potential members

(*Continued*)

2.1.2 *Phylogeny of the Family* Alloherpesviridae

Shortly after the first formal reports of its discovery (Ariav, Tinman, Paperna, & Bejerano, 1999; Bretzinger, Fischer-Scherl, Oumouma, Hoffmann, & Truyen, 1999), CyHV-3 was characterized as a herpesvirus based on virion morphology (Hedrick et al., 2000). Although there was some suggestion, based on early DNA sequence data, that this assignment might not be correct (Hutoran et al., 2005; Ronen et al., 2003), the initial characterization was soon shown to be sound (Waltzek et al., 2005). The subsequent accumulation of extensive sequence data for a range of fish and amphibian herpesviruses provided a solid understanding of the phylogeny and evolution of the family *Alloherpesviridae*.

The ICTV currently lists twelve species in the family *Alloherpesviridae*, distributed among four genera, of which three contain fish viruses (*Cyprinivirus*, *Ictalurivirus*, and *Salmonivirus*, with CyHV-3 in the genus *Cyprinivirus*) and one contains amphibian viruses (*Batrachovirus*) (Table 1). Full genome sequences are available for seven of these viruses, representing three genera (Table 2). Partial sequence data are available for the other five classified, and also several unclassified, fish herpesviruses. A phylogenetic

Figure 1—Cont'd of the family *Alloherpesviridae*. For (A), the sequences (996–1357 amino-acid residues in length) were derived from relevant GenBank accessions. Virus names are aligned at the branch tips in the style that mirrors the species names (e.g., chelonid herpesvirus 5 (Chelonid HV5) is in the species *Chelonid herpesvirus 5*). The names of subfamilies and genera are marked on the left and right, respectively. The branching order in the genus *Rhadinovirus* is typically difficult to determine (McGeoch et al., 2006). For (B), the sequences (1507–1720 residues in length) were derived from the GenBank accessions listed in Table 1 and also from FJ815289.2 (Doszpoly, Somogyi, LaPatra, & Benko, 2011) for AciHV-2 and AAC59316.1 (Davison, 1998) and unpublished data (A.J.D.) for SalHV-1. Abbreviated virus names are shown at the branch tips (see Table 1), and the names of genera are marked on the right. For (C), partial sequences (134–158 residues in length; some truncated from longer sequences) located between the highly conserved DF(A/T/S)(S/A)(L/M)YP and GDTDS(V/T/I)M motifs were derived from EF685904.1 (Kelley et al., 2005) for AciHV-1, HQ857783.1 (Marcos-Lopez et al., 2012) for GadHV-1, KM357278.1 (Doszpoly et al., 2015) for CyHV-4, FJ641907.1 (Doszpoly et al., 2008; Waltzek et al., 2009) for IcHV-2, FJ641908.1 (Waltzek et al., 2009) for SalHV-2, and EU349277.1 (Waltzek et al., 2009) for SalHV-3. Abbreviated virus names are shown at the branch tips (see Table 1), and the names of genera are marked on the right. For (A), (B), and (C), the sequences were aligned by using Clustal Omega (Sievers & Higgins, 2014), and the tree was calculated by using MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) under an LG+G+I model with 100 bootstraps (values shown at the branch nodes). The scale in each panel shows the number of changes per site.

Table 1 Classification of the Family *Alloherpesviridae*

Genus Name	Species Name	Virus Name and Abbreviation	Alternative Virus Name[a]
Batrachovirus	*Ranid herpesvirus 1*	ranid herpesvirus 1 (RaHV-1)	Lucké tumor herpesvirus
	Ranid herpesvirus 2	ranid herpesvirus 2 (RaHV-2)	frog virus 4
Cyprinivirus	*Anguillid herpesvirus 1*	anguillid herpesvirus 1 (AngHV-1)	European eel herpesvirus
	Cyprinid herpesvirus 1	cyprinid herpesvirus 1 (CyHV-1)	carp pox herpesvirus
	Cyprinid herpesvirus 2	cyprinid herpesvirus 2 (CyHV-2)	goldfish hematopoietic necrosis virus
	Cyprinid herpesvirus 3	cyprinid herpesvirus 3 (CyHV-3)	koi herpesvirus
Ictalurivirus	*Acipenserid herpesvirus 2*	acipenserid herpesvirus 2 (AciHV-2)	white sturgeon herpesvirus 2
	Ictalurid herpesvirus 1	ictalurid herpesvirus 1 (IcHV-1)	channel catfish virus
	Ictalurid herpesvirus 2	ictalurid herpesvirus 2 (IcHV-2)	Ictalurus melas herpesvirus
Salmonivirus	*Salmonid herpesvirus 1*	salmonid herpesvirus 1 (SalHV-1)	herpesvirus salmonis
	Salmonid herpesvirus 2	salmonid herpesvirus 2 (SalHV-2)	Oncorhynchus masou herpesvirus
	Salmonid herpesvirus 3	salmonid herpesvirus 3 (SalHV-3)	epizootic epitheliotropic disease virus

[a]From Waltzek et al. (2009). In instances in which a virus is known by several alternative names, a single example is given.

tree of nine of the classified viruses, based on the complete sequence of the viral DNA polymerase, is shown in Fig. 1B. A tree of all 12 viruses, plus 3 others not yet classified (cyprinid herpesvirus 4 [CyHV-4, sichel herpesvirus], acipenserid herpesvirus 1 [AciHV-1, white sturgeon herpesvirus 1], and gadid herpesvirus 1 [GadHV-1, Atlantic cod herpesvirus]), based on a short segment of the same gene, is shown in Fig. 1C. As indicated by the bootstrap values, the robustness of the former tree is greater than that of the latter.

Table 2 Data on Complete Genome Sequences of Members of the Family *Alloherpesviridae*

Species Name	Virus Name and Abbreviation	Genome Size (bp)	Genome G+C (%)	ORFs (No.)[a]	GenBank Accession	References
Anguillid herpesvirus 1	anguillid herpesvirus 1 (AngHV-1)	248,526	53	134	FJ940765.3	van Beurden et al. (2010) van Beurden, Gatherer, et al. (2012)
Cyprinid herpesvirus 1	cyprinid herpesvirus 1 (CyHV-1)	291,144	51	143	JQ815363.1	Davison et al. (2013)
Cyprinid herpesvirus 2	cyprinid herpesvirus 2 (CyHV-2)	290,304	52	154	JQ815364.1	Davison et al. (2013)
Cyprinid herpesvirus 3	cyprinid herpesvirus 3 (CyHV-3)	295,146	59	163	DQ657948.1[b]	Aoki et al. (2007) Davison et al. (2013)
Ictalurid herpesvirus 1	ictalurid herpesvirus 1 (IcHV-1)	134,226	56	90	M75136.2	Davison (1992)
Ranid herpesvirus 1	ranid herpesvirus 1 (RaHV-1)	220,859	55	132	DQ665917.1	Davison, Cunningham, Sauerbier, and McKinnell (2006)
Ranid herpesvirus 2	ranid herpesvirus 2 (RaHV-2)	231,801	53	147	DQ665652.1	Davison et al. (2006)

[a]Predicted to encode functional proteins. Includes ORFs duplicated in repeated sequences.
[b]Additional genome sequences: DQ177346.1 (Aoki et al., 2007), AP008984.1 (Aoki et al., 2007), and KJ627438.1 (Li, Lee, Weng, He, & Dong, 2015).

Nonetheless, the trees are similar in overall shape, and they support the arrangement of the family into the four genera. The phylogeny of two of the unclassified viruses (AciHV-1 and GadHV-1) is not clear from the limited data used in Fig. 1C. However, the positions of these viruses, and others not included in Fig. 1C, have been examined with greater discrimination using sequences from other genes (Doszpoly, Benko, Bovo, Lapatra, & Harrach, 2011; Doszpoly et al., 2008, 2015; Doszpoly, Somogyi, et al., 2011; Kelley et al., 2005; Kurobe, Kelley, Waltzek, & Hedrick, 2008; Marcos-Lopez et al., 2012).

There has been some consideration of establishing subfamilies in the family *Alloherpesviridae*, as has taken place in the family *Herpesviridae*. These could number two (genus *Cyprinivirus* in one subfamily and the other three genera in another (Waltzek et al., 2009)) or three (genus *Cyprinivirus* in one subfamily, genus *Batrachovirus* in another, and the other two genera in the third (Doszpoly, Somogyi, et al., 2011)). For various reasons, this would seem premature at present.

The overall genetic coherence of the family *Alloherpesviridae* is evident from the presence of 12 convincingly conserved genes in fully sequenced members (Davison et al., 2013). This modest number suggests a last common ancestor that is considerably older than that of the family *Herpesviridae*. Patterns of coevolution between virus and host are apparent only toward the tips of phylogenetic trees and therefore are relevant to a more recent evolutionary period (Waltzek et al., 2009). For example, in Fig. 1B and C, the cyprinid herpesviruses 1 and 2 (CyHV-1 and CyHV-2) cluster with CyHV-3, and salmonid herpesviruses 1, 2, and 3 (SalHV-1, SalHV-2, and SalHV-3) cluster together. However, one of the sturgeon herpesviruses (AciHV-1) is deeply separated from the other viruses, whereas the other (AciHV-2) is most closely related to the ictalurid herpesviruses. Also, the branch point of the frog viruses falls within the fish herpesviruses rather than outside. The apparently smaller degree of coevolution of the family *Alloherpesviridae* compared with the family *Herpesviridae* may be due to several factors, not least those relating to the respective environments and the lengths of time the two families have been evolving.

2.2 Main Biological Properties

All members of the order *Herpesvirales* seem to share common biological properties (Ackermann, 2004; Pellett et al., 2011b): (i) they produce virions with the structure described above (Fig. 2A); (ii) they encode their own

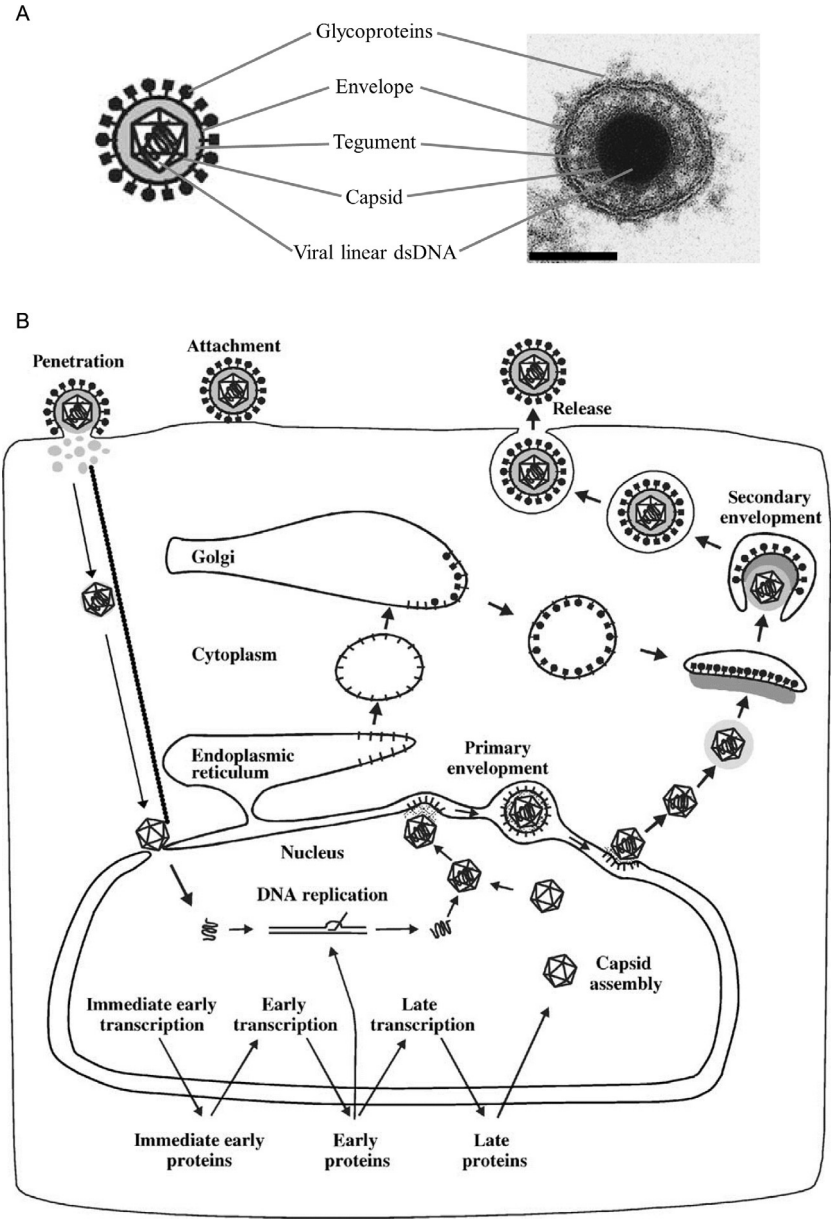

Figure 2 Virion structure and replication cycle of herpesviruses. (A) Schematic representation (left) and electron microscopy examination (right) of CyHV-3 virion. Bar represents 100 nm. (B) Replication cycle of CyHV-3. Diagrammatic representation of the herpesvirus replication cycle, including virus entry and dissociation of the tegument, transport of incoming capsids to the nuclear pore, and release of viral DNA into the nucleus where transcription occurs in a cascade-like fashion, and DNA replication ensues. Capsid assembly, DNA packaging, and primary and secondary envelopment are also illustrated. *Panel (A): Adapted with permission from Mettenleiter (2004) and Mettenleiter, Klupp, and Granzow (2009). Copyright © Elsevier. Panel (B): Reproduced with permission from Mettenleiter (2004). Copyright © Elsevier.*

DNA synthesis machinery, with viral replication as well as nucleocapsid assembly taking place in the nucleus (Fig. 2B); (iii) production of progeny virions is usually associated with lysis of the host cell; (iv) they are able to establish lifelong latent infection, which is characterized by the absence of regular viral transcription and replication and the lack of production of infectious virus particles, but presence of intact viral genomic DNA and the transcription of latency-associated genes. Latency can eventually be interrupted by reactivation that leads to lytic replication and the excretion of infectious particles by infected subjects despite the adaptive immune response developed against the virus; and (v) their ability to establish persistent infection in immunocompetent hosts (Pellett et al., 2011b) is the consequence of immune evasion mechanisms targeting major components of the immune system.

In addition to these properties that are considered to be common to all members of the order *Herpesvirales*, fish alloherpesviruses seem to share several biological properties that differentiate them from *Herpesviridae* (herpesviruses infecting mammals, birds, and reptiles). First, while herpesviruses generally show only modest pathogenicity in their natural immunocompetent hosts, fish herpesviruses can cause outbreaks associated with mortality reaching 100%. The markedly higher virulence of fish herpesviruses could reveal a lower adaptation level of these viruses to their hosts (see Section 2.1.2). However, it could also be explained by other factors such as the high-density rearing conditions and inbreeding promoted by intensive aquaculture. Second, the tropism of members of the family *Herpesviridae* is generally restricted to their natural host species or closely related species. In contrast, whereas some alloherpesviruses induce severe disease in only one or few closely related members of the same genus, others are able to establish subclinical infections in a broader range of hosts. Thus, although CyHV-3 causes a disease only in common and koi carp, its genome has been detected in a wide range of fish species (see Section 3.2.1.1). Third, an age-dependent pathogenesis has been described for several fish herpesviruses, in that AciHV-1, AciHV-2, CyHV-1, CyHV-2, SalHV-2, SalHV-3, and ictalurid herpesvirus 2 (IcHV-2) are particularly pathogenic for young fry (Hanson et al., 2011; van Beurden & Engelsma, 2012). Fourth, a marked difference in the outcome of herpesvirus infection in poikilothermic hosts is related to their temperature dependency, both *in vitro* and *in vivo*. For example, anguillid herpesvirus 1 (AngHV-1), infecting Japanese eel (*Anguilla japonica*) and European eel (*Anguilla anguilla*), only propagates in eel kidney 1 cells between 15 and 30 °C, with an optimum around

20–25 °C (Sano, Fukuda, & Sano, 1990; van Beurden, Engelsma, et al., 2012). *In vivo*, replication of ranid herpesvirus 1 (RaHV-1) is promoted by low temperature, whereas induction of tumor metastasis is promoted by high temperature (McKinnell & Tarin, 1984). In general, fish herpesvirus-induced infection is less severe or even asymptomatic if the ambient water temperature is suboptimal for virus replication, which explains the seasonal occurrence of certain fish herpesviruses, including CyHV-3 (Gilad et al., 2003). In practice, these biological properties have been utilized successfully to immunize naturally carp against CyHV-3 (Ronen et al., 2003) and to reduce the clinical signs and mortality rates of AngHV-1 infections in eel culture systems (Haenen et al., 2002). In addition, temperature could play a role in the induction of latency and reactivation of fish herpesviruses (see Sections 3.2.3.2 and 3.2.3.3).

2.3 Herpesviruses Infecting Fish

The first description of lesions caused by a fish herpesvirus dates from the sixteenth century, when the Swiss naturalist Conrad Gessner described a pox disease of carp. Four hundred years later, the pox-like lesions were found to be associated with herpesvirus-like particles (Schubert, 1966), later designated as CyHV-1 (Sano, Fukuda, & Furukawa, 1985). However, the alloherpesviruses that were first studied in detail originated from the North American leopard frog (*Rana pipiens*). Lucké tumor herpesvirus or RaHV-1 was identified as the etiological agent of renal adenocarcinoma or Lucké tumor (Fawcett, 1956), and frog virus 4 or ranid herpesvirus 2 (RaHV-2) was isolated subsequently from the pooled urine of tumor-bearing frogs (Gravell, Granoff, & Darlington, 1968).

Alloherpesviruses infect a wide range of fish species worldwide, including several of the most important aquaculture species such as catfish, salmon, carp, sturgeon, and eel. As a result of host specificity, the prevalence of specific fish herpesviruses may be restricted to certain parts of the world. For example, pilchard herpesvirus 1 has been described only in wild Australasian pilchards (*Sardinops sagax neopilchardus*) in Australia and New Zealand (Whittington, Crockford, Jordan, & Jones, 2008), whereas CyHV-2 has a worldwide prevalence due to the international trade in goldfish (Goodwin, Sadler, Merry, & Marecaux, 2009).

Currently, 10 herpesviruses infecting fish are included in the family *Alloherpesviridae* (Table 1). At least a dozen of other fish herpesviruses have

been described, but many of these viruses have not been isolated yet, and the availability of limited sequence data hampers their official classification (Hanson et al., 2011; Waltzek et al., 2009). Interestingly, all but one of these viruses occur in bony fish, the exception having been found in a shark. Based on the number of different herpesvirus species recognized in humans (i.e., nine) and domestic animals, it is probable that each of the numerous fish species hosts multiple herpesviruses. It is likely that the alloherpesvirus species currently known are biased toward commercially relevant hosts, and the species that cause significant disease.

Channel catfish virus (IcHV-1) has been the prototypic fish herpesvirus for decades (Hanson et al., 2011; Kucuktas & Brady, 1999). In the late 1960s, the extensive catfish (*Ictalurus punctatus*) industry in the United States experienced high mortality rates among fry and fingerlings (Wolf, 1988). The causative virus was isolated and shown by electron microscopy to possess the distinctive morphological features of a herpesvirus (Wolf & Darlington, 1971). The genome sequence of IcHV-1 revealed that fish herpesviruses have evolved separately from herpesviruses infecting mammals, birds, and reptiles (Davison, 1992; see Section 2.1.2).

In the late 1990s, mass mortalities associated with epidermal lesions, gill necrosis, and nephritis occurred worldwide in koi and common carp aquaculture (Haenen, Way, Bergmann, & Ariel, 2004). This highly contagious and virulent disease was called KHV disease (KHVD) and was shown to be caused by a herpesvirus, which was later designated CyHV-3 (Bretzinger et al., 1999; Hedrick et al., 2000; Waltzek et al., 2005). Due to its economic impact on carp culture and its rapid spread across the world, CyHV-3 was listed as a notifiable disease by the World Organization for Animal Health OIE (Michel, Fournier, Lieffrig, Costes, & Vanderplasschen, 2010). Although IcHV-1 had been the model of fish herpesviruses for more than three decades, the associated problems mainly affected the catfish industry in the USA and could be limited by management practices (Hanson et al., 2011; Kucuktas & Brady, 1999). Meanwhile, the desire to protect common and koi carp from the negative impact of CyHV-3 infection prompted an increased interest to study this virus. In addition, the natural host of CyHV-3, the common carp, has been a traditional species for fundamental research on fish immunology, making it a perfect model to study host–virus interactions (Adamek, Steinhagen, et al., 2014; Rakus et al., 2013). As a consequence, advancement in our understanding of CyHV-3 now far exceeds that of any other alloherpesvirus.

3. CYPRINID HERPESVIRUS 3

3.1 General Description

3.1.1 Morphology and Morphogenesis

Like all members of the order *Herpesvirales*, CyHV-3 virions are composed of an icosahedral capsid containing a single copy of a large, linear, double-stranded DNA genome, a host-derived lipid envelope bearing viral glycoproteins and an amorphous proteinaceous layer termed the tegument, which resides between the capsid and the envelope (Fig. 2A; Mettenleiter, 2004; Mettenleiter et al., 2009). The diameter of CyHV-3 virions varies somewhat according to the infected cell type both *in vitro* (180–230 nm in koi fin cells KF-1 (Hedrick et al., 2000) and 170–200 nm in koi fin derived cells (KF-1, NGF-2, and NGF-3) (Miwa, Ito, & Sano, 2007)) and *in vivo* (167–200 nm in various organs (Miyazaki, Kuzuya, Yasumoto, Yasuda, & Kobayashi, 2008)). Despite the very limited sequence conservation in proteins involved in morphogenesis, members of the families *Herpesviridae*, *Alloherpesviridae*, and *Malacoherpesviridae* exhibit a common structure, suggesting that the mechanisms used are similar (Mettenleiter et al., 2009). Indeed, the structure of the CyHV-3 virion and its morphogenesis are entirely typical of herpesviruses (Figs. 2B and 3). Assembly of the nucleocapsids (size 100 nm) takes place in the nucleus (Miwa et al., 2007; Miyazaki et al., 2008), where marginalization of chromatin occurs at the inner nuclear membrane (Miwa et al., 2007; Miyazaki et al., 2008). Mature nucleocapsids with an electron-dense core composed of the complete viral genome bud at the inner nuclear membrane into the perinuclear space and are then released into the cytoplasm according to the envelopment/de-envelopment model (Miwa et al., 2007; Miyazaki et al., 2008). Viral nucleocapsids in the cytoplasm, prior to envelopment, are surrounded by a layer of electron-dense material composed of tegument proteins (Fig. 3). A similar feature is found in members of the subfamily *Betaherpesvirinae* but not in the subfamilies *Alpha-* and *Gammaherpesvirinae*, where they appear to be naked (Mettenleiter et al., 2009). Finally, the lipid envelope bearing viral glycoproteins is acquired by budding into vesicle membranes derived from the Golgi apparatus (Mettenleiter et al., 2009; Miwa et al., 2007; Miyazaki et al., 2008).

3.1.2 Genome

The complete DNA sequences of four CyHV-3 strains derived from different geographical locations have been determined (Aoki et al., 2007; Li et al.,

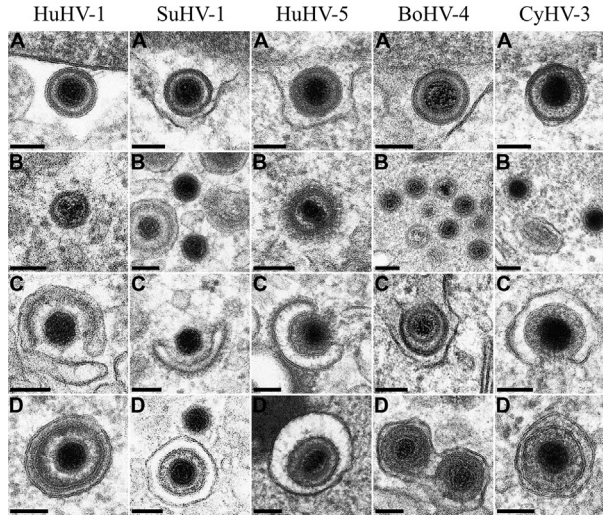

Figure 3 Primary and secondary envelopment of some herpesviruses. (A) Primary-enveloped virions in the perinuclear space. In comparison with Fig. 2, the electron-dense sharply bordered layer of tegument underlying the envelope and the conspicuous absence of envelope glycoprotein spikes are noteworthy. (B) After translocation into the cytosol, capsids of HuHV-1, SuHV-1, and BoHV-4 appear "naked," whereas those of HuHV-5 and CyHV-3 are covered with a visible layer of "inner" tegument. (C) Secondary envelopment and (D) presence of enveloped virions within a cellular vesicle during transport to the plasma membrane. The same stages can be observed for the members of the *Herpesviridae* and *Alloherpesviridae* families. Bars represent 100 nm. HuHV-1, *Human herpesvirus 1* (herpesvirus simplex 1, HSV-1); SuHV-1, *Suid herpesvirus 1* (pseudorabies virus, PrV); HuHV-5, *Human herpesvirus 5* (human cytomegalovirus, HCMV); BoHV-4, *Bovine herpesvirus 4*; CyHV-3, *Cyprinid herpesvirus 3*. *Adapted with permission from Mettenleiter et al. (2009). Copyright © Elsevier.*

2015). CyHV-3 is notable for having the largest known genome among the herpesviruses, at 295 kbp. It is followed by its two closest relatives, CyHV-1 (291 kbp) and CyHV-2 (290 kbp) (Aoki et al., 2007; Davison et al., 2013). Like all other fully sequenced alloherpesvirus genomes, the CyHV-3 genome contains two copies of a terminal direct repeat (TR), which, in the case of CyHV-3, are 22 kbp in size. The arrangement of open reading frames (ORFs) in the CyHV-3 genome that are predicted to encode functional proteins was first described by Aoki et al. (2007), and later refined on the basis of a full comparison with the genomes of other viruses in the genus *Cyprinivirus*, as well as members of the other genera (Davison et al., 2013). A map of the predicted CyHV-3 genes is shown in Fig. 4; the central part of the genome and the TR encode 148 (ORF9–ORF156) and

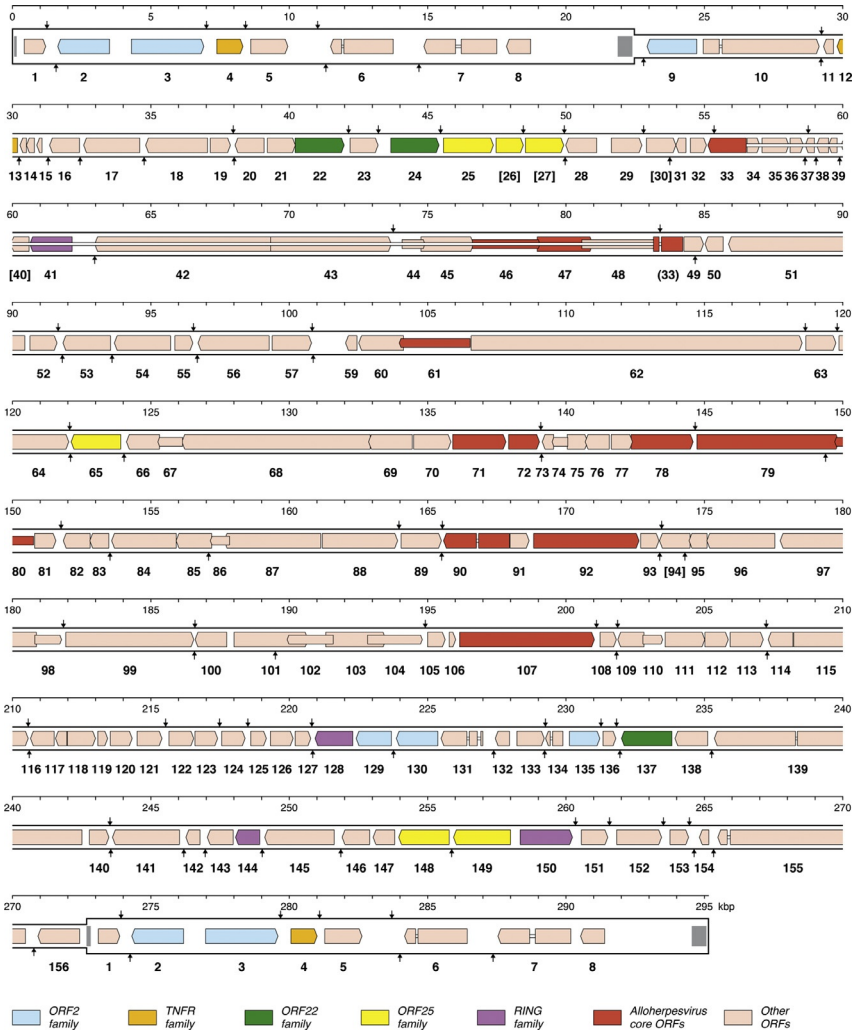

Figure 4 Map of the CyHV-3 genome. The terminal direct repeat (TR) is shown in a thicker format than the rest of the genome. ORFs predicted to encode functional proteins are indicated by arrows (see the key at the foot), with nomenclature lacking the ORF prefix given below. Introns are shown as narrow white bars. The colors (gray shades in the print version) of protein-coding regions indicate core ORFs that are convincingly conserved among members of the family *Alloherpesviridae*, families of related ORFs, and other ORFs. Telomere-like repeats at the ends of TR are shown by gray-shaded blocks. Predicted poly(A) sites are indicated by vertical arrows above and below the genome for rightward- and leftward-oriented ORFs, respectively. *Reproduced with permission from Davison et al. (2013). Copyright © American Society for Microbiology.*

8 (ORF1–ORF8) ORFs, respectively. The latter are therefore duplicated in the copies of TR. One of the unusual features in the sequenced CyHV-3 genomes is the presence of fragmented, and therefore probably non-functional, ORFs. The precise set of such ORFs varies from strain to strain, and there is evidence that at least some originated *in vivo* rather than during viral isolation in cell culture. It is possible that loss of gene functions may have contributed to emergence of disease in carp populations.

Consistent with their close relationships, the 3 cyprinid herpesviruses share 120 conserved genes, of which up to 55 have counterparts in the more distantly related AngHV-1, which is also a member of the genus *Cyprinivirus*. However, as mentioned above, only 12 genes are conserved across the family *Alloherpesviridae* (see Section 2.1.2). The relevant ORFs are marked in Fig. 4, and their characteristics are listed in the upper part of Table 3. There are perhaps two additional genes in this core class (ORF66 and ORF99; not listed in Table 3), but the evidence for their conservation is minimal. Comments may be made on the features or functions of a sizeable number of the remaining gene products, as shown in the lower part of Table 3. This list omits genes that are members of related families and lack other clearly identifiable characteristics, such as incorporation into virions or similarity to other genes. It also excludes genes encoding proteins of which the only identifiable features are those indicating that they might be associated with membranes (e.g., the presence of potential signal peptides or hydrophobic transmembrane regions), which are numerous in CyHV-3. Also, the ancestors of CyHV-3 have evidently captured several genes from the host cell (e.g., the deoxyuridine triphosphatase and interleukin-10 genes) or other viruses (e.g., genes of which the closest relatives are found in iridoviruses or poxviruses) (Ilouze, Dishon, Kahan, & Kotler, 2006).

The CyHV-3 genome also contains five gene families that have presumably arisen by gene duplication, a mechanism for generating diversity that has been used commonly by herpesviruses in all three families. They are shaded in distinguishing colors (gray shades) in Fig. 4. These are the ORF2 family (ORF2, ORF3, ORF9, ORF129, ORF130, and ORF135), the TNFR family (ORF4 and ORF12, encoding proteins related to tumor necrosis factor receptor), the ORF22 family (ORF22, ORF24, and ORF137), the ORF25 family (ORF25, ORF26, ORF27, ORF65, ORF148, and ORF149, encoding potential membrane proteins containing an immunoglobulin domain), and the RING family (ORF41, ORF128, ORF144, and ORF150). Some of the proteins encoded by these genes are virion components (ORF137, ORF25, ORF27, ORF65,

Table 3 Information on Selected CyHV-3 ORFs[a]

ORF Name	Function or Features of Encoded Protein
Conserved among all sequenced members of the family *Alloherpesviridae*	
ORF33	DNA packaging terminase subunit 1
ORF46	Putative helicase–primase primase subunit
ORF47	Putative DNA packaging terminase subunit 2
ORF61	
ORF71	Putative helicase–primase helicase subunit
ORF72	Capsid triplex subunit 2; virion protein
ORF78	Capsid maturation protease; virion protein
ORF79	DNA polymerase catalytic subunit
ORF80	
ORF90	Virion protein
ORF92	Major capsid protein
ORF107	
Additional ORFs with recognizable features	
ORF4	Tumor necrosis factor receptor; member of TNFR gene family
ORF11	*Virion protein*
ORF12	Tumor necrosis factor receptor; member of TNFR gene family
ORF16	Predicted membrane protein; similar to G protein-coupled receptors
ORF19	Deoxyguanosine kinase
ORF23	Ribonucleotide reductase subunit 2
ORF25	Predicted membrane protein; contains an immunoglobulin domain; virion protein; member of ORF25 gene family
ORF27	Predicted membrane protein; contains an immunoglobulin domain; *virion protein*; member of ORF25 gene family
ORF28	Contains an NAD(P)-binding Rossmann-fold domain; similar to bacterial NAD-dependent epimerase/dehydratase
ORF31	Similar to eukaryotic PLAC8 proteins; virion protein
ORF32	Similar to a family of Singapore grouper iridovirus proteins; predicted membrane protein; virion protein
ORF34	*Virion protein*

Table 3 Information on Selected CyHV-3 ORFs—cont'd

ORF Name	Function or Features of Encoded Protein
ORF35	*Virion protein*
ORF36	*Virion protein*
ORF41	Contains a RING-type C3HC4 zinc finger domain; member of RING gene family
ORF42	Virion protein
ORF43	Virion protein
ORF44	*Virion protein*
ORF45	Virion protein
ORF48	Similar to protein kinases
ORF51	Virion protein
ORF54	Contains a putative zinc-binding domain
ORF55	Thymidine kinase
ORF57	Similar to crocodile poxvirus protein CRV155; virion protein
ORF59	Predicted membrane protein; virion protein
ORF60	Virion protein
ORF62	Contains an OTU-like cysteine protease domain; virion protein
ORF64	Predicted membrane protein; similar to equilibrative nucleoside transporter ENT1
ORF65	Predicted membrane protein; contains an immunoglobulin domain; virion protein; member of ORF25 gene family
ORF66	Capsid triplex subunit 1; virion protein
ORF68	Similar to myosin and related proteins; virion protein
ORF69	Virion protein
ORF70	Virion protein
ORF81	Multiple transmembrane protein; virion protein
ORF83	Predicted membrane protein; *virion protein*
ORF84	Virion protein
ORF89	Virion protein
ORF91	*Virion protein*
ORF94	Predicted membrane protein; similar to trypsin-like serine proteases

Continued

Table 3 Information on Selected CyHV-3 ORFs—cont'd

ORF Name	Function or Features of Encoded Protein
ORF95	Virion protein
ORF97	Virion protein
ORF98	Uracil-DNA glycosylase
ORF99	Predicted membrane protein; virion protein
ORF104	Similar to protein kinases
ORF106	*Virion protein*
ORF108	Predicted membrane protein; virion protein
ORF112	Contains a double-stranded nucleic acid-binding domain (helix–turn–helix); virion protein
ORF114	Predicted membrane protein; similar to *Danio rerio* LOC569866
ORF115	Predicted membrane protein; virion protein
ORF116	Predicted membrane protein; *virion protein*
ORF123	Deoxyuridine triphosphatase; *virion protein*
ORF128	Contains a RING-type C3HC4 zinc finger domain; similar to SPRY and TRIM proteins; member of RING gene family
ORF131	Predicted membrane protein; virion protein
ORF132	Predicted membrane protein; virion protein
ORF134	Interleukin-10
ORF136	Predicted membrane protein; virion protein
ORF137	*Virion protein*; member of ORF22 gene family
ORF139	Predicted membrane protein; similar to poxvirus B22R proteins
ORF140	Thymidylate kinase
ORF141	Ribonucleotide reductase subunit 1
ORF144	Contains a RING-type C3HC4 zinc finger domain; member of RING gene family
ORF148	Predicted membrane protein; contains an immunoglobulin domain; virion protein; member of ORF25 gene family
ORF149	Predicted membrane protein; contains an immunoglobulin domain; virion protein; member of ORF25 gene family
ORF150	Contains a RING-type C3HC4 zinc finger domain; member of RING gene family

[a]Data derived from Aoki et al. (2007), Michel, Leroy, et al. (2010), Yi et al. (2014), and Davison et al. (2013).
Italic-type indicates virion proteins detected in only some of the strains tested (Michel, Leroy, et al., 2010; Yi et al., 2014).

ORF148, and ORF149). Members of each of these gene families are also present in CyHV-1 and CyHV-2, whereas AngHV-1 lacks all but the TNFR family, having instead several other families that are absent from the cyprinid herpesviruses (Davison et al., 2013).

Herpesvirus genomes are described as infectious because their transfection into permissive cells is sufficient to initiate replication and the production of progeny virions. This property has been exploited to produce recombinant viruses by using bacterial artificial chromosome (BAC) cloning of the entire viral genome and prokaryotic recombination technologies. Such an approach has been used extensively for members of the *Herpesviridae* family (Tischer & Kaufer, 2012) and has been demonstrated to be also applicable to CyHV-3 (Costes et al., 2008).

3.1.3 Genotypes

Early investigations on CyHV-3 genetic diversity comparing partial DNA polymerase gene and partial major envelope protein gene sequences of CyHV-3 isolates from Japan, the USA, and Israel showed a high degree of nucleotide sequence identity (Ishioka et al., 2005). Similar sequence identities were also found among isolates from Poland and Germany (Antychowicz, Reichert, Matras, Bergmann, & Haenen, 2005; El-Matbouli, Saleh, & Soliman, 2007), suggesting that the virus causing disease in carp worldwide represented a single virus entity. Comparison of the complete genome sequences of three isolates from Japan (CyHV-3 J), the USA (CyHV-3 U), and Israel (CyHV-3 I) also revealed more than 99% identity (Aoki et al., 2007) which was consistent with this scenario.

Despite this close genetic relationship between isolates, the alignment of three complete CyHV-3 sequences revealed numerous minor deletions/insertions and single-nucleotide substitutions. These variations enabled a distinction between the CyHV-3 J lineage and the lineage represented by CyHV-3 U and CyHV-3 I isolates (Aoki et al., 2007; Bercovier et al., 2005). Recently, the full-length sequencing of a fourth strain, CyHV-3 GZ11 (isolated from a mass mortality outbreak in adult koi in China), revealed a closer relationship of this isolate with the CyHV-3 U/I lineage (Li et al., 2015). The existence of two lineages was confirmed on a larger set of European and Asian isolates using a PCR-based approach targeting two distinct regions of the genome (Bigarré et al., 2009). Marker I, located between ORF29 and ORF31 of CyHV-3 (Aoki et al., 2007), was 168 bp in length (designated I^{++}) for CyHV-3 J and only 130 bp (I^{--}) for the CyHV-3 U/I. Marker II, located upstream of ORF133, was 352 bp in

length in the CyHV-3 J sequence (II^{+}) compared to 278 bp (II^{-}) in the other two sequences. These studies also provided the first evidence of other potential genotypes, describing a unique genotype of CyHV-3 in koi carp from Poland that was identical to the CyHV-3 U/I viruses in marker II (II^{-}) but shared features of both the CyHV-3 J and CyHV-3 U/I genotypes in marker I (I^{-+}) (Bigarré et al., 2009). A similar profile was observed for a CyHV-3 strain from Korea and the GZ11 strain from China (Kim & Kwon, 2013; Li et al., 2015). The same markers were used to identify another novel "intermediate" genotype of CyHV-3 in Indonesia that resembled the CyHV-3 J genotype in marker I (I^{++}) but was identical to the CyHV-3 U/I genotype in marker II (II^{-}) (Sunarto et al., 2011). Sunarto et al. (2011) speculated that genotype $I^{--}II^{-}$ has evolved through genetic intermediates, $I^{-+}II^{-}$ and $I^{++}II^{-}$, to give rise to $I^{++}II^{+}$, and that the first genotype $I^{--}II^{-}$ (corresponding to E1 genotype based on the thymidine kinase (TK) gene sequence, see below) may be the origin of CyHV-3. Alternatively, it is suggested that an ancestral form diverged to give rise to two lineages, CyHV-3 J and CyHV-3 U/I (Aoki et al., 2007).

Analysis of the TK gene sequence (Bercovier et al., 2005), particularly the region immediately downstream of the stop codon, provided significantly more resolution (Table 4). In combination with sequence data for the SphI-5 (coordinates 93604–93895, NCBI: DQ657948) and the 9/5 (coordinates 165399–165882, NCBI: DQ657948) regions (Gilad et al., 2002; Gray et al., 2002), nine different genotypes were identified (Kurita et al., 2009). The CyHV-3 from Asia showed a high degree of sequence homology, although two variants were differentiated based on a single-nucleotide polymorphism in the TK gene (A1 and A2). In contrast, seven genotypes were identified in CyHV-3 from outside of Asia (E1–E7).

Interestingly, a study by Han et al. (2013) identified a sequence insertion in a glycoprotein gene (ORF125) of a Korean isolate (CyHV-3 K) compared with the viruses from Japan (CyHV-3 J), the USA (CyHV-3 U), and Israel (CyHV-3 I). This suggests that the CyHV-3 K is distinct from the CyHV-3 A1 and A2 genotypes. However, in the absence of comparable data from the TK gene, marker I or II regions, it is not possible to confirm this hypothesis (Han et al., 2013). In addition, some recent CyHV-3 isolates from Korea, Malaysia, and China were shown to belong to the E4 genotype which suggests the emergence of European lineages in Asia (Chen et al., 2014; Dong et al., 2013; Kim & Kwon, 2013; Li et al., 2015).

Besides the nucleotide mismatches, insertions, or deletions, much of the sequence differences between CyHV-3 isolates occurred at the level of

Table 4 Genotyping Scheme for CyHV-3 Based on Three Distinct Regions of the Genome: the 9/5 Region (Gilad et al., 2002), the SphI-5 Region (Gray, Mullis, LaPatra, Groff, & Goodwin, 2002), and the TK Gene (Bercovier et al., 2005)

		9/5 Region		SphI-5 Region		TK Gene							
Genotype	**Country of Origin**	**184–187**	**212–218**	**209**	**586–588**	**94**	**778**	**813–814**	**849–850**	**877–885**	**945–956**	**957–958**	**961–967**
A1	Japan[a,b], Indonesia[a], Taiwan[a], Philippines[a], South Korea[c], Malaysia[b], China[b]	TTTT	AAAAAA	C	–	C	A	–	AA	TTTTTTT	CTTTAAAAAAAA	–	AGATATT
A2	Indonesia[a], Taiwan[a]	TTTT	AAAAAA	C	–	C	A	–	AA	TTTTTTTT	CTTTAAAAAAAA	–	AGATATT
E1	USA[a], Netherlands[a]	TTTT	AAAAAAA	C	AAC	C	G	AT	–	TTTTTTTTT	CTTTAAAAAAAA	CA	AGATATT
E2	Netherlands[a]	TTTT	AAAAAAA	T	AAC	C	G	AT	–	TTTTTTTTT	CTTTAAAAAAAA	CA	AGATATT
E3	Netherlands[a]	–	AAAAAAA	C	AAC	C	G	AT	–	TTTTTTTTT	CTTTAAAAAAAA	CA	AGATATT
E4	Netherlands[a], South Korea[c], Malaysia[b], China (TK)[d]	TTTT	AAAAAAA	C	AAC	C	G	AT	AA	TTTTTTTTT	CTTTAAAAAAAA	CA	AGATATT
E5	Netherlands[a]	TTTT	AAAAAAA	C	AAC	C	G	AT	–	TTTTTTTTT	–	–	–
E6	Israel[a]	TTTT	AAAAAAA	C	AAC	T	G	AT	–	TTTTTTTTT	CTTTAAAAAAAA	CA	AGATATT
E7	UK[a]	TTTT	AAAAAAA	C	AAC	C	G	AT	–	TTTTTTTT	CTTTAAAAAAAA	CA	AGATATT

[a]From Kurita et al. (2009).
[b]From Chen et al. (2014).
[c]From Kim and Kwon (2013).
[d]From Dong, Li, Weng, Xie, and He (2013).
Adapted from Kurita et al. (2009).

variable number of tandem repeat (VNTR) sequences (Avarre et al., 2011). In agreement with other genetic studies (Bigarré et al., 2009; Kim & Kwon, 2013), analyses using multiple VNTR loci identified two lineages which were equivalent to the Asian and European viruses, but, with the increased discriminatory power of VNTR analysis, allowed the identification of up to 87 haplotypes (Avarre et al., 2011, 2012). As expected, several of the isolates from the Netherlands showed a close relationship to CyHV-3 J and were assigned to the same lineage, but the isolates from France and the Netherlands generally showed a closer relationship to CyHV-3 U/I and were assigned to the European lineage (Bigarré et al., 2009). Surprisingly, the Indonesian isolates, with a $I^{++}II^{-}$ haplotype (Sunarto et al., 2011), are closely related to CyHV-3 J and were assigned to the same lineage. No VNTR data were available for CyHV-3 K and GZ11 strains.

VNTR polymorphism has shown great potential for differentiating isolates of large DNA viruses such as human herpesvirus 1 (Deback et al., 2009). However, since the mechanism of VNTR evolution in CyHV-3 is not fully understood, it remains possible for the different phylogeographic types to share some VNTR features but have acquired them through separate evolutionary routes. Therefore, in future epidemiological studies on CyHV-3, it may be necessary to consider undertaking an initial phylogeographic analysis using the non-VNTR polymorphisms (insertions, deletions, and point mutations) observed throughout the genome and, only after, exploit the power of the VNTR variability to provide resolution to the isolate level.

3.1.4 Transcriptome

Herpesvirus gene expression follows a coordinated temporal pattern upon infection of permissive cells as shown in Fig. 2B (Pellett et al., 2011b). Immediate-early (IE) genes are first transcribed in the absence of *de novo* protein synthesis and regulate the subsequent expression of other genes. Expression of early (E) genes is dependent on IE-gene expression, and they encode enzymes and proteins involved in the modification of host cell metabolism and the viral DNA replication complex. The late (L) genes form the third and last set to be expressed, dependent on viral DNA synthesis, and primarily encode the viral structural proteins. The first indication that fish herpesvirus gene expression follows a similar temporal pattern came from *in vitro* studies on IcHV-1 transcription (Hanson et al., 2011).

More recently, two extensive genome-wide gene expression analyses of CyHV-3 (Ilouze, Dishon, & Kotler, 2012a) and AngHV-1 (van Beurden, Peeters, Rottier, Davison, & Engelsma, 2013) explored the kinetic class of each

annotated ORF following two approaches. First, gene expression was studied by RT-PCR or RT-qPCR during the first hours post-infection (hpi). Second, cycloheximide (CHX), and either cytosine-β-D-arabinofuranoside (Ara-C) or phosphonoacetic acid (PAA), were used to block *de novo* protein synthesis and viral DNA replication, respectively. In the presence of CHX, only IE genes are expressed, whereas in the presence of Ara-C or PAA, the IE and E genes but not the L genes are expressed. For CyHV-3, viral RNA synthesis was evident as early as 1 hpi, and viral DNA synthesis initiated between 4 and 8 hpi (Ilouze et al., 2012a). Transcription of 59 ORFs was detectable from 2 hpi, 63 ORFs from 4 hpi and 28 ORFs from 8 hpi. Transcription of six ORFs was only evident at 24 hpi (Table 5). Expression kinetics for related AngHV-1 genes were analyzed differently, thus hampering direct comparison, but in general followed the same pattern (van Beurden et al., 2013). RNAs from all 156 predicted ORFs of CyHV-3 were detected (including ORF58 which was initially predicted based on a marginal prediction but recently removed from the predicted genome map (Fig. 4; Davison et al., 2013)), and based on the observation that antisense transcription for related AngHV-1 was very low, it is expected that all annotated ORFs indeed code for viral RNAs (Aoki et al., 2007; Ilouze et al., 2012a; van Beurden, Gatherer, et al., 2012).

By blocking protein synthesis or viral DNA replication, 15 IE, 112 E, and 22 L genes were identified for CyHV-3, whereas for 7 ORFs no classification was possible (Ilouze et al., 2012a; Table 5). In general, this classification followed the expression kinetics determined for each ORF, with most IE genes being expressed at 1 or 2 hpi, most E genes between 2 and 4 hpi and most L genes at 8 hpi. For AngHV-1, 4 IE genes, 54 E or E-L genes, and 68 L genes were found (van Beurden et al., 2013). As there is no clear boundary between the E-L (or leaky-late) and L genes, these differences may be explained by sensitivity of the method used to determine the onset of gene expression and data analysis. Similar to mammalian herpesviruses, gene transcripts known to be involved in DNA replication were expressed early, while proteases and enzymes involved in virion assembly and maturation were expressed late (Ilouze et al., 2012a; van Beurden et al., 2013). Inhibition of some E genes involved in DNA replication (e.g., TK and DNA polymerase) by specific siRNA decreased viral release from infected cells (Gotesman, Soliman, Besch, & El-Matbouli, 2014).

Interestingly, in IcHV-1, CyHV-3, and AngHV-1, the IE genes show a clear clustering in or near the TRs, suggesting positional conservation of these regulatory genes (Ilouze et al., 2012a; Stingley & Gray, 2000; van Beurden

Table 5 Transcriptomic Classification of CyHV-3 ORFs

ORF	Putative Function[a]	Kinetic Class[b]	22 °C[c] (hpi)	30 °C[d] (dpi)
1L/R		IE	2	1–8
2L/R		E	2	1
3L/R		IE	2	1
4L/R	Immune regulation	E	2	1–8
5L/R		E	4	1
6L/R		IE	2	–
7L/R		IE	2	1–8
8L/R		IE	2	1
9		IE	2	1
10		IE	2	1–8
11	Virion protein	IE	2	1
12	Immune regulation	L	8	1
13		E	2	1
14		E	2	1
15		E	2	1
16	Intracellular signaling	E	2	1
17		L	2	1
18		E	2	1
19	Nucleotide metabolism	E	4	1
20		E	4	1
21		E	4	1
22		E	4	–
23	Nucleotide metabolism	E	2	1
24		IE	2	1
25	Virion protein	E	4	1
26		E	2	1
27	Virion protein	E	2	1
28		E	4	–

Table 5 Transcriptomic Classification of CyHV-3 ORFs—cont'd

ORF	Putative Function	Kinetic Class	22 °C (hpi)	30 °C (dpi)
29		E	2	1
30		E	4	1–8
31	Virion protein	E	4	1
32	Virion protein	E	2	1–8
33	DNA encapsidation	E	4	1
34	Virion protein	UN	24	–
35	Virion protein	E	4	1
36	Virion protein	E	4	1
37		E	2	1–8
38		E	2	1–8
39		E	2	1–8
40		E	2	1
41		E	2	1
42	Virion protein	E	4	1–8
43	Virion protein	E	4	1
44	Virion protein	L	8	–
45	Virion protein	E	4	1
46	DNA replication	E	4	1
47	DNA encapsidation	L	8	1
48	Protein phosphorylation	E	2	1
49		UN	24	–
50		E	2	1
51	Virion protein	E	4	1
52		E	4	1
53		E	2	1
54		IE	2	1
55	Nucleotide metabolism	E	2	1
56		E	2	1–8

Continued

Table 5 Transcriptomic Classification of CyHV-3 ORFs—cont'd

ORF	Putative Function	Kinetic Class	22 °C (hpi)	30 °C (dpi)
57	Virion protein	UN	8	1
58		E	4	–
59	Virion protein	E	4	1
60	Virion protein	E	4	1
61		E	4	1
62	Virion protein	L	8	–
63		E	4	–
64		E	2	–
65	Virion protein	L	8	–
66	Virion protein/capsid morphogenesis	E	4	1
67		E	4	–
68	Virion protein	L	8	1
69	Virion protein	UN	24	–
70	Virion protein	UN	24	1–8
71	DNA replication	E	4	1
72	Virion protein/capsid morphogenesis	E	4	–
73		E	8	1
74		L	8	–
75		L	8	1
76		L	8	1
77		E	4	–
78	Virion protein/capsid morphogenesis	L	8	–
79	DNA replication	E	4	–
80		E	4	1
81	Virion protein	E	4	1
82		E	4	–

Table 5 Transcriptomic Classification of CyHV-3 ORFs—cont'd

ORF	Putative Function	Kinetic Class	22 °C (hpi)	30 °C (dpi)
83	Virion protein	E	8	1–8
84	Virion protein	E	4	1
85		L	8	1
86		L	8	1
87		E	4	1
88		IE	4	–
89	Virion protein	L	8	–
90	Virion protein	E	8	–
91	Virion protein	E	4	–
92	Virion protein/major capsid protein	E	4	1
93		E	4	1
94		E	4	–
95	Virion protein	L	8	1
96		E	4	1
97	Virion protein	L	8	1
98	DNA repair	E	4	1
99	Virion protein	E	8	–
100		E	4	1
101		E	4	–
102		E	4	–
103		E	4	–
104	Protein phosphorylation	E	4	1
105		UN	24	–
106	Virion protein	L	8	–
107		E	4	–
108	Virion protein	E	4	–
109		E	4	–

Continued

Table 5 Transcriptomic Classification of CyHV-3 ORFs—cont'd

ORF	Putative Function	Kinetic Class	22 °C (hpi)	30 °C (dpi)
110		L	8	–
111		E	2	1
112	Virion protein/immune regulation	IE	2	–
113		E	8	–
114		L	8	1–18
115	Virion protein	L	8	1–18
116	Virion protein	E	4	1
117		E	2	1
118		E	2	1
119		UN	24	–
120		E	2	1
121		E	2	1
122		E	4	1
123	Virion protein/nucleotide metabolism	E	4	1
124		E	4	1–8
125		L	8	1
126		L	8	–
127		E	2	1
128		E	4	1
129		E	4	1
130		E	2	1
131	Virion protein	E	4	1
132	Virion protein	E	2	1
133		E	4	1
134	Immune regulation	E	2	1
135		E	4	–
136	Virion protein	E	4	1

Table 5 Transcriptomic Classification of CyHV-3 ORFs—cont'd

ORF	Putative Function	Kinetic Class	22 °C (hpi)	30 °C (dpi)
137	Virion protein	E	2	1
138		E	2	1
139	Immune regulation	E	2	1–8
140	Nucleotide metabolism	E	4	–
141	Nucleotide metabolism	E	8	–
142		E	2	1
143		E	2	1–8
144		E	4	–
145		E	4	–
146		IE	2	1
147		E	2	–
148	Virion protein	E	4	1
149	Virion protein	IE	2	–
150		E	2	–
151		E	2	1
152		E	2	1
153		E	2	1
154		E	2	1
155		IE	2	1–8
156		E	2	1

[a]Putative gene functions were adapted from Davison et al. (2013).
[b]Kinetic class as determined by transcription analysis in the presence of CHX or Ara-C (adapted from Ilouze et al., 2012a). Light grey: immediate early (IE) gene; intermediate grey: early (E) gene; dark grey: late (L) gene; white: unknown (UN).
[c]Initiation of viral mRNA transcription at permissive temperature (adapted from Ilouze et al., 2012a).
[d]Presence of CyHV-3 transcripts at restrictive temperature (adapted from Ilouze, Dishon, & Kotler, 2012b).
dpi, days post-infection; hpi, hours post-infection.

et al., 2013). The E and L genes are mainly located in the unique long region of the genome, with almost half of the CyHV-3 E genes clustered and transcribed simultaneously (Ilouze et al., 2012a). This observation may be biased, however, by 3′-coterminality of transcripts, which was shown to be abundant in the AngHV-1 genome (van Beurden, Gatherer, et al., 2012).

3.1.5 Structural Proteome and Secretome

Initial predictions of the structural proteome of CyHV-3 were based on comparison with experimental findings obtained for IcHV-1 and bioinformatically predicted properties of the putative CyHV-3 encoded proteins (Aoki et al., 2007; Davison & Davison, 1995). More recently, two independent studies explored the structural proteome of one European and two Chinese CyHV-3 isolates by a combination of virus particle purification, gel electrophoresis, and mass spectrometry-based proteomic approaches (Michel, Leroy, et al., 2010; Yi et al., 2014). A total of 34 structural proteins were identified for all 3 CyHV-3 isolates, and another 12 proteins were found in only 1 or 2 of the 3 studied isolates (Table 6 and Fig. 5). The latter were generally of low abundance, suggesting that these small differences in protein constitution indicate either strain-specific variation or interstudy variation. Overall, the total number of structural proteins of viral origin reported for CyHV-3 (46) corresponds with the number reported for closely related AngHV-1 (40) and is in line with numbers reported for members of the *Herpesviridae* family, e.g., 44 for herpes simplex virus 1 (Loret, Guay, & Lippe, 2008).

Comparisons of homologous genes with similar studies for related alloherpesviruses IcHV-1 and AngHV-1, as well as bioinformatical predictions of protein properties, enabled putative localization of the proteins within the virion (Table 6). Based on these predictions, five capsid proteins were identified, including the highly conserved major capsid protein, capsid triplex subunit 1 and 2, and the capsid maturation protease. Indeed, the architecture and protein composition of fish herpesvirus capsids generally mirror that of mammalian herpesviruses, with the exception of the small protein which forms the hexon tips in mammalian herpesviruses (Booy et al., 1996; Davison & Davison, 1995). Comparison with the closely related AngHV-1 resulted in the identification of 11 tegument or tegument-associated proteins, including the large tegument protein ORF62 (Michel, Leroy, et al., 2010; van Beurden, Leroy, et al., 2011; Yi et al., 2014). Bioinformatical predictions for signal peptides, transmembrane domains, and glycosylation allowed the identification of a total of 16 putative membrane proteins (Aoki et al., 2007; Michel, Leroy, et al., 2010; Yi et al., 2014).

In addition, several studies dedicated to specific virion proteins have been carried out (Aoki et al., 2011; Dong et al., 2011; Fuchs, Granzow, Dauber, Fichtner, & Mettenleiter, 2014; Rosenkranz et al., 2008; Tu et al., 2014; Vrancken et al., 2013; Yi et al., 2014). Some of these proteins have been studied in more detail, notably ORF81, which is a type 3 membrane protein and is thought to be one of the most immunogenic (major) membrane

Table 6 Structural Proteome of CyHV-3

ORF	NCBI ID	Predicted MM (kDa)	Predicted Localization	Protein Description[a]	No. of Peptides[b]		
					FL	GZ11	GZ10
11	131840041	13.1	Unknown	–	–	1	2
25	131840055	67.1	Envelope[c]	Predicted membrane protein; ORF25 gene family	7	6	8
27	380708459	47.9	Envelope[c]	Predicted membrane protein; ORF25 gene family	–	1	1
31	131840058	13.9	Unknown	Similar to eukaryotic PLAC8 proteins	2	3	7
32	131840059	22.3	Envelope[c]	Predicted membrane protein; similar to a family of Singapore grouper iridovirus proteins	3	2	3
34	131840061	17	Unknown	–	2	3	–
35	131840062	36.3	Unknown	–	1	–	1
36	131840063	30.3	Unknown	–	1	–	–
42	131840068	53.5	Tegument[d]	Related to AngHV-1 ORF18	13	18	24
43	131840069	159.4	Unknown	–	48	51	59
44	131840070	97.5	Unknown	–	4	–	–
45	131840045	97.5	Tegument[d]	Related to AngHV-1 ORF20	5	4	6
51	131840077	165.9	Tegument-associated[d]	Related to AngHV-1 ORF34	41	38	48

Continued

Table 6 Structural Proteome of CyHV-3—cont'd

ORF	NCBI ID	Predicted MM (kDa)	Predicted Localization	Protein Description	No. of Peptides		
					FL	GZ11	GZ10
57	131840083	54	Tegument-associated[d]	Similar to crocodile poxvirus CRV155; related to AngHV-1 ORF35	17	11	20
59	131840085	14.6	Envelope[c]	Predicted membrane protein	2	1	2
60	131840086	59.9	Tegument-associated[d]	Related to AngHV-1 ORF81	10	4	12
62	131840088	442.2	Tegument-(associated)[d,e]	Contains an OTU-like cysteine protease domain; related to AngHV-1 ORF83 and IcHV-1 ORF65	76	83	92
65	131840091	63.5	Envelope[c]	Predicted membrane protein; member of ORF25 gene family	10	6	10
66	131840092	45.4	Capsid[d]	Capsid triplex subunit 1; related to AngHV-1 ORF42	13	10	21
68	131840094	253	Unknown	Similar to myosin-related proteins; related to IcHV-1 ORF22, RaHV-1 ORF56 and ORF89, and RaHV-2 ORF126	59	77	75
69	131840095	58.9	Tegument[d]	Related to AngHV-1 ORF39	1	1	3
70	131840096	51.1	Tegument[d]	Related to AngHV-1 ORF38	2	4	3
72	131840098	40.7	Capsid[d,e]	Capsid triplex subunit 2; related to AngHV-1 ORF36, IcHV-1 ORF27, RaHV-1 ORF95, and RaHV-2 ORF131	10	11	13

78	131840104	76.9	Capsid[d,e]	Capsid maturation protease; related to AngHV-1 ORF57, IcHV-1 ORF28, RaHV-1 ORF63, and RaHV-2 ORF88	5	2	5
81	131840107	28.6	Envelope[c–e]	Multiple transmembrane protein; related to AngHV-1 ORF51, positionally similar to IcHV-1 ORF59, RaHV-1 ORF83, and RaHV-2 ORF117	3	5	3
83	131840109	26.9	Envelope[c,d]	Predicted multiple transmembrane protein; related to AngHV-1 ORF49	–	2	3
84	131840110	85.6	Unknown	–	25	21	32
89	131840115	53.5	Unknown	–	7	5	10
90	131840116	86.1	Capsid[d]	Related to AngHV-1 ORF100, IcHV-1 ORF37, RaHV-1 ORF52, and RaHV-2 ORF78	9	11	14
91	131840117	26.4	Tegument[d]	Related to AngHV-1 ORF103	–	–	1
92	131840118	140.4	Capsid[d,e]	Major capsid protein; related to AngHV-1 ORF104, IcHV-1 ORF39, RaHV-1 ORF54, and RaHV-2 ORF80	45	32	45
95	131840121	24.2	Unknown	–	3	1	5

Continued

Table 6 Structural Proteome of CyHV-3—cont'd

ORF	NCBI ID	Predicted MM (kDa)	Predicted Localization	Protein Description	No. of Peptides		
					FL	GZ11	GZ10
97	131840123	117.5	Tegument-associated[d]	Related to AngHV-1 ORF30	19	20	22
99	131840125	170.7	Envelope[c,d]	Predicted membrane protein; related to AngHV-1 ORF67, IcHV-1 ORF46, RaHV-1 ORF46, RaHV-2 ORF72	34	14	16
106	131840132	7.5	Unknown	–	–	1	–
108	131840134	21	Envelope[c]	Predicted membrane protein	2	1	3
112	131840138	31	Unknown	Contains a double-stranded nucleic acid-binding domain (helix–turn–helix)	1	1	1
115	131840141	86.2	Envelope[c]	Predicted membrane protein	14	12	17
116	131840142	30.4	Envelope[c]	Predicted membrane protein	–	–	1
123	131840149	29.5	Tegument[c]	Deoxyuridine triphosphatase; related to AngHV-1 ORF5, IcHV-1 ORF49, and RaHV-2 ORF142; also encoded by some iridoviruses and poxviruses	2	–	4
131	131840157	30.6	Envelope[c]	Predicted membrane protein	5	3	4
132	131840158	19	Envelope[c]	Predicted membrane protein	2	4	1

136	131840162	17	Envelope[c]	Predicted membrane protein	2	3	3
137	131840163	69.7	Unknown	Member of ORF22 gene family	4	–	–
148	131840174	64.8	Envelope[c]	Predicted membrane protein; member of ORF25 gene family	7	4	6
149	131840175	72.8	Envelope[c]	Predicted membrane protein; member of ORF25 gene family	7	9	9

[a]Protein descriptions adapted from Michel, Leroy, et al. (2010).
[b]Number of peptides detected as determined by Michel, Leroy, et al. (2010) (FL strain) and Yi et al. (2014) (GZ11 and GZ10 strains).
[c]Predicted based on bioinformatical predictions, adapted from Aoki et al. (2007).
[d]Predicted based on sequence homology with AngHV-1 as determined by van Beurden, Leroy, et al. (2011).
[e]Predicted based on sequence homology with IcHV-1 as determined by Davison and Davison (1995).
MM, molecular mass.

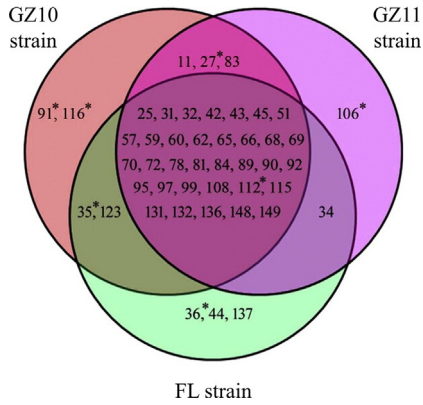

Figure 5 Structural proteome of CyHV-3. Schematic representation of virion-associated proteins from two CyHV-3 Chinese isolates (GZ10 and GZ11) (Yi et al., 2014) and one European isolate (FL) (Michel, Leroy, et al., 2010). Numbers indicate CyHV-3 ORFs. A total of 46 viral proteins were identified from which 34 were consistently identified in the three CyHV-3 isolates. Asterisks indicate viral proteins in which only one matched peptide was detected. *Adapted with permission from Yi et al. (2014). Copyright © Elsevier.*

proteins of CyHV-3 (Rosenkranz et al., 2008). Based on their high abundance and unique locations upon SDS-PAGE of purified proteins, Yi et al. (2014) marked ORF43, ORF51, ORF62, ORF68, ORF72, ORF84, and ORF92 as the major structural proteins of CyHV-3 (Yi et al., 2014). Two of these proteins, namely the large tegument protein encoded by ORF62 and ORF68, had previously been identified as major antigenic CyHV-3 proteins by immunoscreening (Aoki et al., 2011). Moreover, sera from infected carp reacted also against cells transfected with plasmids encoding for ORF25, ORF65, ORF148, ORF149 (four members of the ORF25 family; envelope proteins), ORF99 (envelope protein), and ORF92 (major capsid protein) (Fuchs et al., 2014).

The degree of conservation of the tegument and envelope proteins among fish herpesviruses is limited, with only one large tegument protein and potentially two envelope proteins being conserved between CyHV-3, AngHV-1, and IcHV-1 (van Beurden, Leroy, et al., 2011). For AngHV-1, the distribution of the structural proteins across the different viral compartments resembles that of other herpesviruses, with decreasing numbers for the different proteins from tegument to envelope to capsid (van Beurden, Leroy, et al., 2011). Although the localization of the CyHV-3 structural proteins remains to be demonstrated experimentally, a similar ratio may be expected, implying that most of the yet unclassified proteins could be located in the tegument.

Both studies on the CyHV-3 structural proteome also identified 18–27 cellular proteins associated with extracellular CyHV-3 virions (Michel, Leroy, et al., 2010; Yi et al., 2014). Similar to mammalian herpesviruses, these include proteins involved in stress response, signal transduction, vesicular trafficking, metabolism, cytoskeleton organization, translational control, immunosuppression, and cell-signaling regulation. Except for the so-called virus-induced stress protein identified by Yi et al. (2014), host cellular proteins were generally low in abundance suggesting them as minor components of the virions (Michel, Leroy, et al., 2010; Yi et al., 2014).

The viral secretome of CyHV-3 was examined by analyzing concentrated supernatants of infected cell cultures by mass spectrometry (Ouyang et al., 2013). Five viral proteins were identified, of which the two most abundant were ORF12 encoding a soluble TNF receptor homolog, and ORF134 encoding an IL-10 homolog. Three additional viral proteins (encoded by ORF52, ORF116, and ORF119) had previously been predicted to be potential membrane proteins, but were not convincingly identified as such. Overall, the identification of the viral and cellular protein composition of the virions and viral secretome represents a milestone in fundamental CyHV-3 research and may facilitate the development of diagnostic and prophylactic applications (see, for example, Fuchs et al., 2014; Vrancken et al., 2013).

3.1.6 Viral Replication in Cell Culture

3.1.6.1 Cell Lines Permissive to CyHV-3

CyHV-3 can be cultivated in cell lines derived from common carp brain (CCB) (Davidovich, Dishon, Ilouze, & Kotler, 2007; Neukirch, Böttcher, & Bunnajrakul, 1999), gills (CCG) (Neukirch et al., 1999), and fin (CaF-2, CCF-K104, MFC) (Imajoh et al., 2014; Neukirch & Kunz, 2001; Zhou et al., 2013). Permissive cell lines have also been derived from koi fin: KF-1 (Hedrick et al., 2000), KFC (Hutoran et al., 2005; Ronen et al., 2003), KCF-1 (Dong et al., 2011), NGF-2 and NGF-3 (Miwa et al., 2007), and KF-101 (Lin, Cheng, Wen, & Chen, 2013; Table 7). Other permissive cell lines were developed from snout tissues (MSC, KS) (Wang et al., 2015; Zhou et al., 2013). Non-carp cell lines, such as silver carp fin (Tol/FL) and goldfish fin (Au), were also described as permissive to CyHV-3 (Davidovich et al., 2007). One report showed cytopathic effect (CPE) in a cell line from fathead minnow (FHM cell line) after inoculation with CyHV-3 (Grimmett et al., 2006), but this observation was not confirmed by others (Davidovich et al., 2007; Hedrick et al., 2000; Neukirch et al., 1999). Similarly, Neukirch et al. (1999) and Neukirch and Kunz

Table 7 Cell Lines Susceptible to CyHV-3 Infection

		Cytopathic Effect	
Origin	**Name**	**Yes**	**No**
Cyprinus carpio			
Brain			
Common carp brain	CCB	Davidovich et al. (2007) Neukirch et al. (1999)	
Gills			
Common carp gill	CCG	Neukirch et al. (1999)	
Fins/skin			
Common carp fin	CaF-2	Neukirch and Kunz (2001)	
	CCF-K104	Imajoh et al. (2014)	
	MFC	Zhou et al. (2013)	
Common carp skin tumor	EPC	Neukirch et al. (1999) Neukirch and Kunz (2001)	Hedrick et al. (2000)[a] Hutoran et al. (2005) Davidovich et al. (2007)
Koi carp fin	KF-1	Hedrick et al. (2000)	
	KFC	Hutoran et al. (2005)	
	KCF-1	Dong et al. (2011)	
	NGF-2(-3)	Miwa et al. (2007)	
	KF-101	Lin et al. (2013)	
Common carp snout	MSC	Zhou et al. (2013)	
Koi carp snout	KS	Wang et al. (2015)	
Other species			
Silver carp fin (*Hypophthalmichthys molitrix*)	Tol/FL	Davidovich et al. (2007)	
Goldfish fin (*Carassius auratus*)	Au	Davidovich et al. (2007)	
Fathead minnow connective tissue and muscle (*Pimephales promelas*)	FHM	Grimmett, Warg, Getchell, Johnson, and Bowser (2006)	Neukirch et al. (1999) Hedrick et al. (2000) Davidovich et al. (2007)
Chinook salmon embryo (*Oncorhynchus tshawytscha*)	CHSE-214		Neukirch et al. (1999)
Channel catfish ovary (*Ictalurus punctatus*)	CCO		Davidovich et al. (2007)

[a]Only transient CPE.

(2001) reported CPE in EPC (*epithelioma papulosum cyprini*) cells but this observation was also not confirmed (Davidovich et al., 2007; Hedrick et al., 2000; Hutoran et al., 2005). This discrepancy could be partially explained by the controversial origin of the EPC cell line. This cell line was initially described as originating from common carp but was recently found to derive from fathead minnow (Winton et al., 2010). Other commonly used cell lines such as CHSE-214 (chinook salmon embryo) (Neukirch et al., 1999) and CCO (channel catfish ovary) (Davidovich et al., 2007) are not permissive to CyHV-3 infection. Typical CPE induced by CyHV-3 includes vacuolization and increased cell volume. Infected cells form characteristic plaques that grow according to time post-infection, frequently leading to the formation of syncytia (Ilouze, Dishon, & Kotler, 2006). Finally, infected cells become rounded before they detach from the substrate. Infectious virions are mostly retrieved from the infected cell supernatant (cell-free fraction) (Gilad et al., 2003). Isolation and adaptability of CyHV-3 *in vitro* seem to vary according to the field strain and cell line used. However, well-adapted laboratory strains usually reach titers up to 10^6–10^7 pfu/ml (Ilouze, Dishon, & Kotler, 2006).

3.1.6.2 Temperature Restriction

CyHV-3 replication is restricted by temperature *in vitro* and *in vivo*. *In vitro*, optimal viral growth was observed in KF-1 cell line at temperatures between 15 and 25 °C (Gilad et al., 2003); however, within this range, temperature affected the time at which viral production peaked (e.g., peak of viral titer observed at 7 days post-infection (dpi) and 13 dpi after incubation at 20–25 and 15 °C, respectively) (Gilad et al., 2003).

Virus production, virus gene transcription, and genome replication are gradually turned off when cells are moved from permissive temperature to the nonpermissive temperature of 30 °C (Dishon, Davidovich, Ilouze, & Kotler, 2007; Ilouze et al., 2012b; Imajoh et al., 2014). Although most of the 110 ORFs still transcribed 24 h after the temperature shift are gradually shut off (Table 5), few ORFs such as ORF114 and 115 were still expressed 18 days after temperature shift. However, infected cells maintained for 30 days at 30 °C preserve the potential to reinitiate a productive infection when returned to permissive temperatures (Dishon et al., 2007). This state of abortive infection with the potential to reactivate resembles latency as described for *Herpesviridae*. Putatively, the viral membrane protein encoded by ORF115 may represent an Epstein–Barr virus-like membrane-bound antigen associated with latency.

3.2 CyHV-3 Disease

3.2.1 Epidemiology

3.2.1.1 Fish Species Susceptible to CyHV-3 Infection

There is evidence that CyHV-3 can infect a wide range of species but that it only induces disease in common and koi carp. Hybrids of koi × goldfish and koi × crucian carp can be infected by CyHV-3, with mortality rates of 35% and 91%, respectively (Bergmann, Sadowski, et al., 2010). Common carp × goldfish hybrids have also been reported to show some susceptibility to CyHV-3 infection; however, the mortality rate observed was rather limited (5%) (Hedrick, Waltzek, & McDowell, 2006). PCR detection of CyHV-3 performed on cyprinid and non-cyprinid fish species, but also on freshwater mussels and crustaceans, suggested that these species could act as a reservoir of the virus (Table 8; El-Matbouli et al., 2007; El-Matbouli & Soliman, 2011; Fabian et al., 2013; Kempter & Bergmann, 2007; Kempter et al., 2009, 2012; Kielpinski et al., 2010; Radosavljevic et al., 2012). Cohabitation experiments suggest that some of these fish species (goldfish, tench, vimba, common bream, common roach, European perch, ruffe, gudgeon, rudd, northern pike, Prussian carp, silver carp, and grass carp) can carry CyHV-3 asymptomatically and transmit it to naive carp (Bergmann, Lutze, et al., 2010; El-Matbouli & Soliman, 2011; Fabian et al., 2013; Kempter et al., 2012; Radosavljevic et al., 2012). Recent studies provided increasing evidence that CyHV-3 can infect goldfish asymptomatically (Bergmann, Lutze, et al., 2010; El-Matbouli & Soliman, 2011; Sadler, Marecaux, & Goodwin, 2008), although some discrepancies exist in the literature (Yuasa et al., 2013). Consistent with this observation, *in vitro* studies showed that CyHV-3 can replicate and cause CPE in cell cultures derived not only from common and koi carp but also from silver carp and goldfish (Davidovich et al., 2007). Finally, the World Organization for Animal Health (OIE) lists one KHVD susceptible species (*C. carpio* and its hybrids) and several suspected carrier fish species (goldfish, grass carp, ide, catfish, Russian sturgeon, and Atlantic sturgeon) (OIE, 2012).

3.2.1.2 Geographical Distribution and Prevalence

The geographical range of the disease caused by CyHV-3 has become extensive since the first outbreaks in Germany in 1997 and in the USA and Israel in 1998 (Bretzinger et al., 1999; Hedrick et al., 2000; Perelberg et al., 2003). Worldwide trade in common and koi carp is generally held responsible for the spread of the virus before methods of detection were available and

Table 8 Organisms Tested for CyHV-3 Infection

Common Name (Species)	Detection of CyHV-3			Detection of CyHV-3 Genome in Carp After Cohabitation
	DNA	Transcript	Antigen	
Vertebrates				
Cyprinidae				
Goldfish (*Carassius auratus*)	Yes[a–d,k]/no[e]	Yes[b]	Yes[c]	Yes[b–d]/no[e]
Ide (*Leuciscus idus*)	Yes[a,g]	nt	nt	nt
Grass carp (*Ctenopharyngodon idella*)	Yes[a,d,g]	nt	nt	Yes[d,g]
Silver carp (*Hypophthalmichthys molitrix*)	Yes[d,g]	nt	nt	Yes[d,g]
Prussian carp (*Carassius gibelio*)	Yes[d,g]/no[h]	nt	nt	Yes[d]/no[h]
Crucian carp (*Carassius carassius*)	Yes[g]	nt	nt	nt
Tench (*Tinca tinca*)	Yes[d,g,h]	nt	nt	Yes[d,g,h]
Vimba (*Vimba vimba*)	Yes[f,g]	nt	nt	Yes[g]
Common bream (*Abramis brama*)	Yes[g,h]	nt	nt	Yes[g]
Common roach (*Rutilus rutilus*)	Yes[g,h]	nt	nt	Yes[g]/no[h]
Common dace (*Leuciscus leuciscus*)	Yes[f,g,h]	nt	nt	No[h]
Gudgeon (*Gobio gobio*)	Yes[g,h]	nt	nt	Yes[h]
Rudd (*Scardinius erythrophthalmus*)	Yes[h]	nt	nt	Yes[h]
European chub (*Squalius cephalus*)	Yes[g]/no[h]	nt	nt	nt
Common barbel (*Barbus barbus*)	Yes[g]	nt	nt	nt
Belica (*Leucaspius delineatus*)	Yes[g]	nt	nt	nt

Continued

Table 8 Organisms Tested for CyHV-3 Infection—cont'd

Common Name (Species)	Detection of CyHV-3			Detection of CyHV-3 Genome in Carp After Cohabitation
	DNA	Transcript	Antigen	
Common nase (*Chondrostoma nasus*)	Yes[g]	nt	nt	nt
Acipenseridae				
Russian sturgeon (*Acipenser gueldenstaedtii*)	Yes[i]	nt	nt	nt
Atlantic sturgeon (*Acipenser oxyrinchus*)	Yes[i]	nt	nt	nt
Cobitidae				
Spined loach (*Cobitis taenia*)	Yes[g]	nt	nt	nt
Cottidae				
European bullhead (*Cottus gobio*)	Yes[g]	nt	nt	nt
Esocidae				
Northern pike (*Esox lucius*)	Yes[g,h]	nt	nt	Yes[h]
Gasterosteidae				
Three-spined stickleback (*Gasterosteus aculeatus*)	Yes[h]	nt	nt	No[h]
Ictaluridae				
Brown bullhead (*Ameiurus nebulosus*)	Yes[h]	nt	nt	No[h]
Loricariidae				
Ornamental catfish (*Ancistrus sp.*)	Yes[a]	nt	nt	nt
Percidae				
European perch (*Perca fluviatilis*)	Yes[g,h]	nt	nt	Yes[g]/no[h]
Ruffe (*Gymnocephalus cernua*)	Yes[g]/no[h]	nt	nt	Yes[g,h]

Table 8 Organisms Tested for CyHV-3 Infection—cont'd

Common Name (Species)	Detection of CyHV-3			Detection of CyHV-3 Genome in Carp After Cohabitation
	DNA	Transcript	Antigen	
Invertebrates				
Swan mussels (*Anodonta cygnea*)	Yes[j]	nt	nt	nt
Scud (*Gammarus pulex*)	Yes[j]	nt	nt	nt

[a]Bergmann et al. (2009).
[b]El-Matbouli and Soliman (2011).
[c]Bergmann, Lutze, et al. (2010).
[d]Radosavljevic et al. (2012).
[e]Yuasa, Sano, and Oseko (2012).
[f]Kempter and Bergmann (2007).
[g]Kempter et al. (2012).
[h]Fabian, Baumer, and Steinhagen (2013).
[i]Kempter et al. (2009).
[j]Kielpinski et al. (2010).
[k]El-Matbouli et al. (2007).
nt, not tested.
Adapted with permission from Rakus et al. (2013); Original publisher BioMed Central.

implemented (OIE, 2012). The disease is now known to occur in, or has been reported in fish imported into, at least 28 different countries (OIE, 2012).

In Europe, reports of widespread mass mortality have been notified in carp farms and fisheries in Germany, Poland, and the UK (Bergmann, Kempter, Sadowski, & Fichtner, 2006; Gotesman, Kattlun, Bergmann, & El-Matbouli, 2013; Taylor, Dixon, et al., 2010). The disease is also known to occur in, or has been recorded in fish imported into, Austria, Belgium, Czech Republic, Denmark, France, Hungary, Italy, Luxembourg, The Netherlands, Republic of Ireland, and Switzerland (Haenen et al., 2004; McCleary et al., 2011; Pokorova et al., 2010; Pretto et al., 2013). Most recently, KHVD outbreaks have been reported to the OIE from Romania, Slovenia, Spain, and Sweden (OIE, 2012). Three novel CyHV-3-like viruses were also identified by PCR in The Netherlands, UK, Austria, and Italy, sharing only 95–98% nucleotide identity with the CyHV-3 J, CyHV-3 I, and CyHV-3 U strains. Carp carrying the CyHV-3 variants did not show clinical signs consistent with CyHV-3 infection and originated from locations with no actual CyHV-3 outbreaks. These strains might represent low- or nonpathogenic variants of CyHV-3 (Engelsma et al., 2013).

In Asia, in the Middle East, the first disease outbreaks with mass mortalities were seen in Israel in 1998 and in the following 3 years, the virus had spread to 90% of all carp farms (Perelberg et al., 2003). In southeastern Asia, the first outbreaks of KHVD, with mass mortalities of cultured koi carp, occurred in Indonesia in 2002 and were associated with an importation of koi from Hong Kong (Haenen et al., 2004; Sunarto et al., 2011). Later in 2002, the first occurrence of CyHV-3 infection was reported in koi carp in Taiwan (Tu, Weng, Shiau, & Lin, 2004). In 2003, detection of CyHV-3 was first reported in Japan following mass mortalities of cage-cultured common carp in the Ibaraki prefecture (Sano et al., 2004). Since then, the virus has been confirmed in 90% of the 109 class A natural rivers and in 45 of the 47 prefectures (Lio-Po, 2011; Minamoto, Honjo, Yamanaka, Uchii, & Kawabata, 2012). Similarly, CyHV-3 spread rapidly in Indonesia with disease outbreaks reported on most of the major islands by 2006 (Lio-Po, 2011). CyHV-3 has also been detected in China (Dong et al., 2011), South Korea (Gomez et al., 2011), Singapore (Lio-Po, 2011), Malaysia (Musa, Leong, & Sunarto, 2005), and Thailand (Lio-Po, 2011; Pikulkaew, Meeyam, & Banlunara, 2009).

In North America, the first reports of CyHV-3 infection were from disease outbreaks at koi dealers (Gray et al., 2002; Hedrick et al., 2000). Then, in 2004, CyHV-3 was confirmed from mass mortalities of wild common carp in South Carolina and New York states (Grimmett et al., 2006; Terhune et al., 2004). In Canada, CyHV-3 was first detected during disease outbreaks in wild common carp in Ontario in 2007 and further outbreaks were reported in Ontario and Manitoba in 2008 (Garver et al., 2010). More recently, mass mortalities of common carp have been reported along the US/Canada border in Michigan and Wisconsin (Gotesman et al., 2013) (S. Marcquenski, personal communication).

There are no reports of KHVD or CyHV-3 detections from South America or Australasia, and the only reports from the African continent are from South Africa (OIE, 2012).

Horizontal transmission of the disease is very rapid (see Section 3.2.3.1.3). Several hypotheses were suggested to explain the swift spread of the virus: (i) The practice of mixing koi carp in the same tanks at koi shows has been held responsible for spreading the disease, particularly within a country (Gilad et al., 2002). (ii) In Israel, piscivorous birds are suspected to be responsible for the rapid spread of CyHV-3 from farm to farm (Ilouze, Davidovich, Diamant, Kotler, & Dishon, 2010). (iii) Disposal of infected fish by selling them below the market price was one suspected route of dissemination of

the virus in Indonesia (Sunarto, Rukyani, & Itami, 2005). (iv) It was suggested that the outbreaks of disease in public parks and ponds in Taiwan without recent introduction of fish were the result of members of the public releasing infected fish into the ponds (Tu et al., 2004). (v) Additionally, the virus has also been spread nationally and internationally before regulators were aware of the disease and methods to detect CyHV-3 were available. This is evidenced by the detection of CyHV-3 DNA in archive histological specimens collected during unexplained mass mortalities of koi and common carp in the UK in 1996 and in cultured common carp in South Korea in 1998 (Haenen et al., 2004; Lee, Jung, Park, & Do, 2012).

There are limited published observations of virus prevalence in wild or farmed populations of carp. A PCR survey, performed 2 years after the KHVD outbreaks in Lake Biwa, Japan, found a higher prevalence of CyHV-3 in larger common carp (>3 cm, 54% of seropositive fish) compared to smaller ones (<3 cm, 0% seropositive fish) (Uchii, Matsui, Iida, & Kawabata, 2009). Again in Japan, CyHV-3 DNA was detected in 3.9% (3/76), 5.1% (4/79), and 16.7% (12/72) of brain samples in three rivers of the Kochi prefecture (Fujioka et al., 2015).

In England, three sites experiencing clinical outbreaks of disease in 2006 and having no introduction of fish since that time were revisited in 2007, and found to have detectable serum anti-CyHV-3 antibodies in the surviving carp with a seroprevalence of 85–93% (Taylor, Dixon, et al., 2010). Similarly, studies to determine the prevalence of CyHV-3 in a country's carp farms or natural water bodies have been few. In the UK, common carp positive for CyHV-3 antibodies were found to be widely distributed in fisheries (angling waters) but the majority of carp farms remained negative. The main route of spread of CyHV-3 was determined to be live fish movements but alternative routes, including the stocking of imported ornamental fish, were also suggested (Taylor, Norman, Way, & Peeler, 2011; Taylor, Way, Jeffery, & Peeler, 2010).

Further evidence of widespread dissemination of CyHV-3 is provided by molecular epidemiology studies using the approaches described in Section 3.1.3. Two major lineages, CyHV-3 J and CyHV-3 U/I, have been identified with lineage J representing the major lineage in eastern Asia (Aoki et al., 2007; Kurita et al., 2009). Further studies have identified potential subgenotypes within the European (CyHV-3 U/I) and Asian (CyHV-3 J) lineages, with the European viruses showing the most variation (Kurita et al., 2009). The CyHV-3 J lineage has been detected in samples of infected koi and common carp from France and The Netherlands (Bigarré et al.,

2009), and the same study also identified a unique genotype of CyHV-3, intermediate between J and U/I, in koi carp from Poland. In Austria, the sequence analysis that was undertaken indicates that the CyHV-3 J was the only lineage detected in infected tissues from 15 koi carp from different locations in 2007 and suggests that the presence of the CyHV-3 J lineage in Europe may be linked to imports of Asian koi. In the UK, VNTR analysis similar to that described by Avarre et al. (2011) identified 41 distinct virus VNTR profiles for 68 disease cases studied between 2000 and 2010, and since these were distributed throughout three main clusters, CyHV-3 J, CyHV-3 I, and CyHV-3 U, and an intermediate lineage (D. Stone, personal communication), it suggests multiple incursions of CyHV-3 into the UK during that period.

In eastern and southeastern Asia, the U/I or European lineage has been detected but only at low frequency. In Indonesia, analysis of infected tissues from 10 disease outbreaks, from 2002 to 2007, identified two Asian genotypes and also another intermediate genotype (Sunarto et al., 2011). A study in South Korea identified from disease outbreaks in 2008, both a European genotype in samples of infected common carp and the expected Asian genotype in koi carp (Kim & Kwon, 2013). More recently, a European genotype of CyHV-3 was detected from a disease outbreak in 2011 in China (Dong et al., 2013), and in imported carp from Malaysia in Singapore (Chen et al., 2014).

3.2.1.3 Persistence of CyHV-3 in the Natural Environment

CyHV-3 remains infectious in water for at least 4 h, but not for 21 h, at water temperatures of 23–25 °C (Perelberg et al., 2003). Other studies in Japan have displayed a significant reduction in the infectious titer of CyHV-3 within 3 days in environmental water or sediment samples at 15 °C, while the infectivity remained for more than 7 days when CyHV-3 was exposed to sterilized water samples, thus suggesting the roles of microorganisms in the inactivation of CyHV-3 (Shimizu, Yoshida, Kasai, & Yoshimizu, 2006). Supporting this hypothesis, a recent report showed that bacteria isolated from carp habitat waters and carp intestine contents possessed some anti-CyHV-3 activity (Yoshida, Sasaki, Kasai, & Yoshimizu, 2013). These studies suggest that, in the absence of hosts, CyHV-3 can be rapidly inactivated in environmental water.

In Japan, the detection of CyHV-3 DNA in river water samples at temperatures of 9–11 °C has been reported 4 months before an outbreak of KHVD in a river (Haramoto, Kitajima, Katayama, & Ohgaki, 2007).

Japanese researchers have quantified CyHV-3 in environmental samples by cation-coated filter concentration of virus linked to a quantitative PCR (qPCR) (Haramoto, Kitajima, Katayama, Ito, & Ohgaki, 2009; Honjo et al., 2010). Using this technique, CyHV-3 was detected at high levels in water samples collected at eight sites along the Yura river system during, and 3 months after an episode of mass mortality caused by KHVD, and at water temperatures ranging from 28.4 down to 14.5 °C (Minamoto, Honjo, Uchii, et al., 2009). The seasonal distribution of CyHV-3 in Lake Biwa, Japan, was investigated using qPCR, which found the virus to be distributed all over the lake 5 years after the first KHVD outbreak in 2004. Mean concentrations of CyHV-3 in the lake water showed annual variation, with a peak in the summer and a trough in winter, and also indicated that the virus is more prevalent in reductive environments such as the turbid, eutrophic water found in reed zones (Minamoto, Honjo, & Kawabata, 2009). These areas are the main spawning sites of carp in Lake Biwa and support the hypothesis of increased prevalence of CyHV-3 during spawning (Uchii et al., 2011). The researchers suggested that, in highly turbid water, viruses may escape degradation by attaching to organic or nonorganic particles (Minamoto, Honjo, & Kawabata, 2009). Further studies of carp spawning areas in Lake Biwa reported the detection of CyHV-3 DNA in plankton samples and in particular the *Rotifera* species (Minamoto et al., 2011).

Finally, as explained earlier (see Section 3.2.1.1), other vertebrate and invertebrate species could play a significant role in CyHV-3 persistence in aquatic environments and should be considered as an epidemiological risk for carp farms (Fabian et al., 2013).

3.2.1.4 Use of CyHV-3 for Biological Control of Common Carp

In Australia, common carp is considered as an important invasive pest species. Its population and geographical range drastically expanded after an accidental escape from isolated farms in southeastern Australia due to flooding in the 1970s. In the early 2000s, an integrated pest management plan was developed to counteract common carp invasion. CyHV-3 was proposed as a biological control agent to reduce common carp populations (McColl, Cooke, & Sunarto, 2014). With regard to this goal, CyHV-3 possesses some interesting characteristics such as inducing high morbidity/mortality, high contagiosity, and a narrow host range for induction of the disease (not for asymptomatic carriers). These viral characteristics coupled with some epidemiological conditions specific to Australia, such as the absence of CyHV-3

and other cyprinid fish species together with the relatively low abundance of common and koi carp aquaculture, suggest that CyHV-3 could be a successful biocontrol agent. However, as stated by the authors, the use of exotic viruses as biocontrol agents is not trivial and studies addressing the safety and the efficacy of this measure are essential before applying it to the field (McColl et al., 2014).

3.2.2 Clinical Aspects

KHVD is seasonal, occurring mainly at water temperatures between 18 and 28 °C (Gotesman et al., 2013; Rakus et al., 2013). It is a highly contagious and extremely virulent disease with mortality rates up to 80–100% (Ilouze, Dishon, & Kotler, 2006). The disease can be reproduced experimentally by immersion of fish in water containing the virus, by ingestion of contaminated food, by cohabitation with freshly infected fish, and, more artificially, by injection of infectious material (Fournier et al., 2012; Perelberg et al., 2003). Fish infected with CyHV-3 using these various routes, and kept at permissive temperature, die between 5 and 22 dpi with a peak of mortality between 8 and 12 dpi (Fournier et al., 2012; Hedrick et al., 2000; Perelberg et al., 2003; Rakus, Wiegertjes, Adamek, et al., 2009). Furthermore, CyHV-3-infected fish are more susceptible to secondary infections by bacterial, parasitic, or fungal pathogens, which may contribute to the mortalities observed in the infected population (McDermott & Palmeiro, 2013).

3.2.2.1 Clinical Signs

The first clinical signs usually appear 2–3 dpi, while the first mortalities are frequently delayed to 6–8 dpi (McDermott & Palmeiro, 2013). The course of infection and the clinical signs observed are variable between individual fish, even after simultaneous and controlled experimental CyHV-3 inoculation. Fish can express the following clinical signs: folding of the dorsal fin; increased respiratory frequency; gathering near well-aerated areas; skin changes including gradual hyperemia at the base of fins, increased (sometimes decreased) mucus secretion, hemorrhages and ulcers on the skin, sloughing of scales and fin erosion, sandpaper-like texture of the skin, and skin herpetic lesions; gasping at the water surface; lethargy (lying at the bottom of the tank, hanging in head-down position in the water column) associated with anorexia; sunken eyes; and neurological symptoms with erratic swimming and loss of equilibrium (Hedrick et al., 2000; McDermott & Palmeiro, 2013; Rakus et al., 2013; Walster, 1999). None of these clinical signs are pathognomonic of KHVD.

3.2.2.2 Anatomopathology

The external post-mortem lesions that can be observed on the skin include pale and irregular patches, hemorrhages, fin erosions, ulcers, and peeling away of the epithelium. The main lesion in the gills is a mild to severe necrosis with multifocal or diffuse discoloration, sometimes associated with extensive erosions of the primary lamellae. Some of these anatomopathological lesions are illustrated in Fig. 6a. Other inconsistent necropsy changes include enlarging, darkening, and/or mottling of some internal organs associated with petechial hemorrhages, accumulation of abdominal fluid, and abdominal adhesions (Bretzinger et al., 1999; Hedrick et al., 2000; McDermott & Palmeiro, 2013; Walster, 1999). None of the lesions listed above are pathognomonic of KHVD.

3.2.2.3 Histopathology

Histopathological alterations are observed in the gills, skin, kidneys, heart, spleen, liver, gut, and brain of CyHV-3-infected fish (Hedrick et al., 2000; Miyazaki et al., 2008). In the skin, the lesions can appear as soon as 2 dpi and worsen with time (Fig. 6b; Miwa et al., 2014). The cells exhibiting degeneration and necrosis show various stages of nuclear degeneration (e.g., pale coloration, karyorrhexis, pyknosis), frequently associated with characteristic intranuclear inclusion bodies (Fig. 6b, D). These cells, shown to be infected by CyHV-3 using EM, are characterized by a basophilic material within the nucleus associated with marginal hyperchromatosis (Miyazaki et al., 2008). The number of goblet cells is reduced by 50% in infected fish, and furthermore, they appear mostly slim and slender, suggesting that mucus has been released and not replenished (Adamek et al., 2013). At later stages, erosion of skin epidermis is frequently observed (Adamek et al., 2013; Miwa et al., 2014). A recent report revealed that the damages caused to the skin of the body and fins were the most pronounced lesions (Miwa et al., 2014).

During the course of CyHV-3 infection, important histopathological changes are observed in the two compartments of the gills, the gill lamellae and gill rakers (Fig. 6c; Miyazaki et al., 2008; Pikarsky et al., 2004). The lesions observed in the gill lamellae involve infiltration of inflammatory cells, hyperplasia, hypertrophy, degeneration and necrosis of epithelial cells, congestion, and edema (Miyazaki et al., 2008; Ouyang et al., 2013; Pikarsky et al., 2004). As a consequence of the pronounced hyperplasia, the secondary lamellae interspace is progressively filled by cells. At later stages, the gill lamellae architecture can be completely lost by necrosis, erosion, and fusion of the primary lamellae (Pikarsky et al., 2004). These lesions can be

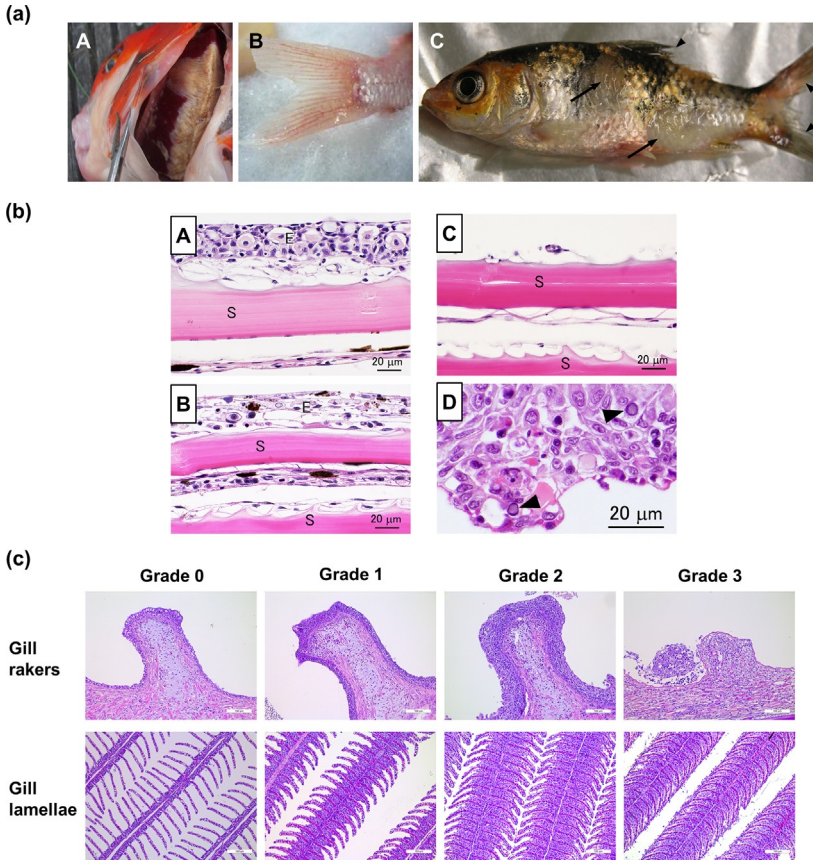

Figure 6 Illustration of anatomopathological and histopathological lesions induced by CyHV-3. (a) Anatomopathological lesions. (A) Severe gill necrosis. (B) Hyperemia at the base of the caudal fin. (C) Extensive necrosis of the skin covering the body (arrows indicate circular herpetic lesion) and fin erosion (arrowheads). (b) Histopathological lesions in the skin. Sections of the skin of carp stained with hematoxylin and eosin. S, scale; E, epidermis. (A) The skin of a mock-infected fish. (B) The skin of a moribund specimen sampled 6 dpi. Most of the cells exhibit degenerescence and necrosis as well as marginalization of the chromatin. (C) The skin of a moribund fish sampled 5 dpi. The epidermis has detached from the underlying dermis probably as a consequence of extensive necrosis. (D) High magnification of the skin of an infected fish 2 dpi. Note the characteristic chromatin marginalization observed in some epithelial cells (arrowheads). (c) Histopathological lesions in the gills. Five-micrometer sections were stained with hematoxylin and eosin. A grading system was developed to characterize the lesions observed in gill rakers and gill lamellae. The grading system evaluates the degree of epithelial hyperplasia, the presence of intranuclear viral inclusions, and cell degeneration. Briefly, grade 0 = physiological state; grade 1 = mild hyperplasia without evidence of degenerated cells and viral inclusions; grade 2 = severe hyperplasia and presence of few degenerated cells and viral inclusions; and grade 3 = presence of abundant degenerated cells and viral inclusions (gill lamellae and gill rakers), massive epithelial hyperplasia filling the entire secondary lamellae interspace (gill lamellae), and ulcerative erosion of the epithelium (gill rakers). Scale bars = 100 µm. *Panel (a): Adapted with permission from Michel, Fournier, et al. (2010). Panel (b): Adapted from with permission from Miwa, Kiryu, Yuasa, Ito, and Kaneko (2014). Copyright © Wiley & Sons, Inc. Panel (c): Adapted with permission from Boutier et al. (2015).*

visualized macroscopically and are frequently associated with secondary infections (Pikarsky et al., 2004). In the gill rakers, the changes are even more recognizable (Pikarsky et al., 2004). These include subepithelial inflammation, infiltration of inflammatory cells, and congestion at early stages (Pikarsky et al., 2004), followed by hyperplasia, degeneration, and necrosis of cells presenting intranuclear inclusion bodies. At ulterior stages, complete erosion of the epithelium can be observed. Based on these histopathological observations, a grading system (Fig. 6c) has been proposed by Boutier et al. (2015). This grading system classifies the lesions according to three criteria, i.e., (i) hyperplasia of epithelial cells, (ii) presence and extent of degeneration and necrosis, and (iii) presence and abundance of intranuclear inclusion bodies. As the number of presumed infected cells does not always correlate with the severity of the lesions, the combination of these criteria is necessary to obtain a reliable histopathological grading system (Boutier et al., 2015; Miwa et al., 2014).

In the kidney, a weak peritubular inflammatory infiltrate is evident as early as 2 dpi and increases with time. It is accompanied by blood vessel congestion and degeneration of the tubular epithelium in many nephrons (Pikarsky et al., 2004). Intranuclear inclusion bodies are mainly found in hematopoietic cells (Miwa et al., 2014; Miyazaki et al., 2008). In the spleen, the main susceptible cells are the splenocytes. In extreme cases, the lesions include large numbers of necrotic splenocytes accompanied by hemorrhages (Miyazaki et al., 2008). In the heart, many myocardial cells exhibit nuclear degeneration and alteration of the myofibril bundles with disappearance of the cross-striation (Miyazaki et al., 2008).

In the intestine and stomach, the lesions induced are mainly the consequence of the hyperplasia of the epithelium, forming projections inside the lumen. Cells of the epithelium expressing intranuclear inclusion bodies and necrosis detach from the mucosa and locate in the lumen of the organ (El-Din, 2011). In the liver, hepatocytes are the most affected cell type (Miyazaki et al., 2008) and mild inflammatory infiltrates can be observed in the parenchyma (Pikarsky et al., 2004).

In the brain, focal meningeal and parameningeal inflammation is observed (Pikarsky et al., 2004). Analysis of brains from fish that showed clear neurologic signs revealed congestion of capillaries and small veins associated with edematous dissociation of nerve fibers in the valvula cerebelli and medulla oblongata (Miyazaki et al., 2008). Infected cells were detected at 12 dpi in all compartments of the brain. These cells were mainly ependymal cells and, to a lesser extent, neurons (Fig. 7; Miwa et al., 2014). At 20 dpi, the

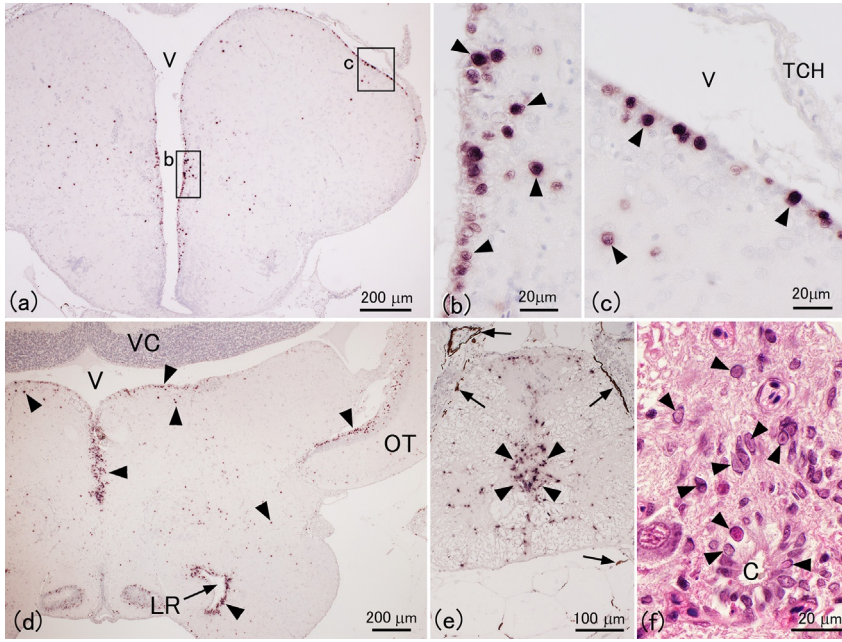

Figure 7 Illustration of histopathological lesions induced in the central nervous system of carp by CyHV-3. (A), (D), and (E) show sections of telencephalon, mesencephalon, and spinal cord hybridized for the viral genome, respectively. Fish were sampled 12 dpi. The hybridization signals (arrowheads) are observed along the ependyma as well as in some neurons in the neuropil and around the central canal. The rectangles in (A) are shown enlarged in (B) and (C). Arrows indicate melanin. (F) A section of the spinal cord stained with hematoxylin and eosin. Arrowheads indicate nuclei of cells presumably infected with CyHV-3. V, ventricle; TCH, tela choroidea; VC, valvula cerebelli; OT, optic tectum; LR, lateral recess; C, central canal. *Reproduced with permission from Miwa et al. (2014). Copyright © Wiley & Sons, Inc.*

lesions are accompanied by perivascular lymphocyte infiltration and gliosis. The peak of nervous lesions coincides in time with the peak of neurological clinical signs (Miwa et al., 2014).

3.2.3 Pathogenesis

All members of the family *Herpesviridae* exhibit two distinct phases in their infection cycle: lytic replication and latency. While lytic replication is associated with production of viral particles, latency entails the maintenance of the viral genome as a nonintegrated episome and the expression of very few viral genes and microRNAs. Upon reactivation, lytic replication ensues. Studies on a few members of the *Alloherpesviridae* family also suggest the

existence of these two types of infection. Most of these studies are on CyHV-3 and suggest that the temperature of the water could regulate the switch between latency and lytic replication and *vice versa*, allowing the virus to persist in the host population throughout the seasons even when the temperature is nonpermissive (Uchii, Minamoto, Honjo, & Kawabata, 2014). Below, we have summarized the data available for CyHV-3 for the two types of infection.

3.2.3.1 Productive Infection

3.2.3.1.1 Portals of Entry In early reports, it has been suggested that CyHV-3 may enter the host through infection of the gills (Hedrick et al., 2000; Ilouze, Dishon, & Kotler, 2006; Miyazaki et al., 2008; Miyazaki, Yasumoto, Kuzuya, & Yoshimura, 2005; Pikarsky et al., 2004; Pokorova, Vesely, Piackova, Reschova, & Hulova, 2005) and the intestine (Dishon et al., 2005; Ilouze, Dishon, & Kotler, 2006). These hypotheses rely on several observations: (i) the gills undergo histopathological lesions early after inoculation by immersion in infectious water (Hedrick et al., 2000; Pikarsky et al., 2004), (ii) viral DNA can be detected in the gills and the gut as early as 1 dpi (as in virtually all organs including skin mucus) (Gilad et al., 2004), and (iii) the gills are an important portal of entry for many fish pathogens. More recent studies using *in vivo* bioluminescent imaging system (IVIS) demonstrated that the skin is the major portal of entry of CyHV-3 after immersion in virus-containing water (Fig. 8; Costes et al., 2009; Fournier et al., 2012). The epidermis of teleost fish is a living stratified squamous epithelium that is capable of mitotic division at all levels (even the outermost squamous layer). The scales are dermal structures and, consequently, are covered by the epidermis (Costes et al., 2009). A discrete luciferase signal was detected as early as 12 hpi in most of the fish, while all fish were clearly positive at 24 hpi with the positive signal preferentially localized on the fins (Costes et al., 2009). This finding is supported by independent reports that show early CyHV-3 RNA expression in the skin as early as 12 hpi (Adamek et al., 2013) and detection of viral DNA in infected cells by *in situ* hybridization (ISH) in the fin epithelium as early as 2 dpi (the earliest positive organ) (Miwa et al., 2014). Fish epidermis has also been shown to support early infection of a Novirhabdovirus (IHNV, infectious hematopoietic necrosis virus) in trout, suggesting that the skin is an important portal of entry of viruses in fish (Harmache, LeBerre, Droineau, Giovannini, & Bremont, 2006).

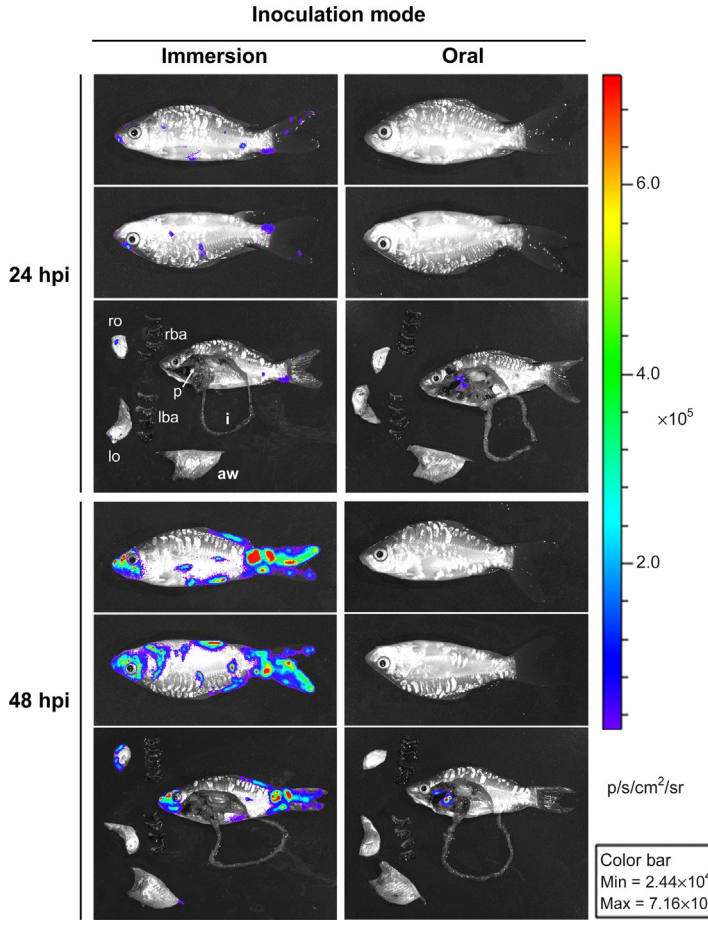

Figure 8 The portals of entry of CyHV-3 in carp analyzed by *in vivo* bioluminescent imaging. Two groups of fish (mean weight 10 g) were infected with a recombinant CyHV-3 strain expressing luciferase as a reporter gene either by bathing them in water containing the virus (immersion, left column) or by feeding them with food pellets contaminated with the virus (oral, right column). At the indicated times post-infection, six fish per group were analyzed by IVIS. Each fish was analyzed lying on its right and left side. The internal signal was analyzed after euthanasia and dissection. Dissected fish and isolated organs were analyzed for *ex vivo* bioluminescence using IVIS. One representative fish is shown for each time point and inoculation mode. Images collected over the course of the experiment were normalized using an identical pseudocolor scale ranging from violet (black in the print version; least intense) to red (dark gray in the print version; most intense) using Living Image 3.2 software. rba, right branchial arches; lba, left branchial arches; ro, right operculum; lo, left operculum; p, pharynx; aw, abdominal wall; i, intestine. *Reproduced with permission from Fournier et al. (2012). Original publisher BioMed Central.*

The data listed above demonstrated that the skin is the major portal of entry after inoculation of carp by immersion in water containing CyHV- 3. While this mode of infection mimics natural conditions in which infection takes place, other epidemiological conditions could favor entry of virus through the digestive tract. To test this hypothesis, carp were fed with material contaminated with a CyHV-3 recombinant strain expressing luciferase as a reporter gene, and bioluminescence imaging analyses were performed at different times post-infection (Fig. 8; Fournier et al., 2012). These experiments demonstrated that the pharyngeal periodontal mucosa is the major portal of entry after oral contamination. This mode of inoculation led to the dissemination of the infection to the various organs tested, inducing clinical signs and mortality rates comparable to the infection by immersion (Fournier et al., 2012). More recently, Monaghan, Thompson, Adams, Kempter, and Bergmann (2015) claimed that the gills and gut represent additional portals of entry by using ISH analysis. In this report, several organs were tested after infection by immersion and positive signal was detected as early as 1–2 hpi in gills, gut, and blood vessels of internal organs. Surprisingly, this early detection occurs far before viral DNA replication, which starts 4–8 hpi *in vitro* (Ilouze et al., 2012a). Moreover, this report is in contradiction with another study that detected positive cells only after 2 days of infection in the fins by using the very same technique (Miwa et al., 2014). Further evidence that the skin, and not the gills, is the major portal of entry after inoculation by immersion in infectious water was recently provided by a study aiming to develop an attenuated recombinant vaccine (Boutier et al., 2015). The study of the tropism of a recombinant strain deleted for ORF56 and 57 (Δ56-57) demonstrated that it also spreads from the skin to all tested organs. However, compared to the wild-type strain, its systemic spread to the other organs was much slower, and its replication was reduced in intensity and duration (Boutier et al., 2015). The slower spread of the Δ56-57 vaccine strain within infected fish allowed better discrimination of the portal(s) of entry from secondary sites of infection. Though the skin of all fish was positive as early as 2 dpi, all of the other tested organs (including gills and gut) were positive in the majority of fish after 6 dpi. These data further demonstrate that the skin is the major portal of entry of CyHV-3 after infection by immersion and suggest that the other organs (including gills and gut) represent secondary sites of replication.

3.2.3.1.2 Secondary Sites of Infection After infection at the portals of entry, CyHV-3 rapidly spreads in infected fish as demonstrated by the

detection of CyHV-3 DNA in almost all tissues as early as 1–2 dpi (Boutier et al., 2015; Gilad et al., 2003; Ouyang et al., 2013; Pikarsky et al., 2004). The tropism of CyHV-3 for white blood cells most probably explains such a rapid spread of the virus within the body (Eide, Miller-Morgan, Heidel, Bildfell, & Jin, 2011). CyHV-3 DNA can be isolated from blood as early as 1 dpi (Pikarsky et al., 2004). During the first days post-infection, most of the organs (including those that act as portals of entry) support increasing viral replication according to time post-infection (Boutier et al., 2015). The cause of death is more controversial. The severe CyHV-3 infection observed in gills and kidneys, together with the associated histopathological alterations, could be responsible for acute death (Gilad et al., 2004; Hedrick et al., 2000). It has also been proposed that the severe skin alterations could lead to hypo-osmotic shock (Miwa et al., 2014).

3.2.3.1.3 Excretion and Transmission Horizontal transmission of CyHV-3 could occur either by direct contact between fish or by indirect transmission. Study of the CyHV-3 portals of entry demonstrated that, according to specific epidemiological conditions, CyHV-3 can enter carp through either infection of the skin or infection of the pharyngeal periodontal mucosa (Fig. 8). Therefore, direct transmission could result from skin to skin contact between acutely infected or carrier fish with naive ones, or from cannibalistic and necrophagous behaviors of carp (Fournier et al., 2012; Raj et al., 2011). Interestingly, horizontal transmission in natural ponds seems accentuated in hot spots of carp breeding behavior and mating (Uchii et al., 2011), which could favor this skin-to-skin mode of transmission (Raj et al., 2011). Several potential vectors could be involved in the indirect transmission of CyHV-3 including fish droppings (Dishon et al., 2005), plankton (Minamoto et al., 2011), sediments (Honjo, Minamoto, & Kawabata, 2012), aquatic invertebrates feeding by water filtration (Kielpinski et al., 2010), and finally the water as the major abiotic vector (Minamoto, Honjo, Uchii et al., 2009). Indeed, virus replication in organs such as the gills, skin, and gut probably represents a source of viral excretion into the water and the ability of CyHV-3 to remain infective in water has been extensively studied experimentally (see Section 3.2.1.3; Adamek et al., 2013; Costes et al., 2009; Dishon et al., 2005; Pikarsky et al., 2004).

The spread of CyHV-3 was recently studied using two experimental settings designed to allow transmission of the virus through infectious water (water sharing) or through infectious water and physical contact between infected and naive sentinel fish (tank sharing) (Boutier et al., 2015). The

difference in transmission kinetics observed between the two systems demonstrated that direct contact between subjects promotes transmission of CyHV-3 as postulated. Nevertheless, transmission through infectious water was still highly efficient (Boutier et al., 2015). To date, there is no evidence of CyHV-3 vertical transmission.

3.2.3.2 Latent Infection

Although latency has not been demonstrated conclusively in members of the *Alloherpesviridae* family as it has been for *Herpesviridae*, increasing evidence supports the existence of a latent phase. The evidence related to CyHV-3 is discussed in this section.

Low amounts of CyHV-3 DNA have been detected 2 months post-infection in the gills, kidneys, and brain of fish that survived primary infection and no longer showed clinical signs (Gilad et al., 2004). Independent studies confirmed the presence of CyHV-3 DNA in the brain of fish as late as 1 year post-infection (Miwa et al., 2014; Yuasa & Sano, 2009). In addition, CyHV-3 DNA, but no infectious particles, has been detected in several organs of fish after CyHV-3 infection (Eide, Miller-Morgan, Heidel, Bildfell, et al., 2011). Finally, CyHV-3 DNA can be routinely detected in apparently healthy fish (Cho et al., 2014).

CyHV-3 can persist in farmed (Baumer, Fabian, Wilkens, Steinhagen, & Runge, 2013) or wild carp populations (Uchii et al., 2009, 2014). At least 2 years after an initial outbreak, CyHV-3 DNA was detected in the brain of both large-sized seropositive fish and small-sized seronegative fish from a wild population of common carp (Uchii et al., 2009). These data suggest that transmission occurred between latently infected fish that survived previous outbreaks and the new naive generation (Uchii et al., 2009). In a more recent report, Uchii et al. (2014) suggests that it is the seasonal reactivation that enables CyHV-3 to persist in a wild population. Indeed, they were able to detect RNA expression of CyHV-3 replicative-related genes in the brain of seropositive fish, suggesting reactivation, while some fish expressed only presumed latency-related genes (Ilouze et al., 2012a; Uchii et al., 2014).

St-Hilaire et al. (2005) described that fish can express symptoms and die from CyHV-3 infection following a temperature stress several months after the initial exposure to the virus. Reactivation of infectious virions was demonstrated by contamination of naive fish. In another report, a netting stress induced viral reactivation without symptoms 81 days after initial infection as detected by qPCR on gill samples (Bergmann & Kempter, 2011).

Recent studies suggested that white blood cells could support CyHV-3 latency (Eide, Miller-Morgan, Heidel, Bildfell, et al., 2011; Eide, Miller-Morgan, Heidel, Kent, et al., 2011; Reed et al., 2014; Xu, Bently, et al., 2013). First, koi carp with previous exposure to the virus displayed CyHV-3 DNA in white blood cells in the absence of any clinical signs or detectable infectious viral particles (Eide, Miller-Morgan, Heidel, Bildfell, et al., 2011). Similar results were found in wild carp collected from ponds in Oregon with no history of CyHV-3 outbreaks (Xu, Bently, et al., 2013). Interestingly, Eide, Miller-Morgan, Heidel, Kent, et al. (2011) detected low amounts of CyHV-3 DNA ranging from 2 to 60 copies per μg of isolated DNA in white blood cells of previously infected koi. These numbers are similar to those reported during the latency of *Herpesviridae*. Notably, similar viral DNA copies were found in all other tissues with no evidence of whether this widespread tissue distribution reflects detection of latently infected circulating white blood cells or latently infected resident cells (Eide, Miller-Morgan, Heidel, Kent, et al., 2011). Among white blood cells, it seems that the IgM^+ B cells are the main cell type supporting CyHV-3 latency (Reed et al., 2014). Indeed, the amount of CyHV-3 DNA copies was 20 times higher in IgM^+-purified B cells compared to the remaining white blood cells. However, it has to be noted that the latter still contained 10% of IgM^+ B cells due to lack of selectivity of the IgM^+-sorting method. Therefore, it is still not known whether the low amount of CyHV-3 DNA found in the remaining white blood cells could be explained by the existence of another cell type also supporting latent infection or by the IgM^+ B cell contamination. This study also investigated the CyHV-3 transcriptome in latently infected IgM^+ B cells (Reed et al., 2014). It demonstrated that CyHV-3 ORF6 transcription was associated with latent infection of IgM^+ B cells (ORF1–5 and 7–8 were not transcribed). Interestingly, one domain of ORF6 (aa 342–472) was found to be similar to the consensus sequences of EBNA-3B (EBV nuclear antigen) and the N-terminal regulator domain of ICP4 (infected-cell polypeptide 4). The EBNA-3B is one of the proteins expressed by the gammaherpesvirus EBV during latency and is potentially involved in regulation of cellular gene expression, while ICP4 is found in alphaherpesviruses and acts also as a transcriptional regulator (Reed et al., 2014).

A hallmark of herpesviruses is their capacity to establish a latent infection. Recent studies on CyHV-3 highlighted potential latency in white blood cells and, more precisely, in the B cell fraction as observed for some gammaherpesviruses. On the other hand, CyHV-3 DNA was found in

various tissues of long-term infected fish and especially in the brain. Whether the nervous system represents an additional site of latency as observed in alphaherpesviruses requires further investigation.

3.2.3.3 Effect of Water Temperature

KHVD occurs naturally when the water temperature is between 18 and 28 °C (Gotesman et al., 2013; Rakus et al., 2013). Experimentally, KHVD has been reproduced in temperatures ranging from 16 to 28 °C (Gilad et al., 2003, 2004; Yuasa, Ito, & Sano, 2008) and the lowest temperature associated with a CyHV-3 outbreak was 15.5 °C in a field survey in Japan (Hara, Aikawa, Usui, & Nakanishi, 2006). Interestingly, CyHV-2 induces mortalities in goldfish at a slightly enlarged temperature range from 15 to 30 °C (Ito & Maeno, 2014) suggesting a similar but adaptable temperature range in cyprinid herpesviruses. In CyHV-3 infections, the onset of mortality was affected by the water temperature; the first mortalities occurred between 5–8 and 14–21 dpi when the fish were kept between 23–28 and 16–18 °C, respectively (Gilad et al., 2003; Yuasa et al., 2008). Moreover, daily temperature fluctuations of ±3 °C induce important stress in fish, which increases cortisol release in the water and also their susceptibility to CyHV-3 (higher mortality rate and viral excretion) (Takahara et al., 2014).

Several studies demonstrated that transfer of recently infected fish (between 1 and 5 dpi) to nonpermissive low (≤13 °C) (St-Hilaire, Beevers, Joiner, Hedrick, & Way, 2009; St-Hilaire et al., 2005; Sunarto et al., 2014) or high temperatures (30 °C) (Ronen et al., 2003) significantly reduces the mortality. Some observations suggest that the virus can replicate at low temperatures without inducing mortalities. Indeed, relatively high amounts of CyHV-3 DNA, together with the detectable expression of viral genes encoding structural proteins (ORF149 (glycoprotein member of the ORF25 family), ORF72 (Capsid triplex subunit 2)), and nonstructural proteins (ORF55 (TK), ORF134 (vIL-10)) were detected in fish maintained at low temperature (Baumer et al., 2013; Gilad et al., 2004; Sunarto et al., 2012, 2014), while no infectious particles could be isolated (Sunarto et al., 2014). In addition, CyHV-3-infected fish maintained at low temperature (≤13 °C) and then returned to permissive temperature frequently expressed the disease (Eide, Miller-Morgan, Heidel, Kent, et al., 2011; Gilad et al., 2003; St-Hilaire et al., 2005, 2009; Sunarto et al., 2014) and were able to contaminate naive cohabitants (St-Hilaire et al., 2005). Together, these observations suggest that the temperature of the water could

regulate the switch between latency and lytic replication and *vice versa*, thus allowing the virus to persist in the host population throughout the seasons even when the temperature is nonpermissive for productive viral replication.

The studies described above suggest that the effect of temperature on the biological cycle of CyHV-3 *in vivo* is twofold. First, it could control the switch from latency to lytic infection and *vice versa*. Second, it clearly regulates the amplitude of viral replication during lytic infection. Further studies are required to clarify the relative importance of these two effects and their putative interactions.

3.2.4 Host–Pathogen Interactions

3.2.4.1 Susceptibility of Common Carp According to the Developmental Stage

Carp of all ages are affected by CyHV-3, but younger fish (1–3 months, 2.5–6 g) seem to be more susceptible to infection than mature fish (1 year, ≈230 g) (Perelberg et al., 2003). Ito, Sano, Kurita, Yuasa, and Iida (2007) suggested that carp larvae are not susceptible to CyHV-3 since larvae (3 days post-hatching) infected with the virus showed no mortality, whereas most of the carp juveniles (>13 days post-hatching) died after infection. This conclusion was challenged recently. Using a CyHV-3 recombinant strain expressing luciferase as a reporter gene and IVIS, Ronsmans et al. (2014) demonstrated that carp larvae are sensitive and permissive to CyHV-3 infection immediately after hatching and that their sensitivity increases with the developmental stages (Ronsmans et al., 2014). However, the sensitivity of the two early stages (embryo and larval stages, 1–21 days post-hatching) was limited compared to the older stages (juvenile and fingerling stages; >21 days post-hatching) (Ronsmans et al., 2014).

3.2.4.2 Susceptibility of Common Carp According to Host Genetic Background

Common carp originated from the Eurasian continent and consist of at least two subspecies *C. carpio carpio* (Europe) and *C. carpio haematopterus* (East Asia) (Chistiakov & Voronova, 2009). During the long history of domestication, common carp of multiple origins have been intensively submitted to selective breeding which led to a high variety of breeds, strains, and hybrid fish (Chistiakov & Voronova, 2009). In addition, domesticated common carp were spread worldwide by human activities (Uchii, Okuda, Minamoto, & Kawabata, 2013). Fish from genetically distant populations may differ in their resistance to diseases. Traditional selective breeding methods as well as marker-associated selection proved to be a relevant

approach to reduce the economic losses induced by infectious diseases (Midtlyng, Storset, Michel, Slierendrecht, & Okamoto, 2002).

Differences in resistance to CyHV-3 have been described among different carp strains and crossbreeds. Zak, Perelberg, Magen, Milstein, and Joseph (2007) reported that the crossbreeding of some Hungarian strains (Dinnyes and Szarvas-22 bred at the research Institute for Fisheries, Aquaculture and Irrigation (HAKI) in Szarvas) with the Dor-70 strain (bred in Israel) does not improve the resistance to CyHV-3. On the other hand, independent research groups demonstrated that resistance to CyHV-3 can be significantly increased by crossbreeding domesticated carp strains with wild carp strains. Shapira et al. (2005) reported that crossing the domesticated carp Dor-70 (bred in Israel) and Našice (introduced in Israel from ex-Yugoslavia in the 1970s) with a wild carp strain Sassan (originated from the Amur river) significantly increases the resistance to CyHV-3 (Shapira et al., 2005). Carp genetic resistance to CyHV-3 has also been investigated using 96 carp families derived from diallelic crossbreeding of two wild carp strains (Amur and Duna, native of the Amur and Danube rivers) and two domesticated Hungarian strains (Tat, and Szarvas 15) (Dixon et al., 2009; Ødegård et al., 2010). These studies showed that overall the more resistant families derived from wild-type strains, even if important variations were observed according to the pair of genitors used (Dixon et al., 2009). Similarly, Piackova et al. (2013) demonstrated that most of the Czech strains and crossbreeds which are genetically related to wild Amur carp were significantly more resistant to CyHV-3 infection than strains with no relation to Amur carp.

In Japan, common carp of two different genetic origins inhabit Lake Biwa: an ancient Japanese indigenous type and an introduced domesticated Eurasian type (Mabuchi, Senou, Suzuki, & Nishida, 2005). During the CyHV-3 outbreak in Lake Biwa in 2004, mortalities were mainly recorded in the Japanese indigenous type (Ito, Kurita, & Yuasa, 2014; Uchii et al., 2013). This higher susceptibility of the Japanese indigenous type to CyHV-3 was later confirmed experimentally (Ito et al., 2014) and is supposed to be one factor responsible for the important decline of this ancient lineage in Lake Biwa (Uchii et al., 2013).

Recently, resistance to CyHV-3 among common carp strains has also been linked to the polymorphism of genes involved in the immune response, i.e., the MHC class II *B* genes (Rakus, Wiegertjes, Jurecka, et al., 2009) and carp IL-10 gene (Kongchum et al., 2011). All together, these findings support the hypothesis that the outcome of the disease can

be correlated to some extent to genetic factors of the host, and consequently, that selection of resistant carp breeds is one of the potential ways to reduce the negative impact of CyHV-3 on carp aquaculture.

3.2.4.3 Common Carp Innate Immune Response Against CyHV-3

CyHV-3 enters fish through infection of the skin and/or the pharyngeal periodontal mucosa (Fig. 8; Costes et al., 2009; Fournier et al., 2012). These mucosal epithelia are covered by mucus that acts as a physical, chemical, and immunological barrier against pathogens. The mucus layer contains numerous proteins, such as antimicrobial peptides, mucins, immunoglobulins, enzymes, and lytic agents, capable of neutralizing microorganisms (Ellis, 2001; Shephard, 1994; van der Marel et al., 2012). Interestingly, Raj et al. (2011) demonstrated that skin mucus acts as an innate immune barrier and inhibits CyHV-3 binding to epidermal cells at least partially by neutralization of viral infectivity as shown by *in vitro* assay. Recently, the low sensitivity of carp larvae to CyHV-3 infection was circumvented by a mucus removal treatment suggesting a critical role of skin mucus in protecting larvae against infectious diseases (Fig. 9). Such an innate protection is likely to play a key role in the immune protection of this developmental stage which does not yet benefit from a mature adaptive immune system (Ronsmans et al., 2014). The anti-CyHV-3 immune response has been studied in the skin and the intestine of common carp (Adamek et al., 2013; Syakuri et al., 2013). In the skin, CyHV-3 infection leads to downregulation of genes encoding several important components of the skin mucosal barrier, including antimicrobial peptides (beta defensin 1 and 2), mucin 5B, and tight junction proteins (claudin 23 and 30). This probably contributes to the disintegration of the skin (downregulation of claudins), the decreased amount of mucus, and the sandpaper-like surface of the skin (downregulation of mucins), as well as changes in the cutaneous bacterial flora and subsequent development of secondary bacterial infections (Adamek et al., 2013). These studies also revealed an upregulation of proinflammatory cytokine IL-1β, the inducible nitric oxide synthase, and activation of interferon (IFN) class I pathways (Adamek et al., 2013; Syakuri et al., 2013).

IFNs are secreted mediators that play essential roles in the innate immune response against viruses. *In vitro* studies demonstrated that CCB cells can secrete IFN type I in response to spring viremia of carp virus (SVCV) but not CyHV-3 infection, suggesting that CyHV-3 can inhibit this critical antiviral pathway *in vitro* (Adamek et al., 2012). Poly I:C stimulation of CCB cells prior to CyHV-3 infection activates the IFN type I response and

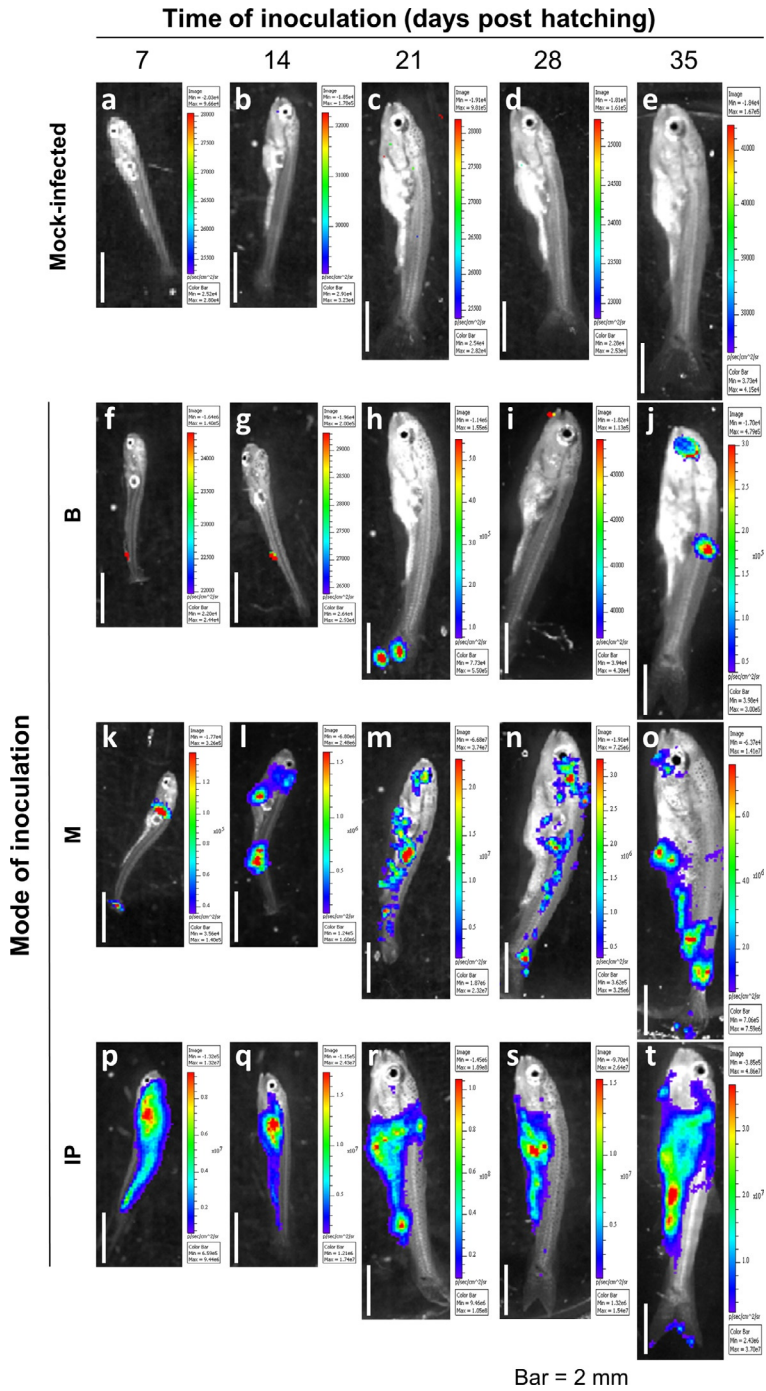

Figure 9 See legend on next page.

reduces CyHV-3 spreading in the cell culture (Adamek et al., 2012). *In vivo*, CyHV-3 induces a systemic IFN type I response in carp skin, intestine, and head kidney, and the magnitude of IFN type I expression is correlated with the virus load (Adamek, Rakus, et al., 2014; Adamek et al., 2013; Syakuri et al., 2013). However, no significant difference in the IFN type I response could be observed between two carp lines with different susceptibility to CyHV-3 (i.e., R3 and K carp lines) (Adamek, Rakus, et al., 2014). Additional *in vitro* studies demonstrated that CyHV-3 does not induce apoptosis, unlike SVCV (Miest et al., 2015), and that CyHV-3 inhibits activity of stimulated macrophages and proliferative response of lymphocytes, in a temperature-dependent manner (Siwicki, Kazuń, Kazuń, & Majewicz-Zbikowska, 2012). Finally, stimulation of the apoptosis intrinsic pathway *in vivo* following CyHV-3 infection, as determined by the expression of proapoptotic proteins (Apaf-1, p53, and Caspase 9), was delayed to 14 dpi (Miest et al., 2015).

Recently, a transcriptomic study uncovered the wide array of immune-related genes involved in the systemic anti-CyHV-3 immune response of carp by sampling the head kidney and the spleen (Rakus et al., 2012). The response of two carp lines with different resistance to CyHV-3 (i.e., R3 and K carp lines) was studied using DNA microarray and real-time PCR. Significantly higher expression of several immune-related genes including a number of those that are involved in pathogen recognition, complement activation, MHC class I-restricted antigen presentation, and development of adaptive mucosal immunity, was noted in the more resistant carp line. In this same line, further real-time PCR-based analyses provided evidence for higher activation of $CD8^+$ T cells. Thus, differences in resistance to CyHV-3 can be correlated with differentially expressed immune-related genes (Rakus et al., 2012). Concerning the acute-phase response following CyHV-3 infection, an upregulation of complement-associated proteins and C-reactive proteins was also detected by 72 hpi, suggesting a

Figure 9 Sensitivity of common carp to CyHV-3 during the early stages of development. At different times post-hatching, carp were inoculated with a recombinant CyHV-3 strain expressing luciferase as a reporter gene, according to three modes of inoculation: by immersion in infectious water (B), by immersion in infectious water just after removing the epidermal mucus (M), and by IP injection of the virus (IP). At 24 hpi, 30 carp were analyzed individually by IVIS. Mock-infected fish (A–E) and representative positive-infected fish (F–T) are shown for each time point of analysis. Images are presented with a relative photon flux scale automatically adapted to each image in order to use the full dynamic range of the pseudocolor scale. Scale bars = 2 mm. *Reproduced with permission from Ronsmans et al. (2014). Original publisher BioMed Central.*

strong and quick innate immune response (Pionnier et al., 2014). A summary of immune responses of common carp against CyHV-3 is shown in Table 9.

3.2.4.4 Common Carp Adaptive Immune Response Against CyHV-3

The systemic immune response to CyHV-3 has been evaluated by measuring anti-CyHV-3 antibodies in the serum of infected carp (Adkison et al., 2005; Perelberg et al., 2008; Ronen et al., 2003; St-Hilaire et al., 2009). Some studies reported slight cross-reaction by enzyme-linked immunosorbent assay (ELISA) and Western blot of anti-CyHV-3 antibodies to CyHV-1, probably due to shared epitopes between these two closely related viruses (Adkison et al., 2005; Davison et al., 2013; St-Hilaire et al., 2009). Detection of anti-CyHV-3 antibodies starts between 7 and 14 dpi, rises till 20–40 dpi, and finally progressively decreases with significant titers still detected at 150 dpi (Perelberg et al., 2008; Ronen et al., 2003). During these periods, the anti-CyHV-3 antibody response correlates with protection against CyHV-3 disease. On the other hand, at 280 dpi, the titer of anti-CyHV-3 antibodies in previously infected fish is only slightly higher or comparable to that of naive fish. Nevertheless, immunized fish, even those in which antibodies are no longer detectable, are resistant to a lethal challenge, possibly because of the subsequent rapid response of B and T memory cells to antigen restimulation (Perelberg et al., 2008).

Temperature strongly influences the adaptive immune response of fish (Bly & Clem, 1992). The cutoff between permissive and nonpermissive temperature for effective cellular and humoral immune response of carp is 14 °C (Bly & Clem, 1992). Therefore, fish kept below this temperature are supposed to be less immunocompetent than fish kept at higher temperature. This has been shown in CyHV-3 infection with a temperature-dependent expression of anti-CyHV-3 antibodies from 14 (slow antibody response shown at 40 dpi) to 31 °C (quick antibody response at 10 dpi) (Perelberg et al., 2008). In another study, only 40% of CyHV-3 exposed fish were able to seroconvert when kept at 12 °C and experienced mortalities due to CyHV-3 disease when brought back to permissive temperature, suggesting a reduced immunocompetence in low-temperature conditions (St-Hilaire et al., 2009).

Recently, the knowledge on mucosal immune response of teleost fish increased with the discovery of a new immunoglobulin isotype, IgT (or IgZ) (Hansen, Landis, & Phillips, 2005; Ryo et al., 2010), specialized in mucosal immunity (Xu, Parra, et al., 2013; Zhang et al., 2010). This specific

Table 9 Immune Responses of *Cyprinus carpio* to Cyprinid Herpesvirus 3 Infection

Immune Response	Antiviral Action	Organ/Cell Type	Phenotype	References
Antimicrobial peptides	Destroying virus particles	Skin	Downregulated/no response	Adamek et al. (2013)
Mucins	Physical protection	Skin	Downregulated	Adamek et al. (2013)
		Gut	No response	Syakuri et al. (2013)
Claudins	Physical protection, tissue permeability	Skin	Downregulated	Adamek et al. (2013)
		Gut	Upregulated	Syakuri et al. (2013)
Type I IFNs and IFN-stimulated genes	Limiting virus replication, inducing antiviral state of the cell	Fibroblasts	No response	Adamek et al. (2012)
		Head kidney leukocytes	Upregulated	Adamek et al. (2012)
		Head kidney	Upregulated	Adamek, Rakus, et al. (2014) Rakus et al. (2012)
		Skin	Upregulated	Adamek et al. (2013) Adamek, Rakus, et al. (2014)
		Gut	Upregulated	Syakuri et al. (2013)
Apoptosis	Death of infected cell	Gills Head kidney Spleen	Upregulated (delay)	Miest et al. (2015)

Proinflammatory cytokines/ chemokines	Activating the immune response, proinflammatory action	Skin	Upregulated	Adamek et al. (2013)
		Gut	Upregulated	Syakuri et al. (2013)
		Spleen	Upregulated/ downregulated	Rakus et al. (2012) Ouyang et al. (2013)
Anti-inflammatory cytokines	Regulation of inflammatory response	Spleen	Upregulated	Rakus et al. (2012) Ouyang et al. (2013)
Acute-phase response (CRP and complement)	Neutralizing viral particles, lysis of infected cells	Serum	Upregulated/no response	Rakus et al. (2012)
		Serum Liver Head kidney Spleen Gills	Upregulated	Pionnier et al. (2014)
MHC class I	Antigen presentation	Head kidney	Upregulated	Rakus et al. (2012)
Cytotoxic $CD8^+$ T cells	Killing infected cells	Spleen	Upregulated	Rakus et al. (2012)
Antibody response	Coating, neutralizing of virus particles	Serum	Upregulated	Perelberg, Ilouze, Kotler, and Steinitz (2008) Ronen et al. (2003) Adkison, Gilad, and Hedrick (2005)
Genetic markers associated with resistance				
Cyprinus carpio IL-10a				Kongchum et al. (2011)
Cyprinus carpio MHC class II *B*				Rakus, Wiegertjes, Adamek, et al. (2009)

Adapted with Permission from Adamek, Steinhagen, et al. (2014).

mucosal adaptive immune response further supports the importance of antigen presentation at the pathogen's portal of entry to induce topologically adequate immune protection capable of blocking pathogen entry into the host (Gomez, Sunyer, & Salinas, 2013; Rombout, Yang, & Kiron, 2014). In a recent study, a CyHV-3 recombinant attenuated vaccine candidate used by immersion was shown to infect the skin mucosa and to induce a strong immune response at this CyHV-3 portal of entry. Indeed, the vaccine induced a protective mucosal immune response capable of preventing the entry of wild-type CyHV-3 expressing luciferase as a reporter (Fig. 10; Boutier et al., 2015). Whether this protection is related to the stimulation of the IgZ-secreting B cells associated with a higher concentration of IgZ in the mucus represents an interesting fundamental research question that could be addressed in the future using this CyHV-3 mucosal immunity model.

3.2.4.5 CyHV-3 Genes Involved in Immune Evasion

In silico analyses but also *in vitro* and *in vivo* experiments suggest that CyHV-3 may express immune evasion mechanisms that could explain the acute and dramatic clinical signs associated with KHVD. Members of the *Herpesviridae* family have developed sophisticated immune evasion mechanisms (Horst, Ressing, & Wiertz, 2011). Bioinformatics analysis of the CyHV-3 genome revealed several genes encoding putative homologs of host or viral immune-related genes (Aoki et al., 2007). These genes are ORF4 and ORF12 encoding TNF receptor homologs, ORF16 encoding a G protein-coupled receptor homolog, ORF112 encoding a Zalpha domain-containing protein, ORF134 encoding an IL-10 homolog, and ORF139 encoding a poxvirus B22R protein homolog (Aoki et al., 2007). The potential roles of some of these genes in immune evasion mechanisms have been addressed in a few studies. Their main results are summarized below.

Ouyang et al. (2013) characterized the secretome of CyHV-3 and demonstrated that ORF12 was the most abundant secreted viral protein in the supernatant of infected CCB cells. Recently, it was established that infected carp produce antibodies raised against the ORF12 protein (Kattlun, Menanteau-Ledouble, & El-Matbouli, 2014). These observations are consistent with the hypothesis that ORF12 could act *in vivo* as a soluble TNFα receptor as suggested by bioinformatics analyses.

When exploring the usefulness of a CyHV-3 BAC clone to produce recombinant viruses, a CyHV-3 ORF16 deleted strain was produced (Costes et al., 2008). No significant reduction of virulence was observed, suggesting a minor role of this gene in the pathogenesis of the infection at least in the experimental conditions tested.

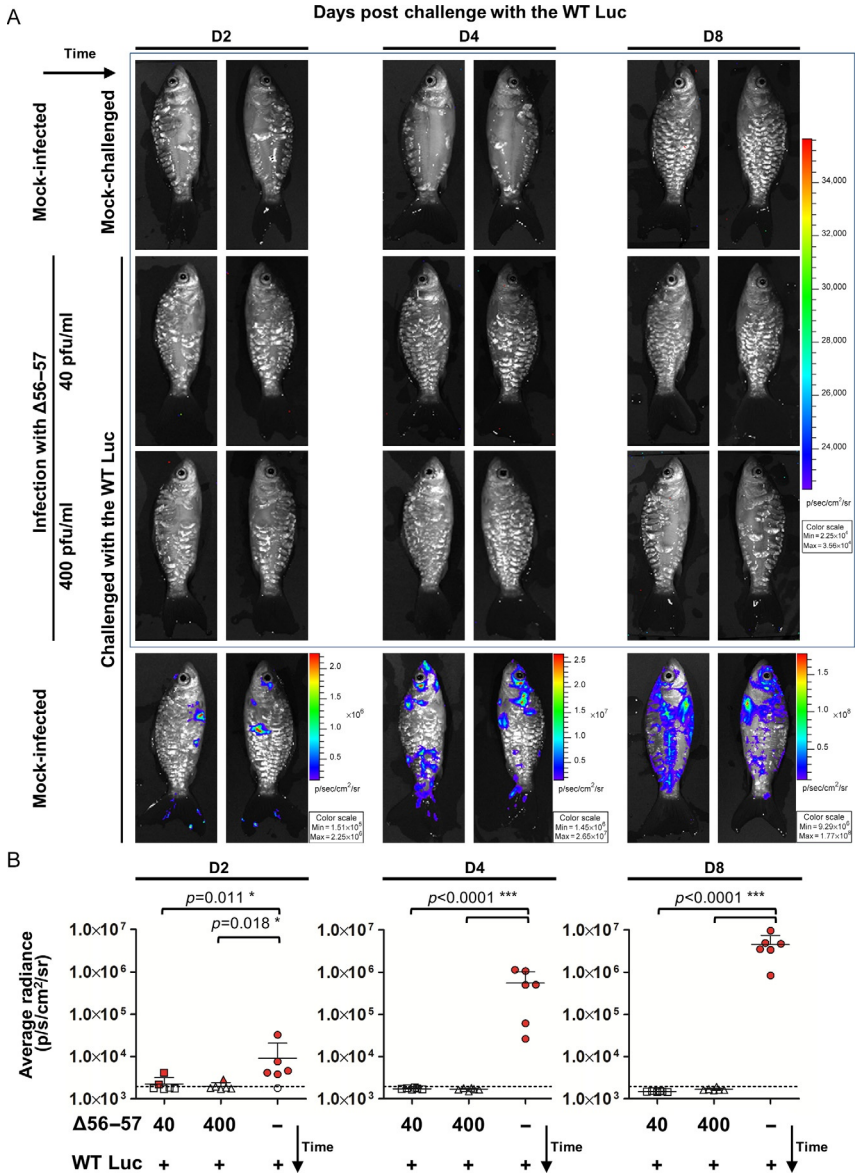

Figure 10 Immune protection conferred by the Δ56–57 attenuated CyHV-3 vaccine revealed by *in vivo* bioluminescent imaging. Common carp (mean ± SD weight 13.82 ± 5.00 g, 9 months old) were infected for 2 h by immersion in water containing 40 or 400 pfu/ml of the Δ56–57 attenuated CyHV-3 strain or mock-infected. None of the fish died from primary infection. Forty-two days post-primary infection, fish were challenged by immersion for 2 h in water containing 200 pfu/ml of the WT Luc strain. At the indicated times post-challenge, fish ($n = 6$) were analyzed using the IVIS.

(*Continued*)

CyHV-3 ORF112 is expressed as an IE gene (Ilouze et al., 2012a) and its 278 amino-acid expression product is incorporated into the virion (structural protein; Fig. 5; Michel, Leroy, et al., 2010). No homology has been detected for the N-terminal part of the protein. In contrast, its C-terminal end encodes a functional Zalpha domain. Zalpha domains are 66 amino acid-long domains which bind to left-handed dsDNA (Z-DNA) or left-handed dsRNA (Z-RNA) (Athanasiadis, 2012). Zalpha domains have been described in three cellular proteins (ADAR1, DAI, and PKZ) belonging to the host innate immune system and in two viral proteins (E3L encoded by most *Chordopoxvirinae* and ORF112 encoded by CyHV-3), acting as immune evasion factors. These data suggest that unusual conformation of nucleic acids detected by Zalpha domain-containing proteins could be interpreted by the innate immune system as pathogen (PAMP) or host cell damage (DAMP). In cells, Z-DNA formation is induced by negative supercoiling generated by moving RNA polymerases. One of the three cellular proteins containing Zalpha domains is PKZ encoded by Cypriniforms and Salmoniforms (Rothenburg et al., 2005). PKZ is a paralog of the dsRNA-dependent protein kinase (PKR) expressed by all vertebrates. PKR is an IFN-induced protein that plays an important role in antiviral innate immunity, mainly (but not exclusively) by phosphorylation of the eukaryotic initiation factor 2 alpha and consequent protein synthesis shutdown when detecting right-handed dsRNA in the cell. PKZ induces the same effects when detecting Z-DNA and/or Z-RNA in infected cells. The demonstration that CyHV-3 encodes a Zalpha domain-containing protein able to over-compete the binding of PKZ to Z-DNA (Tome et al., 2013) suggests that the latter protein plays a significant role in the innate immune response of carp against CyHV-3 and that this immune reaction needs to be evaded by the virus. However, the potential function of ORF112 in virus pathogenesis *in vivo* has not been studied yet.

Figure 10—Cont'd (A) Representative images. Images within the blue frame (light gray in the print version) were normalized using the same scale. (B) Average radiance (individual values, mean + SD) measured on the entire body surface of the fish (individual values represent the mean of the left and right sides obtained for each fish). The discontinuous line represents the cutoff for positivity, which is the mean + 3 SD ($p < 0.00135$) of the values obtained (not presented) for mock-infected and mock-challenged fish (negative control). Positive fish are represented by red filled dots (dark gray in the print version). Significant differences in the mean of the average radiance were identified by post hoc *t*-test after two-way ANOVA analysis taking the treatment and the time post-challenge as variables. *Reproduced with permission from Boutier et al. (2015).*

CyHV-3 ORF134 encodes a viral homolog of cellular IL-10 (Aoki et al., 2007). Cellular IL-10 is a pleiotropic cytokine with both immunostimulating and immunosuppressive properties (Ouyang et al., 2014). Herpesviruses and poxviruses encode orthologs of cellular IL-10, called viral IL-10s, which appear to have been acquired from their host on multiple independent occasions during evolution (Ouyang et al., 2014). Common carp IL-10 was recently shown to possess the prototypical activities described in mammalian IL-10s such as anti-inflammatory activities on macrophages and neutrophils, stimulation of $CD8^+$ memory T cells, stimulation of the differentiation and antibody secretion by IgM^+ B cells (Piazzon, Savelkoul, Pietretti, Wiegertjes, & Forlenza, 2015). Whether CyHV-3 ORF134 exhibits similar properties to carp IL-10 still needs to be investigated. The CyHV-3 ORF134 expression product is a 179 amino-acid protein (Sunarto et al., 2012) which exhibits 26.9% identity (67.3% similarity) with the common carp IL-10 over 156 amino acids (van Beurden, Forlenza, et al., 2011). Transcriptomic analyses revealed that ORF134 is expressed from a spliced transcript belonging to the early (Ilouze et al., 2012a) or early-late class (Ouyang et al., 2013). Proteomic analyses of CyHV-3-infected cell supernatant demonstrated that the ORF134 expression product is the second most abundant protein of the CyHV-3 secretome (Ouyang et al., 2013). In CyHV-3-infected carp, ORF134 is highly expressed during acute and reactivation phases, while it is expressed at a low level during low-temperature-induced persistent phase (Sunarto et al., 2012). *In vivo* study using a zebrafish embryo model suggested that CyHV-3 ORF134 encodes a functional IL-10 homolog. Indeed, injection of mRNA encoding CyHV-3 IL-10 into zebrafish embryos increased the number of lysozyme-positive cells to a similar degree as observed with zebrafish IL-10. Moreover, this effect was abrogated when downregulation of the IL-10 receptor long chain (IL-10R1) was performed using a specific morpholino (Sunarto et al., 2012). Recently, a CyHV-3 strain deleted for ORF134 and a derived revertant strain were produced using BAC cloning technologies (Ouyang et al., 2013). The recombinant ORF134 deleted strain replicated comparably to the parental and the revertant strains both *in vitro* and *in vivo*, leading to a similar mortality rate. These results demonstrated that the IL-10 homolog encoded by CyHV-3 is essential neither for viral replication *in vitro* nor for virulence *in vivo*. In addition, quantification of carp cytokine expression by RT-qPCR at different times post-infection did not reveal any significant difference between the groups of fish infected with the three virus genotypes (Ouyang et al., 2013).

3.2.5 Diagnosis

Diagnosis of KHVD in clinically affected fish can be achieved by numerous methods. The manual of diagnostic tests for aquatic animals lists gross clinical signs, histopathological alterations, and transmission electron microscopy as suitable for presumptive diagnosis of KHVD and descriptions of these can be found earlier in this review (OIE, 2012). However, final diagnosis must rely on direct detection of viral DNA or virus isolation and identification (OIE, 2012). The manual details virus detection methods that include single-round conventional PCR assays, virus isolation in cell culture, indirect fluorescent antibody tests (FATs) on kidney imprints, and formalin-fixed paraffin wax sections followed by confirmatory identification using PCR and nucleotide sequencing. However, none of the tests are fully validated and the manual suggests that diagnosis of KHVD should not rely on just one test but rather a combination of two or three that include clinical examination as well as virus detection (OIE, 2012).

3.2.5.1 PCR-Based Methods

A number of conventional PCR assays have been published, which have been shown to detect CyHV-3 DNA in cell culture supernatant or directly in fish tissues (Bercovier et al., 2005; Gilad et al., 2002; Gray et al., 2002; Hutoran et al., 2005; Ishioka et al., 2005). A PCR based on amplification of the TK gene of CyHV-3 was reported to be more sensitive than other published PCR assays (Gilad et al., 2002; Gray et al., 2002) and could detect 10 fg of CyHV-3 DNA (Bercovier et al., 2005), while the PCR of Ishioka et al. (2005), based on the DNA polymerase gene, detected 100 fg of CyHV-3 DNA. The PCR developed by Gray et al. (2002) was improved by Yuasa, Sano, Kurita, Ito, and Iida (2005) and has been incorporated in the official Japanese guidelines for the diagnosis of KHVD. The Yuasa et al. (2005) and Bercovier et al. (2005) assay protocols are recommended by, and detailed in, the manual of diagnostic tests for aquatic animals (OIE, 2012).

Alternatively, many diagnostic laboratories favor the use of qPCR assays for detection of CyHV-3. The most commonly used quantitative assay for detection of CyHV-3 is the Gilad Taqman real-time PCR assay (Gilad et al., 2004), which has been shown to detect and quantitatively assess very low copy numbers of target nucleic acid sequences and is widely acknowledged to be the most sensitive published PCR method available (OIE, 2012). There are a small number of studies that have compared the sensitivity of the published PCR assays, and different primer sets, for detection of

CyHV-3 (Bergmann, Riechardt, Fichtner, Lee, & Kempter, 2010; Monaghan, Thompson, Adams, & Bergmann, 2015; Pokorova et al., 2010). Conventional PCR assays that include a second round with nested primers have also been shown to be comparable in sensitivity to real-time assays (Bergmann, Riechardt, et al., 2010).

Loop-mediated isothermal amplification (LAMP) is a rapid single-step assay which does not require a thermal cycler, and is widely favored for pond-side diagnosis. LAMP of the TK gene has been developed for detection of CyHV-3 and shown to be more or equally sensitive as conventional PCR assays (Gunimaladevi, Kono, Venugopal, & Sakai, 2004; Yoshino, Watari, Kojima, & Ikedo, 2006; Yoshino, Watari, Kojima, Ikedo, & Kurita, 2009). An assay incorporating DNA hybridization technology and antigen–antibody reactions in combination with LAMP has also been developed and reported to have improved sensitivity and specificity (Soliman & El-Matbouli, 2010).

3.2.5.2 Virus Isolation in Cell Culture

Cell lines permissive to CyHV-3 replication have been described earlier in this review (see Section 3.1.6.1). The CCB and KF-1 cell lines are recommended for isolation of CyHV-3, but cell culture isolation is not considered to be as sensitive as the published PCR-based methods for detecting CyHV-3 DNA. Consequently, virus isolation in cell culture is not a reliable diagnostic method for KHVD (OIE, 2012). Furthermore, viruses isolated in cell culture must be definitively identified, as a number of different viruses have been isolated from carp exhibiting clinical signs resembling those of KHVD (Haenen et al., 2004; Neukirch et al., 1999; Neukirch & Kunz, 2001). The most reliable method for confirmatory identification of a CPE is by PCR and nucleotide sequence analysis (OIE, 2012). A variety of tissues in different combinations have been used for inoculation of cell cultures, such as gill, kidney, spleen, liver, skin, and encephalon (Gilad et al., 2003; Gilad et al., 2002; Hedrick et al., 2000; Neukirch & Kunz, 2001; Sano et al., 2004; Yuasa, Sano, et al., 2012). There is no definitive study that has demonstrated the advantages of certain tissues over others but in the early stages of clinical infection, before clinical signs are observed, virus levels are higher in gill tissue than in kidney tissue (Yuasa, Sano, et al., 2012).

3.2.5.3 Immunodiagnostic Methods

Immunodiagnostic (antibody-based) assays have been little used for the diagnosis of KHVD. Pikarsky et al. (2004) identified the virus in touch

imprints of liver, kidney, and brain of infected fish by FAT; positive immunofluorescence (IF) was the highest in the kidney. The same FAT method was subsequently used by Shapira et al. (2005) who followed the course of KHVD in different strains of fish and detected virus on a kidney imprint 1 dpi. Pikarsky et al. (2004) also detected virus antigen in infected tissues by an immunoperoxidase-staining method. The virus antigen was detected at 2 dpi in the kidney and also observed in the gills and liver. However, the results of antibody-based identification methods must be interpreted with care, as positive cells were seen in a small number of control fish which could have originated from a serologically related virus, or a cross-reaction with nonviral proteins (Pikarsky et al., 2004). ELISA-based methods have not been widely favored by diagnostic laboratories. Currently, one published ELISA method is available to detect CyHV-3 in fish droppings (Dishon et al., 2005). Recently, a CyHV-3-detection kit (The FASTest Koi HV kit) adapted to field conditions has been developed and proved to detect 100% of animals which died from CyHV-3. This lateral flow device relies on the detection of the ORF65 glycoprotein of CyHV-3. It is recommended to be performed on gill swabs and takes 15 min (Vrancken et al., 2013).

3.2.5.4 Other Diagnostic Assays

Assays developed for research applications include a primer probe designed against an exonic mRNA-coding sequence that allows the detection of replicating CyHV-3 (Yuasa, Kurita, et al., 2012). IF and ISH methods, performed on separated fish leucocytes obtained by nondestructive (nonlethal) techniques, have also been used in research applications for detection or identification of CyHV-3 (Bergmann, Lutze, et al., 2010; Bergmann et al., 2009). ISH has also been applied to successfully detect CyHV-3 DNA in archive paraffin-embedded tissue specimens collected during unexplained mass mortalities of koi and common carp in the UK in 1996, and in cultured common carp in South Korea in 1998 (Haenen et al., 2004; Lee et al., 2012).

3.2.6 Vaccination

The economic losses induced by CyHV-3 stimulated the development of prophylactic measures. Passive immunization by administration of pooled sera from immunized fish (Adkison et al., 2005) and addition of anti-CyHV-3 IgY antibodies to fish food (Liu et al., 2014) showed partial effect on the onset of clinical signs but did not significantly reduce mortalities. In contrast,

several vaccine candidates conferring efficient protection were developed. They are reviewed in this section.

3.2.6.1 Natural Immunization

Soon after the identification of CyHV-3 as the causative agent of KHVD, an original protocol was developed to induce a protective adaptive immune response in carp (Ronen et al., 2003). This approach relied on the fact that CyHV-3 replication is drastically altered at temperatures above 30 °C (Dishon et al., 2007). According to this protocol, healthy fingerlings are exposed to the virus by cohabitation with sick fish for 3–5 days at permissive temperature (22–23 °C). After that, the fish are transferred to ponds for 25–30 days at nonpermissive water temperature (≈30 °C). Despite its ingenuity, this protocol has several disadvantages. (i) Fish that are infected with this protocol become latently infected carriers of a fully virulent strain and are therefore likely to represent a potential source of CyHV-3 outbreaks if they later cohabit with naive carp. (ii) The increase of water temperature to nonpermissive is costly and correlates with increasing susceptibility of the fish to secondary infections. (iii) Finally, after this procedure, only 60% of infected fish were sufficiently immunized to be resistant to a CyHV-3 challenge (Ronen et al., 2003).

3.2.6.2 Vaccine Candidates

In addition to the safety/efficacy issues that apply to all vaccines independent of the target species (humans or animals), vaccines for fish and production animals in general are under additional constraints (Boutier et al., 2015). First, the vaccine must be compatible with mass vaccination and administered via a single dose as early as possible in life. Second, the cost–benefit ratio should be as low as possible, implying the lowest cost for vaccine production and administration (Sommerset, Krossoy, Biering, & Frost, 2005). Ideally, cost-effective mass vaccination of young fish is performed by immersion vaccination, meaning that the fish are bathed in water containing the vaccine. This procedure allows vaccination of a large number of subjects when their individual value is still low and their susceptibility to the disease is the highest (Brudeseth et al., 2013). Immersion vaccination is particularly adapted to common carp culture that is a low-cost and low industrial scale production compared to other sectors (Brudeseth et al., 2013). The use of injectable vaccines for mass vaccination of fish is restricted to limited circumstances, i.e., when the value of individual subject is relatively high and when

vaccination can be delayed until an age when the size of the fish is compatible with their manipulation (Plant & Lapatra, 2011).

Various anti-CyHV-3 vaccine candidates have been developed. An inactivated vaccine candidate was described which consists of formalin-inactivated CyHV-3 trapped within a liposomal compartment. This vaccine could be used for oral immunization by addition to fish food. It reduced by 70% the mortality induced by a challenge (Yasumoto, Kuzuya, Yasuda, Yoshimura, & Miyazaki, 2006). Injectable DNA vaccines consisting of plasmids encoding envelope glycoproteins ORF25 and ORF81 were shown efficacious under experimental conditions (Zhou, Wang, et al., 2014; Zhou, Xue, et al., 2014) but are unfortunately incompatible with most of the field constraints described above. Nevertheless, they could represent a solution for individual vaccination of koi carp.

Attenuated vaccines could meet the constraints of mass vaccination listed above. However, they raise safety concerns, such as residual virulence, reversion to virulence, and spread from vaccinated to naive subjects (Boutier et al., 2015). A conventional anti-CyHV-3 attenuated vaccine has been developed by serial passages in cell culture and UV irradiation (O'Connor et al., 2014; Perelberg et al., 2008; Perelberg, Ronen, Hutoran, Smith, & Kotler, 2005; Ronen et al., 2003; Weber et al., 2014). This vaccine is commercialized in Israel for the vaccination of koi and common carp by immersion in water containing the attenuated strain. Recently launched in the US market, it was withdrawn from sale after just a year. This vaccine has two major disadvantages. First, the attenuated strain has residual virulence for fish weighing less than 50 g (Weber et al., 2014; Zak et al., 2007), which restricts the use of this vaccine. Second, the determinism of the attenuation is unknown, and consequently, reversions to a pathogenic phenotype cannot be excluded (Meeusen, Walker, Peters, Pastoret, & Jungersen, 2007).

Due to scientific advances in molecular biology and molecular virology, the development of attenuated vaccines is evolving from empirical to rational design (Rueckert & Guzman, 2012). A viral genome can be edited to delete genes encoding virulence factors in such a way that reversion to virulence can be excluded. This approach has been tested for CyHV-3 by targeting different genes thought to encode virulence factors, such as ORF16, ORF55, ORF123, and ORF134, which encode a G protein-coupled receptor, TK, deoxyuridine triphosphatase, and an IL-10 homolog, respectively. Unfortunately, none of the recombinants expressed a safety/efficacy profile compatible with its use as an attenuated recombinant vaccine (Costes et al., 2008; Fuchs, Fichtner, Bergmann, & Mettenleiter, 2011; Ouyang et al., 2014).

Recently, a vaccine candidate based on the double deletion of ORF56 and ORF57 was produced using BAC cloning technology (Boutier et al., 2015). This strain exhibited properties compatible with its use as an attenuated recombinant vaccine for mass vaccination of carp by immersion in water containing the virus: (i) it replicates efficiently *in vitro* (essential for vaccine production); (ii) the deletion performed makes reversion impossible; (iii) it expresses a safe attenuated phenotype as demonstrated by the absence of residual virulence even for young subjects and by its limited spreading from vaccinated to naive subjects; (iv) it induces a protective mucosal immune response against a lethal challenge by blocking viral infection at the portal of entry (Fig. 10). Although the two ORFs deleted in this vaccine candidate are of unknown function, they are both conserved in cyprinid herpesviruses (CyHV-1 and CyHV-2) and ORF57 is additionally conserved in AngHV-1 and crocodile poxvirus (Davison et al., 2013). These homologs represent evident targets for further development of attenuated recombinants for these pathogenic viruses (Boutier et al., 2015).

4. CONCLUSIONS

It is generally accepted that fundamental research precedes and stimulates applied research. Work on CyHV-3 has demonstrated that events can take place in the reverse order. Since the first description of CyHV-3 in the late 1990s, this virus has been inducing important economic losses in the common and koi carp industries worldwide. It is also producing negative environmental implications by affecting wild carp populations. These negative impacts and the importance of the host species have stimulated studies aimed directly or indirectly at developing diagnostic and prophylactic tools to monitor and treat CyHV-3 disease. Unexpectedly, the data generated by these applied studies have created and highlighted interest in CyHV-3 as a fundamental research model. The CyHV-3/carp model has the advantages that large amounts of information and reagents are available for both the virus and its host, and that it permits the study of the entire biological cycle (including transmission) of an alloherpesvirus during infection of its natural host (i.e., a virus/host homologous model). As highlighted throughout this review, there are many fascinating topics that can be addressed by using the CyHV-3/carp model as the archetype for studying the family *Alloherpesviridae*. These include, for example, how viruses in this family express key biological properties that are shared with members of the family *Herpesviridae* while having relatively few genes in common with

them, and how the temperature of the poikiloterm host affects and possibly regulates the switch between lytic and latent infection.

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