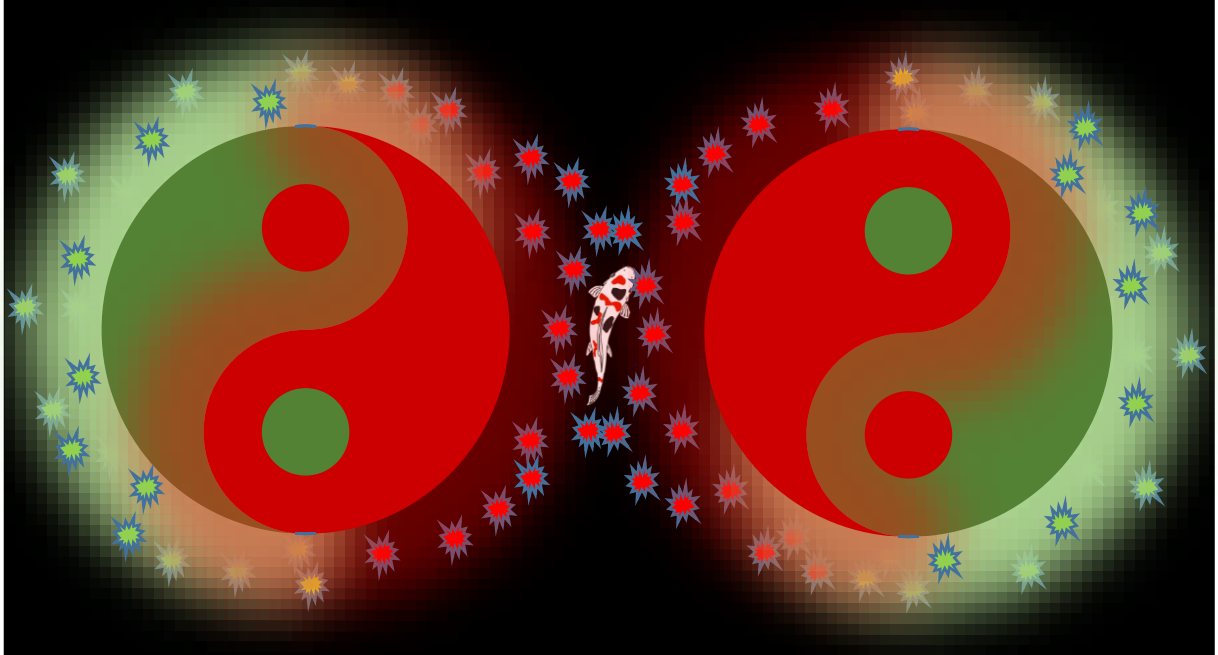
Université de Liège
Faculté de Médecine Vétérinaire
Département des Maladies Infectieuses et Parasitaires
Laboratoire d'Immunologie et de Vaccinologie

# Comparaisons génomiques et biologiques de souches du Cyprinid herpèsvirus 3: un voyage à la découverte de son évolution *in vitro* et *in vivo*

# Genomic and biologic comparisons of Cyprinid herpesvirus 3 strains: a journey into virus evolution *in vitro* and *in vivo*



**Yuan GAO**

**THESE PRESENTEE EN VUE DE L'OBTENTION DU GRADE DE**

**Docteur en Sciences Vétérinaires**

**ANNEE ACADEMIQUE 2020-2021**

**UNIVERSITE DE LIEGE**
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«横看成岭侧成峰，It's a range viewed in face and peaks viewed from the side,

远近高低各不同。Assuming different shapes viewed from far and wide.

不识庐山真面目，Of Mountain Lu we cannot make out the true face,

只缘身在此山中。For we are lost in the heart of the very place. »

出自宋代诗人苏轼〈题西林壁 Written on the Wall of West Forest Temple〉

苏轼 Shi SU (1037-1101)

# *Remerciements*

*Liège, 14 June, 2021*

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# *Abbreviations*

| | |
|---|---|
| **aa** | Amino acid |
| **AciHV-1** | Acipenserid herpesvirus 1 |
| **AciHV-2** | Acipenserid herpesvirus 2 |
| **ADAR1** | Adenosine deaminase acting on RNA 1 |
| **AngHV-1** | Anguillid herpesvirus 1 |
| **ANOVA** | Analysis of variance |
| **Apaf-1** | Apoptotic peptidase activating factor 1 |
| **Ara-C** | Cytosine-β-D-arabinofuranoside |
| **BAC** | Bacterial Artificial Chromosome |
| **Bp** | Base pair |
| **CCB** | *Cyprinus carpio* brain cell |
| **CHX** | Cycloheximide |
| **CPE** | Cytopathic effect |
| **CRV (CrPV)** | Crocodile poxvirus |
| **CyHV-1** | Cyprinid herpesvirus 1 |
| **CyHV-2** | Cyprinid herpesvirus 2 |
| **CyHV-3** | Cyprinid herpesvirus 3 |
| **CyHV-3 J, U, I, GZ11** | Cyprinid herpesvirus 3 Japan, United States, Israel, China isolates, respectively |
| **CyHV-4** | Cyprinid herpesvirus 4 |
| **DAI** | DNA-dependent activator of IFN regulatory factors |
| **DAPI** | 4',6-diamidino-2-phenylindole |
| **Del** | Deleted |
| **DEPP** | Disorder Enhanced Phosphorylation Predictor |
| **DMEM** | Dulbecco's modified essential medium |
| **dpi** | Days post-infection |
| **dsDNA** | Double-stranded deoxyribonucleic acid |
| **E gene** | Early gene |
| **EBNA** | Epstein-Barr virus nuclear antigen |
| **EBV** | Epstein-Barr virus (Human herpesvirus 4) |
| **EGFP** | Enhanced green fluorescent protein |
| **EK-1** | Eel kidney 1 cell line |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **EM** | Electron microscopy |
| **FAO** | Food and Agriculture Organization of the United Nations |
| **FCS** | Fetal calf serum |
| **GadHV-1** | Gadid herpesvirus 1 |
| ***galK*** | Galactokinase cassette |
| ***gB*** | Glycoprotein B |
| **HCMV** | Human cytomegalovirus (Human herpesvirus 5) |
| **hpi** | Hours post-infection |
| **IcHV-1** | Ictalurid herpesvirus 1 |
| **IcHV-2** | Ictalurid herpesvirus 2 |
| **ICTV** | International Committee on Taxonomy of Viruses |
| **ICP4** | Infected cell polypeptide 4 |
| **IE gene** | Immediate early gene |

| | |
|---|---|
| **IFN** | Interferon |
| **IHNV** | Infectious hematopoietic necrosis virus |
| **IL-1β** | Interleukin 1β |
| **IL-10** | Interleukin 10 |
| **iNOS** | Inducible nitric oxide synthase |
| **IVIS** | *In vivo* bioluminescent imaging system |
| **kb (p)** | kilobase (pair) |
| **kDa** | Kilodalton |
| **KF-1** | Koi fin 1 cell line |
| **KHV** | Koi herpesvirus |
| **KHVD** | Koi herpesvirus disease |
| **L genes** | Late genes |
| **LAMP** | Loop-mediated isothermal amplification |
| **Luc** | Luciferase gene |
| **mAb** | Monoclonal antibody |
| **MEM** | Minimum essential medium |
| **MHC** | Major histocompatibility complex |
| **MM** | Molecular mass |
| **MOI** | Multiplicity of infection |
| **MS** | Marker size |
| **NGF-2** | Koi fin derived cell line |
| **NGF-3** | Koi fin derived cell line |
| **ns** | Not significant |
| **OIE** | World Organization for Animal Health |
| **ORF** | Open reading frame |
| **OTU-like** | Ovarian tumor unit like domain |
| **PAA** | Phosphonoacetic acid |
| **pAb** | polyclonal antibody |
| **PAF** | Paraformaldehyde |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **PEI** | Polyethylenimine |
| **PFU** | Plaque forming unit |
| **PKR** | dsRNA-dependent protein kinase |
| **PKZ** | Z-DNA-dependent protein kinase |
| **PLAC8** | Placenta specific 8 protein |
| **pORF** | Amino acid product of an open reading frame |
| **qPCR** | Quantitative PCR |
| **RaHV-1** | Ranid herpesvirus 1 |
| **RaHV-2** | Ranid herpesvirus 2 |
| **RDP** | Recombination detection program |
| **Rev** | Revertant |
| **RFLP** | Restriction fragment length polymorphism |
| **Rh-PCR** | RNase H-dependent PCR |
| **ROI** | Region of interest |
| **RT** | Room temperature |
| **RT-qPCR** | Reverse transcriptase quantitative PCR |
| **RT-PCR** | Reverse transcriptase polymerase chain reaction |
| **SalHV-1** | Salmonid herpesvirus 1 |

| | |
|---|---|
| **SalHV-2** | Salmonid herpesvirus 2 |
| **SalHV-3** | Salmonid herpesvirus 3 |
| **SD** | Standard deviation |
| **SEM** | Standard Error of Mean |
| **siRNA** | Small interfering RNA |
| **SNP** | Single nucleotide polymorphism |
| **SP(s)** | Signal peptide(s) |
| **SVCV** | Spring viremia of carp virus |
| **TK** | Thymidine kinase |
| **TMD(s)** | Transmembrane domain(s) |
| **TNF** | Tumor necrosis factor |
| **TNFR** | Tumor necrosis factor receptor |
| **Trunc** | Truncated |
| **TR** | Terminal direct repeat |
| **UV** | Ultra violet |
| **v/v** | Volume/volume |
| **VLPs** | Virus-like particles |
| **VNTR** | Variable number of tandem repeat |
| **VTP** | Virion transmembrane protein |
| **w/v** | Weight/volume |
| **WB** | Western blot |
| **WT** | Wild type |
| **Z-DNA (-RNA)** | Double stranded left handed DNA (-RNA) |

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# Résumé - Abstract

# *Résumé*

Le « *Cyprinid herpesvirus 3* » (CyHV-3), également connu sous le nom d'herpèsvirus de la carpe koï (KHV), appartient au genre *Cyprinivirus*, famille des *Alloherpesviridae* et l'ordre des *Herpesvirales*. Le CyHV-3 est responsable d'une maladie mortelle chez la carpe commune et la carpe koï (*Cyprinus carpio*) qui sont des espèces de poissons économiquement importantes. Au cours des deux dernières décennies, plusieurs souches de CyHV-3 ont été isolées dans différents pays. Les séquences complètes du génome de 4 souches (KHV-U, KHV-J, KHV-I et KHV GZ11) étaient disponibles dans la Genbank au début de ce projet de thèse, mais aucune comparaison des propriétés biologiques de ces souches n'avait été rapportée. Le premier objectif de cette thèse a été de séquencer les génomes de sept souches additionnelles de CyHV-3 ayant diverses origines géographiques et de comparer certaines propriétés de ces souches *in vitro* et leur virulence *in vivo*. Cette première étude a démontré l'importance de coupler comparaisons génomiques et biologiques de souches virales. Nous avons notamment mis en évidence une relation inversément proportionnelle entre le pouvoir de réplication en culture de cellules et la virulence *in vivo*. Cette observation a suggéré l'existence d'allèles conférant une adaptation à la culture cellulaire mais associés à une réduction de l'adaptation du virus *in vivo* et *vice versa*. Le but de la seconde étude a été d'identifier et d'étudier ce type d'allèles.

En comparant les souches virales *in vitro*, nous avons identifié la formation de syncytium comme un trait commun des souches de CyHV-3 adaptées à la culture cellulaire. La comparaison des protéines transmembranaires du virion codées par les souches de CyHV 3 et la production de divers recombinants ont démontré que le déterminisme génétique de la formation de syncytium est lié au polymorphisme d'un seul nucléotide (SNP pour single nucleotide polymorphism) de l'ORF131 codant pour une glycoprotéine structurelle. Des souches recombinantes différant uniquement par ce SNP (183A et 183T) ont été comparées *in vitro* et *in vivo*. L'allèle 183A de l'ORF131 associé à la formation de syncytium a révélé la meilleure adaptation *in vitro* mais la plus faible *in vivo*. Des expériences de co-infection par les deux recombinants codant pour les SNP 183A et 183T de ORF131 ont révélé, à la fois *in vitro* et *in vivo*, que le variant ayant la meilleure aptitude contribuait à la sélection purificatrice du variant moins adapté par inhibition de la superinfection. Cette observation pourrait expliquer du moins en partie, la faible diversité génétique observée au sein de l'espèce CyHV-3.

# *Abstract*

Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), is a member of the genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales*. CyHV-3 is responsible of a lethal disease in common and koi carp (*Cyprinus carpio*) which are economically important fish species. Over the last two decades, several CyHV-3 strains have been isolated in different countries. Complete genome sequences of 4 strains (KHV-U, KHV-J, KHV-I and KHV-GZ11) were available in the Genbank at the beginning of this PhD project, but no comparisons of the biologic properties of these strains have been reported. In the first part of the thesis, we have sequenced the genomes of a further seven strains from various geographical sources, and have compared their growth *in vitro* and virulence *in vivo.* This part illustrates the importance of coupling genomic and biologic comparisons of viral strains in order to enhance understanding of viral evolution and pathogenesis. It revealed a negative correlation among strains between viral growth *in vitro* and virulence *in vivo*. This observation suggested the existence of alleles conferring adaptation to cell culture but reduced fitness *in vivo* and *vice versa*. The objective of the second part of this thesis was to identify such alleles.

We identified the formation of syncytium *in vitro* as a common trait of cell culture adapted CyHV-3 strains. Comparison of virion transmembrane proteins encoded by CyHV-3 strains and production of various recombinants demonstrated that the genetic determinism of this trait is linked to a single nucleotide polymorphism (SNP) of ORF131 encoding a structural glycoprotein. Recombinant strains differing only by this SNP (183A and 183T) were compared for their fitness *in vitro* and *in vivo.* The ORF131 183A allele associated with syncytium formation revealed the highest fitness *in vitro* but the lowest *in vivo*. Interestingly, co-infection experiments by the two recombinants encoding ORF131 SNP revealed both *in vitro* and *in vivo* that the variant with the highest fitness contributed to the purifying selection of the less fit variant through the inhibition of superinfection, which could contribute the low diversity observed in CyHV-3 species.

# Preamble

# *Preamble*

The structure of this manuscript is as follows. It starts with an introduction on the *Herpesvirales* order and on CyHV-3. The objectives of the thesis are then described, followed by the experimental section. The latter is composed of two chapters. The first study was devoted to genomic and biologic comparisons of CyHV-3 strains. This chapter has been published in Veterinary Research. The second study entitled "Virus-induced inhibition of superinfection as a means for accelerating fitness-based selection of Cyprinid herpesvirus 3 SNP variants *in vitro* and *in vivo"* was under revision in Virus evolution when this thesis was printed. In the last section of this manuscript, the main results will be discussed together with perspectives.

# Introduction

# Introduction

## Cyprinid herpesvirus 3, an archetype of fish alloherpesviruses

Note: most of the structure of this introduction is based on a review from the host lab published in *Advances in Virus Research doi:10.1016/bs.aivir.2015.03.001*

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## 1. Preamble

The order *Herpesvirales* contains a large group of viruses that are widely distributed in nature. Members of this order share structural, genetic and biological properties. Phylogenetically, it is divided into three related families infecting a various range of animals (Pellett *et al.*, 2012). The family *Herpesviridae* comprises viruses infecting mammals, birds or reptiles. It is by far the most important family, in terms both of the number of its members and the volume of studies that devoted to them. The family *Malacoherpesviridae* encompasses viruses infecting molluscs. Finally, the family *Alloherpesviridae* consists of viruses infecting amphibians and fishes. To date, twelve alloherpesviruses have been described with ten of them infecting fish species (Hanson *et al.*, 2011).

Over the last decade, an increasing number of studies have been devoted to alloherpesviruses infecting fish. Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), has emerged as the archetype of fish alloherpesviruses The interest of the scientific community for this virus relies on its impact on wildlife, the economic losses it causes to the aquaculture industry, and its importance as a fundamental research object (Adamek *et al.*, 2014; Rakus *et al.*, 2013).

Since the first outbreak in the late 1990s, CyHV-3 has caused severe ecological impact and induced important financial losses in the common and koi carp industries (Bondad-Reantaso *et al.*, 2005; Perelberg *et al.*, 2003). The common carp (*Cyprinus carpio*) is one of the oldest cultivated freshwater fish species (Balon 1995). It is nowadays one of the most economically valuable species in aquaculture. It is widely cultivated for human consumption, with a worldwide production of 4.6 million tons in 2016 representing US$6.9 billion (FAO, 2017). Moreover, its colourful ornamental variety (koi carp), grown for personal leisure activities and competitive exhibitions, is one of the most expensive markets for individual fish. Due to its economic impact, CyHV-3 has rapidly stimulated research efforts aiming at building essential knowledge for the development of diagnostic and prophylactic tools (Ilouze *et al.*, 2006a; Rakus *et al.*, 2013). These studies also stimulated an interest in CyHV-3 as an object of fundamental research. Nowadays, CyHV-3 is considered as the archetype of fish alloherpesviruses and is the subject of an increasing number of studies. Most of the studies published are summarised in this introduction.

This introduction is structured as follows. It starts with a phylogenetic description of the family *Alloherpesviridae* of the order *Herpesvirales* also and with a review of the main properties of herpesviruses and the specific properties of fish alloherpesviruses. Finally, it provides an exhaustive overview of current knowledge on CyHV-3 with exception of the data that were produced in the context of the present thesis.

## 2. The order *Herpesvirales*

### 2.1. Phylogeny

#### 2.1.1. Phylogeny of the order *Herpesvirales*

The recognition of a virus relies mainly on its virion morphology and its genome structure. In herpesviruses, the virion consists of a linear, double-stranded DNA (dsDNA) genome packed into a T=16 icosahedral capsid, embedded in two complex protein layers known as the inner tegument and outer tegument, wrapped in a glycoprotein-containing lipid membrane. With the development of sequencing and bioinformatics, classification of a virus as a herpesvirus relies mainly on the analysis of its primary sequence data.

For many years, the International Committee on Taxonomy of Viruses (ICTV, http://www.ictvonline.org) reported several fish viruses as being likely members of the family *Herpesviridae* based on morphology. In 1998, the first fish herpesvirus was described in this family, namely *Ictalurid herpesvirus 1* (Ictalurid herpesvirus 1 [IcHV-1], also known as channel catfish virus). Later, the genus in which this species was classified was named *Ictalurivirus*. But before the adoption of the order *Herpesvirales*, it was obvious that this virus was only very distantly related to mammalian herpesviruses (Davison 1992). In 2008, the order *Herpesvirales* was created containing three families, namely 1) the already existing family *Herpesviridae*, encompassing herpesviruses of mammals, birds and reptile, 2) the new families *Alloherpesviridae*, comprising herpesviruses of amphibians and fish, and 3) *Malacoherpesviridae*, consisting of herpesviruses of invertebrates (Pellett *et al.*, 2012; Davison *et al.*, 2009).

The ICTV currently lists 115 species in the family *Herpesviridae.* There are distributed into one unassigned species and three subfamilies, *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae.* These families encompass five, five and seven genera, respectively (https://talk.ictvonline.org/taxonomy/). Establishment of this taxonomical structure has been fostered by a very large number of phylogenetic analyses (McGeoch & Gatherer, 2005; McGeoch *et al.*, 2000; McGeoch *et al.*, 2006). A phylogenetic description of 65 viruses classified in this family is shown in Fig. 1a. It is based on their complete sequences of the highly conserved viral DNA polymerase gene. The overall genetic coherence of this family is apparent due to the fact that 43 genes are conserved among members of the family. These genes are speculated to have been present in the last common ancestor, which has existed some 400 million years ago (McGeoch *et al.*, 2006).

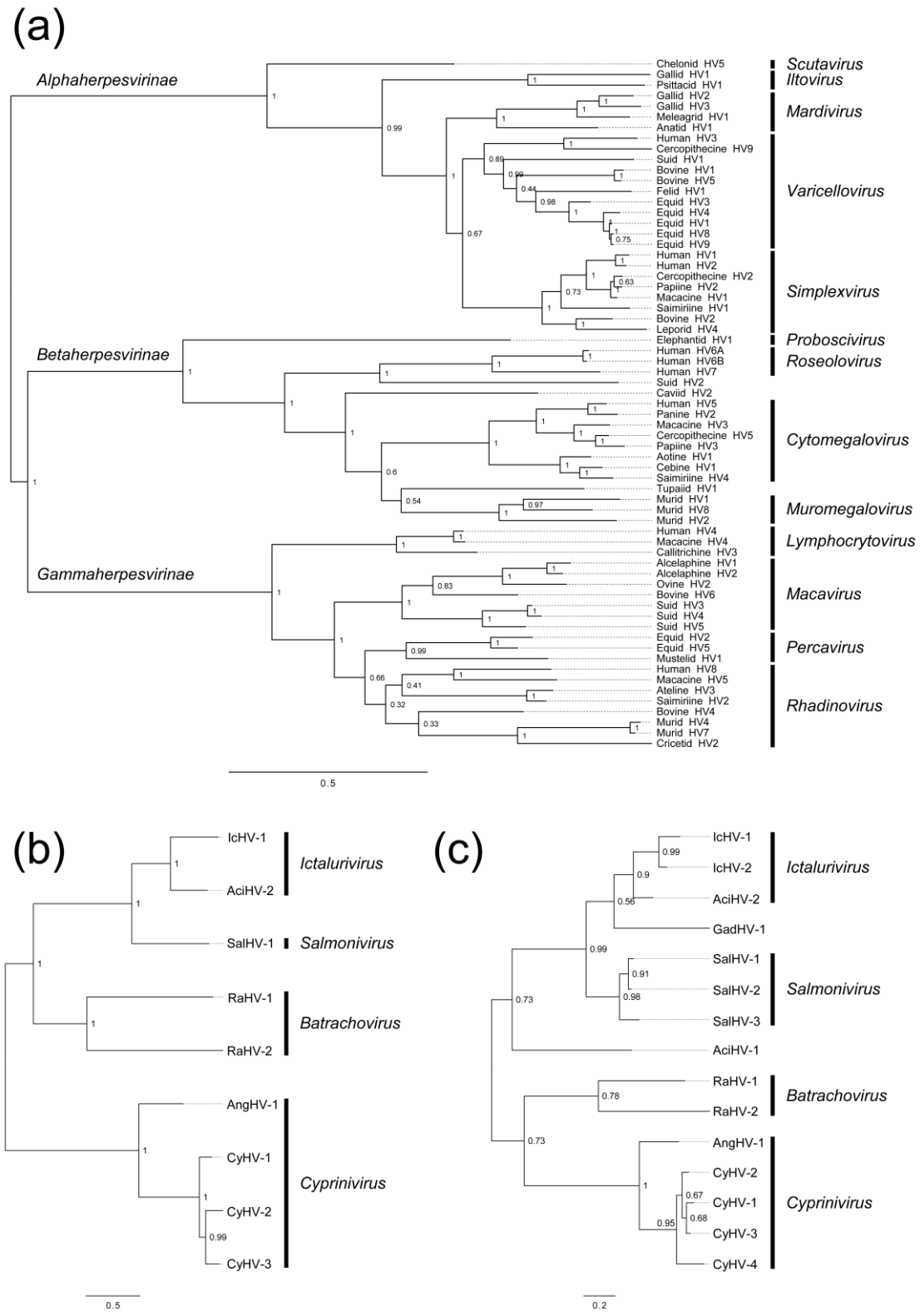


**Figure 1 - Phylogenetic analysis of the *Herpesviridae* and *Alloherpesviridae* families.**

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 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 *et al.*, 2011) for AciHV-2 and AAC59316.1 (Davison 1998) and unpublished data (A.J. Davison) 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 *et al.*, 2013) under a 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.

*Malacoherpesviridae*, the second family in the order *Herpesvirales*, consists of two genera, *Aurivirus*, which contains the species *Haliotid herpesvirus 1* (Haliotid herpesvirus 1 or Abalone herpesvirus), and *Ostreavirus*, which comprises the species *Ostreid herpesvirus 1* (Ostreid herpesvirus 1 or Oyster herpesvirus). Although the coherence of the order is apparent from morphological conservation of the virion, particularly the capsid, among the families (Booy *et al.*, 1996; Davison *et al.*, 2005), detectable genetic similarities are very low. The most convincingly conserved gene is that encoding DNA packaging terminase subunit 1, a subunit of an enzyme complex responsible for incorporating genome DNA into pre-formed 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 and Schmid 2014), point to an origin of all herpesviruses from ancient precursors having existed in bacteria. A description of the phylogeny of the family *Alloherpesviridae*, the third family in the order *Herpesvirales*, to which CyHV-3 belongs, is presented in the following section.

### 2.1.2. Phylogeny of the family *Alloherpesviridae*

Shortly after the first formal report of its discovery (ARIAV 1998; Bretzinger *et al*., 1999), CyHV-3 was characterized as a herpesvirus based on its virion morphology (Hedrick *et al*., 2000). Based on early DNA sequence data, although there were some suggestions that this assignment might not be correct (Hutoran *et al*., 2005; Ronen *et al*., 2003), the initial characterization was soon shown to be confirmed (Waltzek *et al*., 2005). The subsequent accumulation of extensive sequence data for a range of fish and amphibian herpesviruses provided a solid evidence of the phylogeny and evolution of the family *Alloherpesviridae*.

There are currently 12 species listed by the ICTV in the family *Alloherpesviridae*. They are distributed into four genera. Three genera encompass fish viruses (*Cyprinivirus* which contains CyHV-3, *Ictalurivirus* and *Salmonivirus*). The last one regroups amphibian viruses (*Batrachovirus*) (Table 1). Complete genome sequences are available in NCBI for seven of these viruses, representing all genera (Table 2). For the other five classified, and also several unclassified fish herpesviruses, only

partial sequence data are available. A phylogenetic tree of the nine classified viruses, based on the complete DNA sequence of the viral DNA polymerase, is shown in Fig. 1b. Fig. 1c shows a phylogenetic tree of all 12 species, plus three 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 viral DNA polymerase gene. Bootstrap values of these analyses demonstrated that the robustness of the former tree was greater than that of the latter. However, the trees exhibited the same topology, and they were consistent with the arrangement of the family into four genera. The phylogeny of the two unclassified viruses (AciHV-1 and GadHV-1) is not that clear from the limited data used in Fig. 1c. However, the phylogenetic relationship of these viruses, including others not presented in Fig. 1c, has been examined with greater discrimination using sequences from other genes (Doszpoly *et al*., 2008; 2011; 2015; 2011; Kelley *et al*., 2005; Kurobe *et al*., 2008; Marcos-Lopez *et al*., 2012). Recently, a novel unclassified cyprinid herpesvirus in roach (*Rutilus rutilus*) and asp (*Leuciscus aspius*), named CyHV-5, was detected by a molecular technique, which was not included in the phylogeny analyses (Sellyei *et al*., 2020).

There have been some suggestions to establish two or three subfamilies in the *Alloherpesviridae* family. The two-subfamilies option relies on one subfamily encompassing members of the genus *Cyprinivirus* and one subfamily regrouping members of the three other genera. The three-subfamilies option relies on one subfamily encompassing the genus *Cyprinivirus,* one subfamily regrouping members of the genus *Batrachovirus*, and a third subfamily regrouping the two last genera (Doszpoly *et al*., 2011)).

The general genetic coherence of the family *Alloherpesviridae* is apparent from the presence of 12 convincingly conserved genes in full-length genome sequenced members (Davison *et al*., 2013). This limited number suggests the existence of a last common ancestor that is considerably older than that of the family *Herpesviridae*. Patterns of coevolution between virus and host are not supported at deeper nodes but are apparent at the tips of the phylogenetic trees, and therefore are relevant to a more recent evolutionary period (Waltzek *et al*., 2009). Fig. 1 b and c reveal that 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 genus *Ictalurivirus*. Also, the branching point of frog herpesviruses falls within the fish herpesviruses closer to genus *Cyprinivirus*. The lower degree of coevolution of the family *Alloherpesviridae* compared with the family *Herpesviridae* may be due to various factors, such as those related to the respective environments and the lengths of time these two families have been evolving.

**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 haematopoietic 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.

**Table 2 - Data on complete genome sequences of members of the family *Alloherpesviridae*.**

| Virus name | Abbrev. | Genome size (bp) | Genome G+C (%) | ORFs (no.)[a] | GenBank accession | Reference |
|---|---|---|---|---|---|---|
| Anguillid herpesvirus 1 | AngHV-1 | 248,526 | 53 | 134 | FJ940765.3 | van Beurden *et al.* (2010)<br>van Beurden *et al.* (2012b) |
| Cyprinid herpesvirus 1 | CyHV-1 | 291,144 | 51 | 143 | JQ815363.1 | Davison *et al.* (2013) |
| Cyprinid herpesvirus 2 | CyHV-2 | 290,304 | 52 | 154 | JQ815364.1 | Davison *et al.* (2013) |
| Cyprinid herpesvirus 3 | CyHV-3 | 295,146 | 59 | 163 | DQ657948.1[b] | Aoki *et al.* (2007)<br>Davison *et al.* (2013) |
| Ictalurid herpesvirus 1 | IcHV-1 | 134,226 | 56 | 90 | M75136.2 | Davison (1992) |
| Ranid herpesvirus 1 | RaHV-1 | 220,859 | 55 | 132 | DQ665917.1 | Davison *et al.* (2006) |
| 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 *et al.*, 2015).

## 2.2. Genome features

All members of the order *Herpesvirales* contain a genome consisting of a single dsDNA with a length ranging from 124 to 295 kilobase pairs (kb) and a guanine plus cytosine (G+C) percentage varying from 31 to 77% (Aoki *et al.*, 2007). The genome contains internal and terminal reiterated sequences that vary in number of copies, as well as sequences that can be lost or duplicated during passage in cell culture. For years, the arrangement of these sequences has led to the description of 6 classes of genome structure called A to F (Fig. 2 A-F) (Davison 2014). Recently, sequencing of Testudinid herpesvirus 3 (TeHV-3) by the host lab revealed a new class of genome consisting of a long unique region ($U_L$), extended at its right end by a short unique region ($U_S$) flanked by an inverted repeat ($IR_S$ and $TR_S$) and at its left end by a third unique region ($U_T$) also flanked by inverted repeats ($TR_T$ and $IR_T$), yielding the overall configuration $TR_T$-$U_T$-$IR_T$-$U_L$-$IR_S$-$U_S$-$TR_S$ (Fig. 2, class G) (Gandar *et al.*, 2015). All herpesvirus genomes comprise conserved signals for packaging of the DNA into capsids and cleavage of concatemeric genomes to unit length at their termini (Davison 2014).

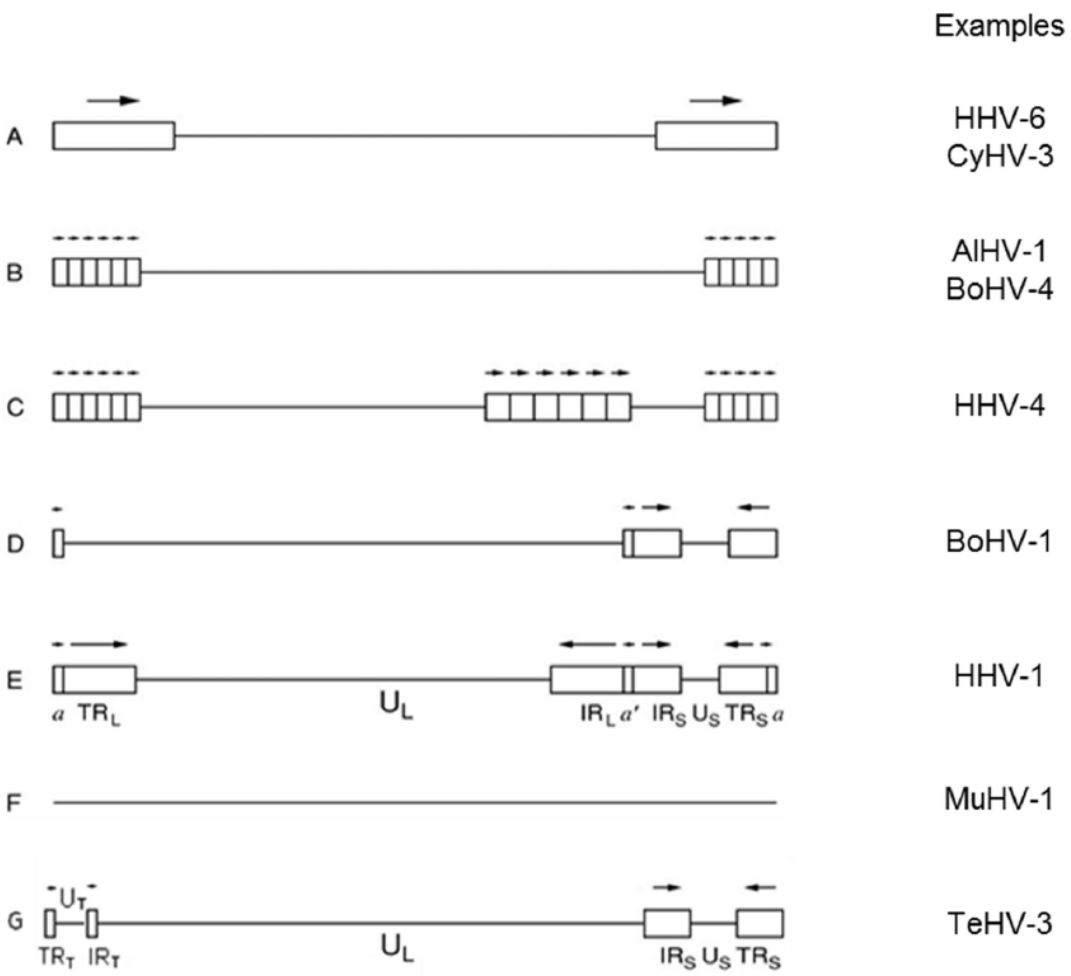


**Figure 2 - Classes of herpesvirus genome structure**. (See legend on next page).

Unique and repeat regions are shown as horizontal lines and rectangles, respectively. The orientation of the repeats are illustrated by arrows. The nomenclature of unique and repeat regions are indicated for classes E and G. (A) The genome consists of a unique sequence flanked by a direct repeat. (B) The genome has directly repeated sequences at the termini which consisting of variable number copies of a tandemly repeated sequence of 0.8–2.3 kb. (C) This structure derives from the class B structure, in which an internal set of direct repeats is present but is unrelated to the terminal set. (D) The genome contains two unique regions (long and short unique regions $U_L$ and $U_S$), each flanked by inverted repeats ($TR_L$/$IR_L$ and $TR_S$/$IR_S$). $U_S$ can be inverted compared to the $U_L$ giving two different isomers. (E) This class is similar to class D, except that $TR_L$/$IR_L$ is much larger and segment inversion gives rise to four equimolar genome isomers. Also, class E genomes are terminally redundant, containing a sequence of a few hundred bp that is repeated directly at the genome termini (a) and inversely at the $IR_L$-$IR_S$ junction (a'). (F) Terminal repeats were not described in the F class. (G) New genome structure recently described for *Testudinid herpesvirus 3* (TeHV-3) with a third unique region ($U_T$) (Gandar *et al.*, 2015). *Human herpesvirus 1* (HHV-1), *4* (HHV-4) and *6* (HHV-6), *Alcelaphine herpesvirus 1* (AlHV-1), *Bovine herpesvirus 1* (BoHV-1) and *4* (BoHV-4), *Murine herpesvirus 1* (MuHV-1), *Cyprinid herpesvirus 3* (CyHV-3) and *Testudinid herpesvirus 3* (TeHV-3) were chosen as examples (adapted from Gandar *et al*., 2015; Davison 2014).

## 2.3. Main biological properties

All members of the order *Herpesvirales* share significantly common biological properties (Ackermann, 2004; Pellett *et al.*, 2012): (*i*) they produce progeny virions with the structure described above (Fig. 3a); (*ii*) the general viral infection includes attachment, penetration, DNA replication, capsid assembly, envelopment and release (Fig. 3b); (*iii*) they encode their own DNA synthesis machinery, with viral replication as well as nucleocapsid assembly taking place in the nucleus (Fig. 3b); (*iv*) production of progeny viral particles is usually accompanied by the lysis of the host cells; (*v*) they are also able to establish lifelong latent infection, which is characterized by the absence of regular viral replication, viral transcription and production of infectious virus particles, but presence of intact viral genomic DNA and the transcription of latency-associated genes. Latency can be interrupted by reactivation to lead to lytic replication and the excretion of virions by infected subjects despite the existence of adaptive immune response developed against the virus; and (*vi*) their ability to establish persistent infection in immunocompetent hosts is the consequence of immune evasion mechanisms targeting major components of the immune system (Pellett *et al.*, 2012).

In addition to these properties which are considered to be common to all members of the order *Herpesvirales*, fish herpesviruses seem to share some biological properties that differentiate them from *Herpesviridae* (herpesviruses infecting mammals, birds and reptiles). Firstly, while herpesviruses generally show only modest pathogenicity in their natural immunocompetent hosts, fish herpesviruses can lead to outbreaks with mortality reaching close to 100%. The markedly higher virulence of some fish herpesviruses could reveal a lower adaptation level of these viruses to their natural hosts (see section 2.1.2.). However, it could also be explained by other reasons such as the high-density rearing conditions and inbreeding promoted by intensive aquaculture. Secondly, compared to the tropism of members of the family *Herpesviridae* which is generally restricted to their natural host species or closely related

species, some alloherpesviruses induce severe disease in only one or few closely related members in the same genus, and 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.). Thirdly, an age-dependent sensitivity and permissivity has been described for several fish herpesviruses, in which AciHV-1, AciHV-2, CyHV-1, CyHV-2, SalHV-2, SalHV-3 and Ictalurid herpesvirus 2 (IcHV-2) are particularly pathogenic for recently hatched fish (Hanson *et al.*, 2011; van Beurden & Engelsma, 2012). Fourthly, a significant difference in the outcome of alloherpesvirus 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 replicates in eel kidney 1 (EK-1) cell line between 15 and 30 °C, with an optimal temperature at 20-25 °C (Sano *et al.*, 1990; van Beurden *et al.*, 2012a). *In vivo*, propagation of Ranid herpesvirus 1 (RaHV-1) is promoted by low temperature, whereas induction of tumor metastasis is promoted by high temperature (McKinnell & Tarin, 1984). Overall, fish herpesvirus-induced infection is less severe or even asymptomatic if the ambient water temperature is suboptimal for virus propagation, which explains the seasonal outbreaks of certain fish herpesviruses, including CyHV-3 (Gilad *et al.*, 2003). Practically, these biological properties have been utilized successfully to immunize naturally carp against CyHV-3 (Ronen *et al.*, 2003), and to decrease the morbidity and mortality rates of AngHV-1 infection in eel culture systems (Haenen *et al.*, 2002). In addition, temperature also plays an important role in inducing latency and reactivation of fish herpesviruses (see sections 3.2.3.2. and 3.2.3.3.).

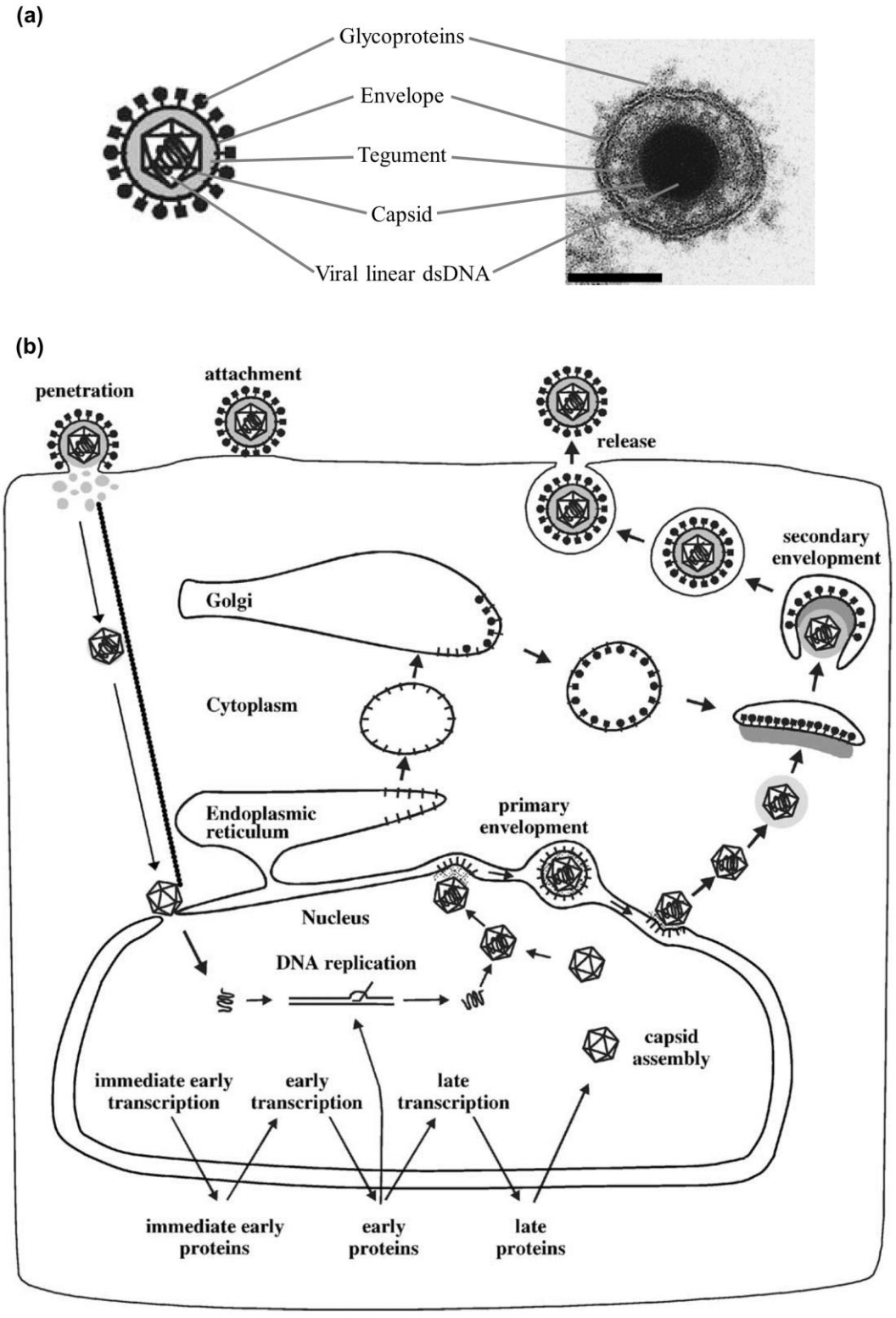


**Figure 3 - Virion structure and replication cycle of herpesviruses.**

(a) Schematic representation (left) and electron microscopy examination (right) of CyHV-3 virion. Bar represents 100 nm. Adapted with permission from Mettenleiter (2004) and Mettenleiter *et al.* (2009). Copyright © Elsevier. (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, primary and secondary envelopment are also illustrated. Reproduced with permission from Mettenleiter (2004). Copyright © Elsevier.

## 2.4. Herpesviruses infecting fish

The first description of fish herpesvirus infection can be traced to the 16th century, when the Swiss naturalist Conrad Gessner described a pox disease of carp inducing skin lesions. After four hundred years, the pox-like lesions were associated with herpesvirus-like particles (Schubert, 1966), later designated as CyHV-1 (Sano *et al.*, 1985). However, the alloherpesvirus that was 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 later, frog virus 4, or Ranid herpesvirus 2 (RaHV-2), was isolated from the pooled urine of tumor-bearing frogs (Gravell *et al.*, 1968).

Alloherpesviruses can infect a wide range of fish species worldwide, including several of the most important species of aquaculture such as catfish, salmon, carp, sturgeon and eel. As a consequence of their restricted host range, the prevalence of specific fish herpesvirus species may be restricted to certain regions of the world. For instance, Pilchard herpesvirus 1 has been reported only in wild Australasian pilchards (*Sardinops sagax neopilchardus*) in Australia and New Zealand (Whittington *et al.*, 2008), whereas CyHV-2 has a worldwide prevalence probably due to the international trade in goldfish (Goodwin *et al.*, 2009).

There are currently ten herpesviruses infecting fish reported in the family *Alloherpesviridae* (see Table 1). In addition, at least a dozen of other fish herpesviruses have been described, but most of these viruses have not been isolated yet, and lack of the primary sequence data prevents their official classification (Hanson *et al.*, 2011; Waltzek *et al.*, 2009). Interestingly, all of these viruses occur in bony fish with one exception having been found in a shark. Based on the number of different herpesvirus species recognized in humans (i.e. nine species) and domestic animals, it is probable that each of the numerous fish species carries various herpesviruses. It is likely that the alloherpesvirus species currently known are biased towards commercially relevant hosts and viruses.

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

In the late 1990s, massive mortalities associated with epidermal lesions, gill necrosis and nephritis occurred worldwide in koi and common carp aquaculture (Haenen *et al.*, 2004). This highly contagious and severe disease was called koi herpesvirus disease (KHVD). Its causative agent was later renamed

CyHV-3 (Bretzinger *et al.*, 1999; Hedrick *et al.*, 2000; Waltzek *et al.*, 2005). Due to its economic impact on carp farming and its rapid spreading across the world, CyHV-3 was listed as a notifiable disease by OIE (World Organization for Animal Health) (Michel *et al.*, 2010a). IcHV-1 had been the prototypic fish herpesvirus for several decades. However, this virus affected the catfish industry only in limited regions and the disease could be controlled 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 prompted an increasing interest to study this virus. In addition, common carp, which is a natural host of CyHV-3, has also been a traditional species for fundamental research in fish immunology, making it a perfect model to study host-virus interactions (Adamek *et al.*, 2014b; Rakus *et al.*, 2013). As a consequence, the CyHV-3-carp homologous model became the most studied model of alloherpesviruses, and CyHV-3 is now considered as the archetype of the *Cyprinivirus* genus.

## 3. Cyprinid herpesvirus 3

### 3.1. General description

#### 3.1.1 Morphology and morphogenesis

Like all members of the order *Herpesvirales*, CyHV-3 virion is composed of a T=16 icosahedral symmetric capsid containing a single copy of a large, linear, double-stranded DNA genome, a host-derived lipid envelope imbedding viral glycoproteins complexes and an amorphous tegument which resides in between the capsid and the envelope (Fig. 2a) (Mettenleiter, 2004; Mettenleiter *et al.*, 2009). The diameter of CyHV-3 virions varies slightly according to the infected cell types 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 *et al.*, 2007)) and *in vivo* (167–200 nm in various organs (Miyazaki *et al.*, 2008)). Despite quite limited conservation of the sequence in proteins involved in virion morphogenesis, members of the families *Herpesviridae*, *Malacoherpesviridae* and *Alloherpesviridae* exhibit a common structure, which implies that the mechanisms of morphogenesis are similar (Mettenleiter *et al.*, 2009). Indeed, the structure of the CyHV-3 virion and its morphogenesis are entirely typical of herpesviruses (Fig. 2b and Fig. 4). Assembly of the nucleocapsid (size 100 nm) takes place in the nucleus and marginalization of chromatin occurs at the inner nuclear membrane (Miwa *et al.*, 2007; Miyazaki *et al.*, 2008). Mature nucleocapsids with the electron-dense core composed of the entire 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 models (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. 4). Finally, the lipid envelope imbedding viral glycoproteins is acquired through budding into vesicle membranes derived from the Golgi apparatus (Mettenleiter *et al.*, 2009; Miwa *et al.*, 2007; Miyazaki *et al.*, 2008).

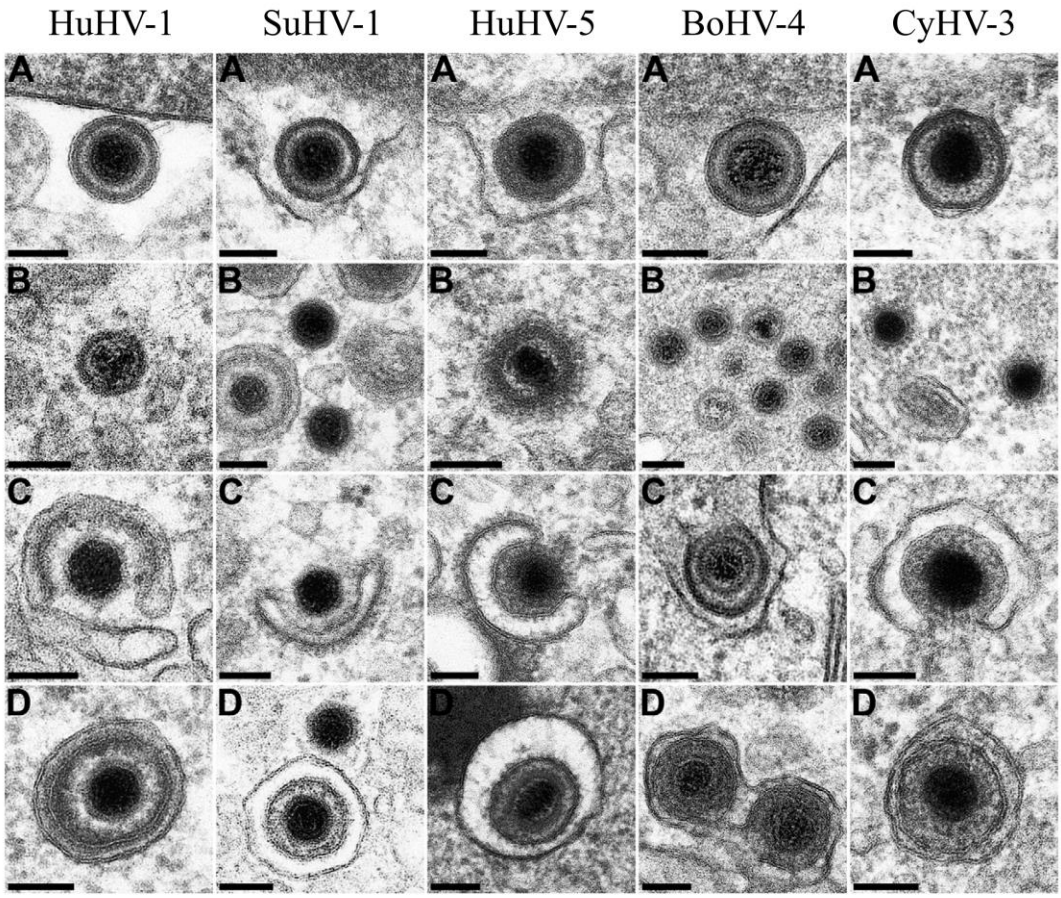


**Figure 4 - Primary and secondary envelopment of some herpesviruses.**

(A) Primary enveloped virions in the perinuclear space. In comparison with Fig. 3, the electron-dense sharply bordered layer of tegument underlying the envelope and the conspicuous absence of envelope glycoprotein spikes is 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.

### 3.1.2. Genome

The complete viral genome sequences of four CyHV-3 strains derived from different geographical origins have been published (Aoki *et al.*, 2007; Li *et al.*, 2015). CyHV-3 is notable for having the largest known genome in herpesviruses, whose size is 295 kbp 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 completely sequenced alloherpesvirus genomes, CyHV-3 genome contains two copies of the terminal direct repeat (TR), which are 22 kbp in the case of CyHV-3. As mentioned in the genome features of the order *Herpesvirales* (see section 2.2), CyHV-3 genome possesses a typical class A genome structure. The annotation of open reading frames (ORFs) in the CyHV-3 genome that are predicted to encode

functional proteins was first reported by Aoki *et al.* (2007), and later refined on the basis of a completely genomic comparison with other viruses of the genus *Cyprinivirus*, as well as members of the other genera (Davison *et al.*, 2013). A feature map of the predicted CyHV-3 genes is shown in Fig. 5, in which the central part of the genome and the two duplicated TR regions encode 148 (ORF9-156) and 8 (ORF1-8) ORFs, respectively. One of the unusual features in the sequenced CyHV-3 genomes is the presence of some fragmented ORFs which are therefore probably non-functional. The precise set of such ORFs varies from strain to strain. There is evidence that at least some originated *in vivo* rather than in cell culture during viral propagation and isolation (Davison *et al.*, 2013). It has been proposed that loss of specific gene functions have contributed to emergence of the disease in common and koi carp populations.

Consistent with their close relationships in the same genus, the three 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 highlighted in red in Fig. 5 (alloherpesvirus core ORFs: ORF33, 46, 47, 61, 71, 72, 78, 79, 80, 90, 92 and 107). There are perhaps two additional genes in this core group (ORF66 and 99) but their conservation is less obvious. Potential features and functions of CyHV-3 ORFs have been described in detail (Boutier *et al.*, 2015). Interestingly, the ancestors of CyHV-3 have evidently captured several genes from the host cells (e.g. the deoxyuridine triphosphatase and interleukin 10 (IL-10) genes) or other viruses (e.g. genes of which the closest relatives are found in iridoviruses or poxviruses) (Ilouze *et al.*, 2006a).

The CyHV-3 genome also comprises five gene families that have presumably arisen by gene duplication, a mechanism for generating genetic diversity that has been reported for herpesviruses in all three families. They are shaded in distinguishingly different colours in Fig. 5. These are the ORF2 family (ORF2, 3, 9, 129, 130 and 135), the tumor necrosis factor receptor (TNFR) family (ORF4 which is in duplicate and 12), the ORF22 family (ORF22, 24 and 137), the ORF25 family (ORF25, 26, 27, 65, 148 and 149, encoding potential type I transmembrane proteins containing an immunoglobulin domain) and the RING family (ORF41, 128, 144 and 150). Some of these ORFs encode proteins related to virion components (ORF25, 27, 65, 137, 148 and 149). Members of these gene families are also present in CyHV-1 and CyHV-2, whereas more distant species, AngHV-1 lacks all but the TNFR family, having instead several other families that are different from the cyprinid herpesviruses (Davison *et al.*, 2013).

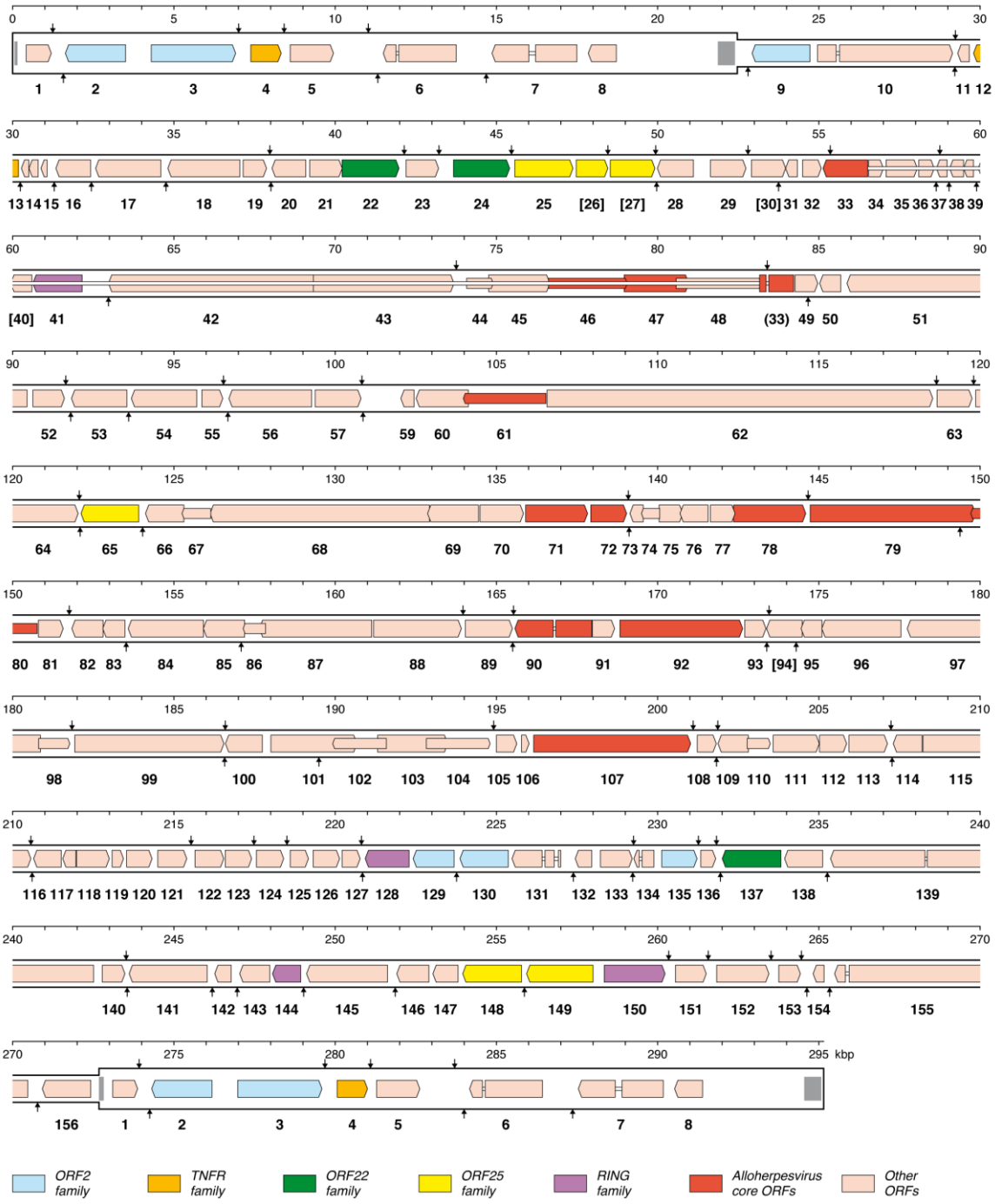


**Figure 5 - Map of the CyHV-3 genome.**

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 colored arrows (see the key at the foot), with nomenclature lacking the ORF prefix given below. Introns are shown as narrow white bars. The colors 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 grey-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, Journal of Virology (2013) 87:2908-2922. doi: 10.1128/JVI.03206-12.

Herpesvirus genomes are described as infectious because their transfection into permissive cells is sufficient to initiate viral DNA replication and production of progeny virions. This property had been

exploited to produce recombinant viruses by recombination in eukaryotic and prokaryotic cells. The latter approach relies on BAC (bacterial artificial chromosome) cloning of the entire viral genome and prokaryotic recombination technologies. This approach has been used extensively for members of the family *Herpesviridae* (Tischer & Kaufer, 2012) and applied by the host laboratory to CyHV-3 (Costes *et al.*, 2008).

### 3.1.3. Genetic diversity, genotypes and evolution

Early investigations on CyHV-3 genetic diversity were based on comparing partial DNA sequences from the DNA polymerase and the major envelope protein genes isolates from the USA, Japan, and Israel. These sequences revealed a high degree of identity (Ishioka *et al.,* 2005). Similar DNA sequence identities were also found between isolates from Poland and Germany (Antychowicz *et al.*, 2005; El-Matbouli, Saleh & Soliman, 2007), suggesting that the virus causing disease in carp worldwide originated from a single virus entity. Later, the complete genome sequences of the three isolates from the United States (CyHV-3 U), Japan (CyHV-3 J), and Israel (CyHV-3 I) revealed a high level of genomic identity (more than 99%), which was consistent with this scenario (Aoki *et al.*, 2007). Despite this very close genetic relationship among isolates, the alignment of the three complete CyHV-3 genome sequences also revealed numerous minor indels (insertions or deletions) and single nucleotide substitutions. These variations enabled a distinction between the CyHV-3 J lineage and the lineage represented by CyHV-3 U and I isolates (Aoki *et al.*, 2007; Bercovier *et al.*, 2005). Furthermore, the full-length sequencing of a fourth strain, CyHV-3 GZ11 (isolated from a mortality outbreak of adult koi carp in Guangzhou, China), revealed a closer relationship of this isolate with the CyHV-3 U/I lineage (Li *et al.*, 2015).

Initially, 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 (Marker I and II) (Bigarré *et al.,* 2009). Analysis of the thymidine kinase (TK, encoded by ORF55) gene sequences (Bercovier *et al*., 2005), particularly the region immediate downstream of the stop codon, provided a significantly higher resolution. In combination with sequence data for the Sph I-5 (coordinates 93604–93895, NCBI: DQ657948) and the 9-5 (coordinates 165399–165882, NCBI: DQ657948) regions (Gilad *et al*., 2002; Gray *et al.*, 2002), 9 different genotypes were identified with 2 from Asia and 7 from Europe (Kurita *et al.*, 2009). The CyHV-3 from Asia showed a high degree of sequence homology, although two variants were differentiated based on a SNP (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). However, preliminary data suggested that the situation might be more complex, as some strains originating from Europe resembled the Asian lineage, and *vice versa* (Li *et al.*, 2015; Bigarré *et al.*, 2009; Dong *et al.*, 2013). Moreover, the coexistence in a single genome of loci belonging to both

lineages suggested that recombination may have occurred (Sunarto *et al.*, 2011). However, definitive conclusions regarding these issues are difficult to make, since partial sequencing covering only a few loci was performed.

Besides the nucleotide mismatches and insertions/deletions (indels), many sequence differences between CyHV-3 isolates were related to number of tandem repeat (VNTR) sequences (Avarre *et al.*, 2011). VNTR polymorphism has shown some utility for differentiating strains of large DNA viruses, such as Human herpesvirus 1. Analysis of multiple VNTR loci in CyHV-3 provided further support for the existence of the two main lineages described above, and, in addition, 87 haplotypes were identified (Avarre *et al.*, 2011; 2012). The discriminatory power of VNTRs is nonetheless more suited to fine tuning the tips of phylogenetic trees based on non-VNTR polymorphisms (SNPs and indels). Unfortunately, robust phylogenetic classification of this sort is lacking for CyHV-3 strains due to the low number of complete genome sequences available. This deficiency could be overcome using genome-wide analysis of multiple viral strains by high-throughput sequencing. This approach has been used successfully for several other herpesviruses, including Human herpesvirus 1 (Szpara *et al.*, 2014), to give a more detailed description of strain diversity (Renner & Szpara, 2018). Recently, this approach was applied to sequence CyHV-3 genome directly from infected fish tissues, without virus isolation (Hammoumi *et al.*, 2016). However, multiple strains were detected in each tissue, potentially causing the derived genome sequences to be artificial composites of the individual strains present.

Current knowledge of herpesvirus evolution relies almost entirely on the study of members of the family *Herpesviridae*. These studies suggest that, despite the proof-reading activity of herpesvirus DNA polymerases, the large size of herpesvirus genomes facilitates a degree of tolerance towards genetic drift (e.g. accumulation of SNPs and indels) in successive rounds of viral replication (Renner & Szpara, 2017). Larger scale changes due to genetic shift also occur, with inter-strain recombination resulting in individual strains acquiring combinations of advantageous genes or alleles initially present in different parental genomes (Kolb *et al.*, 2017; Law *et al.*, 2018; Umene 1999). This process requires co-infection of the same host cell by two parental viruses, and can result either from simultaneous infection or from infection by one strain followed by superinfection by another. The diversity generated by genetic drift and shift is subject to constant fitness-based selection dictated by environmental pressures, which may differ markedly *in vitro* (in cell culture) and *in vivo* (in the natural host). Understanding the key factors that determine how purifying (negative) selection operates on herpesvirus genomes would provide useful insights into the evolution of these viruses. Compared to the family *Herpesviridae* there are relatively few studies regarding evolution within the family *Alloherpesviridae* (Waltzek *et al.*, 2009; 2005; Aoki *et al.*, 2007; Davison *et al.*, 2013). Recently, we investigated the diversity of core gene sequences within species clades of the genus *Cyprinivirus* (referred to as cypriniviruses and comprising alloherpesviruses infecting cyprinids and eels) (Donohoe *et al.*, 2021). This revealed significantly less

genetic diversity within cyprinivirus species clades than within species in the family *Herpesviridae*, which may be linked, at least in part, to different environmental pressures.

### 3.1.4. Transcriptome

Herpesvirus gene transcription and expression follow a coordinated temporal pattern upon infection of permissive cells as shown in Fig. 2b (Pellett *et al.*, 2012). IE (Immediate-early) genes are first transcribed in the absence of *de novo* protein synthesis, and regulate the subsequent expression of following genes. Transcription of E (early) genes is dependent on IE-gene expression, and they encode enzymes and proteins involved in the interaction with host cell metabolism and the viral DNA replication complex. The L (late) genes form the third and last set to be transcribed, dependent on viral DNA synthesis, and primarily encode the viral structural proteins. The first report of fish herpesvirus gene transcription revealed a similar temporal pattern which came from *in vitro* studies on IcHV-1 transcription (Hanson *et al.*, 2011).

Two genome-wide gene transcription analyses of CyHV-3 (Ilouze *et al.*, 2012a) and AngHV-1 (van Beurden *et al.*, 2013) explored the kinetic study of each annotated ORF using two approaches. Firstly, gene transcription was studied by RT-PCR or RT-qPCR during the early hours post-infection (hpi). Secondly, cycloheximide (CHX) and 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 the immediated early genes (IE genes) are transcribed 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 from 4 and 8 hpi (Ilouze *et al.*, 2012a). Transcription of 59 ORFs was detectable at 2 hpi, 63 ORFs at 4 hpi and 28 ORFs at 8 hpi. Transcription of 6 ORFs was only evident at 24 hpi. RNAs from all 156 predicted ORFs of CyHV-3 were detected during infection. This also includes ORF58 which was initially predicted based on a marginal prediction but later removed from the predicted genome map (Fig. 5) (Davison *et al.*, 2013). Expression kinetics of AngHV-1 genes were analyzed in a different way, thus not suitable for direct comparison. However, gene transcription follows a similar pattern (van Beurden *et al.*, 2013).

By inhibiting protein synthesis or viral DNA replication, 15 IE, 112 E and 22 L genes were identified in CyHV-3. Seven ORFs were not classified (Ilouze *et al.*, 2012a). In general, this classification followed the transcription kinetics determined for each ORF, with most IE genes being transcribed at 1 or 2 hpi, most E genes between 2 and 4 hpi and most late genes (L genes) after 8 hpi. In AngHV-1, 4 IE genes, 54 E or E-L genes and 68 L genes were identified (van Beurden *et al.*, 2013). Similar to mammalian herpesviruses, gene transcripts known to be involved in DNA synthesis were expressed earlier, while proteases and enzymes involved in capsid assembly and virion maturation were

expressed later (Ilouze *et al.*, 2012a; van Beurden *et al.*, 2013). Inhibition of some early genes involved in DNA replication (e.g. TK and DNA polymerase) by specific siRNA decreased viral egress from infected cells (Gotesman *et al.*, 2014).

Interestingly, in IcHV-1, AngHV-1and CyHV-3, the IE genes show a clear clustering in or near the terminal direct repeats, which suggests positional conservation of these regulatory elements (Ilouze *et al.*, 2012a; Stingley & Gray, 2000; van Beurden *et al.*, 2013). The E and L genes are mainly located in the unique long region of the viral genome, with almost half of the E genes clustered and transcribed simultaneously in CyHV-3 (Ilouze *et al.*, 2012a). However, this observation may be biased by 3'-coterminality of transcripts, which was shown to be abundant in the AngHV-1 genome (van Beurden *et al.*, 2012b).

### 3.1.5. Structural proteome and secretome

Initial predictions of the structural proteome of CyHV-3 were based on comparison with experimental findings obtained with IcHV-1 and bioinformatically predicted properties of the putative proteins in CyHV-3 (Aoki *et al.*, 2007; Davison & Davison, 1995). Later, two independent studies investigated 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 (Table 3) (Michel *et al.*, 2010b; Yi *et al.*, 2014). More recently, one more study revisited the structural proteome of CyHV-3. Together, the three studies identified 50 structural proteins, 39 of which were detected by the three studies (Fig. 6) (Vancsok *et al.*, 2017). Overall, the total number of structural proteins reported in CyHV-3 corresponds with the number reported for closely related AngHV-1 which has 40 and is in line with the number reported in members of the family *Herpesviridae*, e.g. 44 in Human herpesvirus 1 (Loret *et al.*, 2008).

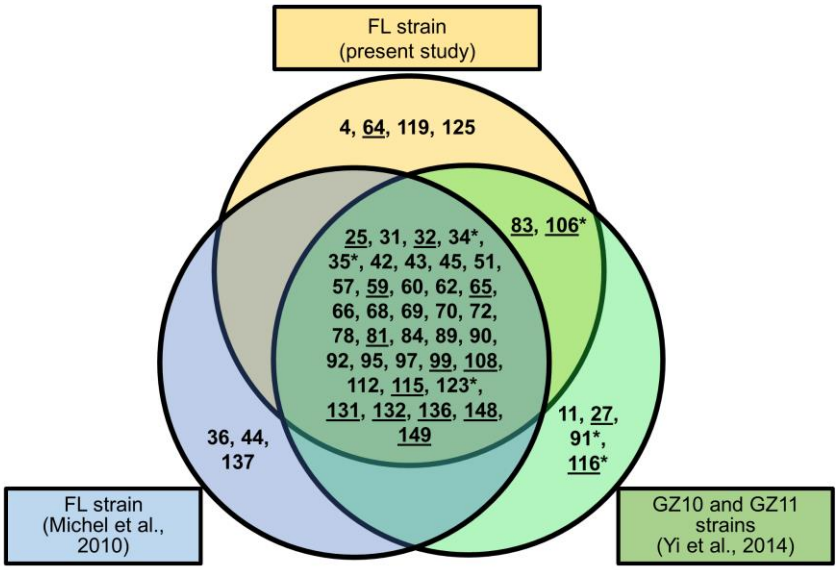


**Figure 6 - Structural proteome of CyHV-3.**

Schematic representation of CyHV-3 virion associated proteins identified in independent studies: upper circle, analyses of the European FL strain performed by Vancsok *et al.* (2017); lower left circle, analyses of the FL strain performed by Michel *et al.* (2010b); and lower right circle, analyses of two Chinese strains (GZ10 and GZ11). Numbers represent CyHV-3 ORFs. Asterisks indicate viral proteins that were detected in only one of the two Chinese isolates. Predicted transmembrane proteins are underlined. Adapted with permission from Vancsok *et al.* (2017).

Comparisons of orthologous ORFs between alloherpesviruses, as well as bioinformatical predictions of protein properties enabled putative localization of the proteins within the virion (Table 3). Based on these predictions, five capsid proteins were identified, including the highly conserved major capsid proteins, capsid triplex subunit 1 and 2, and the capsid maturation protease. Indeed, the architecture and protein compositions of fish herpesvirus capsids generally reflect that of mammalian herpesviruses, with the exception of the small proteins which form the hexon tips in mammalian herpesviruses (Booy *et al.*, 1996; Davison & Davison, 1995). Comparison with AngHV-1 resulted in the identification of 11 tegument or tegument-associated proteins in CyHV-3 (Michel *et al.*, 2010b; van Beurden *et al.*, 2011b; Yi *et al.*, 2014). Bioinformatical predictions for signal peptides (SPs), transmembrane domains (TMDs) and glycosylation enabled the identification of a total of 16 putative virion transmembrane proteins (VTP) (Aoki *et al.*, 2007; Michel *et al.*, 2010b; Yi *et al.*, 2014).

**Table 3 - Structural proteome of CyHV-3.**

| ORF | NCBI ID | Predicted MM (kDa) | Predicted localization | Protein description[d] | No. of peptides[e] | | |
|---|---|---|---|---|---|---|---|
| | | | | | FL | GZ11 | GZ10 |
| **11** | 131840041 | 13.1 | Unknown | - | - | 1 | 2 |
| **25** | 131840055 | 67.1 | Envelope[a] | Predicted membrane protein; ORF25 gene family | 7 | 6 | 8 |
| **27** | 380708459 | 47.9 | Envelope[a] | 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[a] | 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[b] | 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[b] | Related to AngHV-1 ORF20 | 5 | 4 | 6 |
| **51** | 131840077 | 165.9 | Tegument-associated[b] | Related to AngHV-1 ORF34 | 41 | 38 | 48 |
| **57** | 131840083 | 54 | Tegument-associated[b] | Similar to Crocodile poxvirus CRV155; related to AngHV-1 ORF35 | 17 | 11 | 20 |
| **59** | 131840085 | 14.6 | Envelope[a] | Predicted membrane protein | 2 | 1 | 2 |
| **60** | 131840086 | 59.9 | Tegument-associated[b] | Related to AngHV-1 ORF81 | 10 | 4 | 12 |
| **62** | 131840088 | 442.2 | Tegument-(associated)[b, c] | Contains an OTU-like cysteine protease domain; related to AngHV-1 ORF83 and IcHV-1 ORF65 | 76 | 83 | 92 |
| **65** | 131840091 | 63.5 | Envelope[a] | Predicted membrane protein; member of ORF25 gene family | 10 | 6 | 10 |
| **66** | 131840092 | 45.4 | Capsid[b] | 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[b] | Related to AngHV-1 ORF39 | 1 | 1 | 3 |
| **70** | 131840096 | 51.1 | Tegument[b] | Related to AngHV-1 ORF38 | 2 | 4 | 3 |
| **72** | 131840098 | 40.7 | Capsid[b,c] | 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[b,c]** | **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[a,b,c] | 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[a,b] | 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[b] | Related to AngHV-1 ORF100, IcHV-1 ORF37, RaHV-1 ORF52 and RaHV-2 ORF78 | 9 | 11 | 14 |
| **91** | 131840117 | 26.4 | Tegument[b] | Related to AngHV-1 ORF103 | - | - | 1 |
| **92** | 131840118 | 140.4 | Capsid[b,c] | 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 |
| **97** | 131840123 | 117.5 | Tegument-associated[b] | Related to AngHV-1 ORF30 | 19 | 20 | 22 |
| **99** | 131840125 | 170.7 | Envelope[a,b] | 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[a] | 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[a] | Predicted membrane protein | 14 | 12 | 17 |
| **116** | 131840142 | 30.4 | Envelope[a] | Predicted membrane protein | - | - | 1 |
| **123** | 131840149 | 29.5 | Tegument[a] | 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[a] | Predicted membrane protein | 5 | 3 | 4 |
| **132** | 131840158 | 19 | Envelope[a] | Predicted membrane protein | 2 | 4 | 1 |
| **136** | 131840162 | 17 | Envelope[a] | Predicted membrane protein | 2 | 3 | 3 |
| **137** | 131840163 | 69.7 | Unknown | Member of ORF22 gene family | 4 | - | - |
| **148** | 131840174 | 64.8 | Envelope[a] | Predicted membrane protein; member of ORF25 gene family | 7 | 4 | 6 |
| **149** | 131840175 | 72.8 | Envelope[a] | Predicted membrane protein; member of ORF25 gene family | 7 | 9 | 9 |

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

The conservation of the tegument and envelope proteins in 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 *et al.*, 2011b). In AngHV-1, the distribution of the structural proteins across the different viral compartments suggests that in comparison to other herpesviruses, AngHV-1 expresses less protein in envelope and far less in capsid (van Beurden *et al.*, 2011b). Although the localization of the CyHV-3 structural proteins remains to be investigated experimentally, a similar ratio may be expected.

Recently, the roles of the identified 16 VTPs (ORF25, 32, 59, 64, 65, 81, 83, 99, 106, 108, 115, 131, 132, 136, 148, and 149) in FL strain were demonstrated using BAC cloning and recombination technologies. The major findings were: i) 8 of these proteins are essential for viral growth *in vitro*; (ii) 7 are non-essential proteins but their deletion affects viral growth *in vitro*, and 2 proteins affect virulence

*in vivo*; and (iii) a mutant lacking ORF25 is highly attenuated but induces moderate immune protection (Vancsok *et al.*, 2017).

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 secreted in the supernatants were identified (ORF12, 52, 116, 119 and 134), of which the two most abundant were ORF12 encoding a soluble TNF alpha receptor homolog (Rakus *et al.*, 2017), and ORF134 encoding an IL-10 homolog (Ouyang *et al.*, 2013; Piazzon *et al.*, 2015b).

### 3.1.6. Viral replication in cell culture

#### 3.1.6.1. Cell lines permissive to CyHV-3

CyHV-3 can grow in different cell lines derived from tissues of koi and common carp brain, gills and fin. An exhaustive list of the cell lines known to be permissive or not permissive to CyHV-3 replication is available in Boutier *et al.* (2015a). Typical cytopathic effect (CPE) induced by CyHV-3 includes cytoplasmic vacuolization and increased cell volume (Pikarsky *et al.*, 2004). Finally, infected cells become rounded before they detach from the substrate. Infectious viral particles are mostly retrieved from the infected cell supernatant (cell-free fraction) (Gilad *et al.*, 2003). Fitness of CyHV-3 *in vitro* seems to vary between field strains and laboratory strains. Well adapted laboratory strains usually can reach titres up to $10^6$ to $10^7$ pfu/ml (Ilouze *et al.*, 2006b) or even higher as demonstrated in a recent study on the effect of parameters such as multiplicity of infection, time of infection and time of harvest (Mletzko *et al.*, 2017).

#### 3.1.6.2. Viral plaque morphology

Viral replication in permissive cell lines normally induces CPE. Infected cells form characteristic plaques that grow according to time post-infection. Various plaque morphologies have been reported. The *in vitro* propagation rate of a virus is related to its fitness in cell culture. A rapid replicating virus in cell culture is interpreted as a high fitness. Correspondingly, the size of plaques, which are caused by necrosis or apoptosis upon infection of a cell monolayer, is also a common measurement of viral fitness (Goh *et al.*, 2016). Viral plaque morphology was demonstrated in different viruses, in which HSV-1 taken as an example expresses distinct plaque morphologies (Parsons *et al.*, 2015). Parsons *et al.* found that HSV-1 stocks induce different plaque morphologies when they are propagated in cell culture. Plaque morphologies range from small foci of infection to larger zones of CPE. They can also include fusiform or syncytial plaques of different sizes (see Fig. 7A). Syncytium is formed by fusion of infected cells leading to the formation of multi-nucleate enlarged cells. This event is induced by surface expression of viral fusion proteins that are fusogenic. Syncytia can only occur with viruses able to

directly fuse at the cellular surface without the need of endocytosis (Salsman *et al.*, 2005). To determine the genetic variation in HSV-1 viral stocks, Parsons *et al.* separated and purified 3 strains based on plaque morphology, HSV-1 F, KOS and H166 (see Fig. 7B-E). Based on Illumina high-throughput sequencing and viral genome assembly, they decoded that the syncytial plaque morphology was correlated with a mutation in glycoprotein B which is also a fusogenic protein (Fan *et al.*, 2018; Parsons *et al.*, 2015). In CyHV-3, different plaque sizes were reported in recombinant strains deleted for non-essential glycoproteins and an essential virulent gene (Vancsok *et al.*, 2017; Boutier *et al.*, 2017). CyHV-3 was also reported to form syncytial viral plaque (Ilouze *et al.*, 2006b).

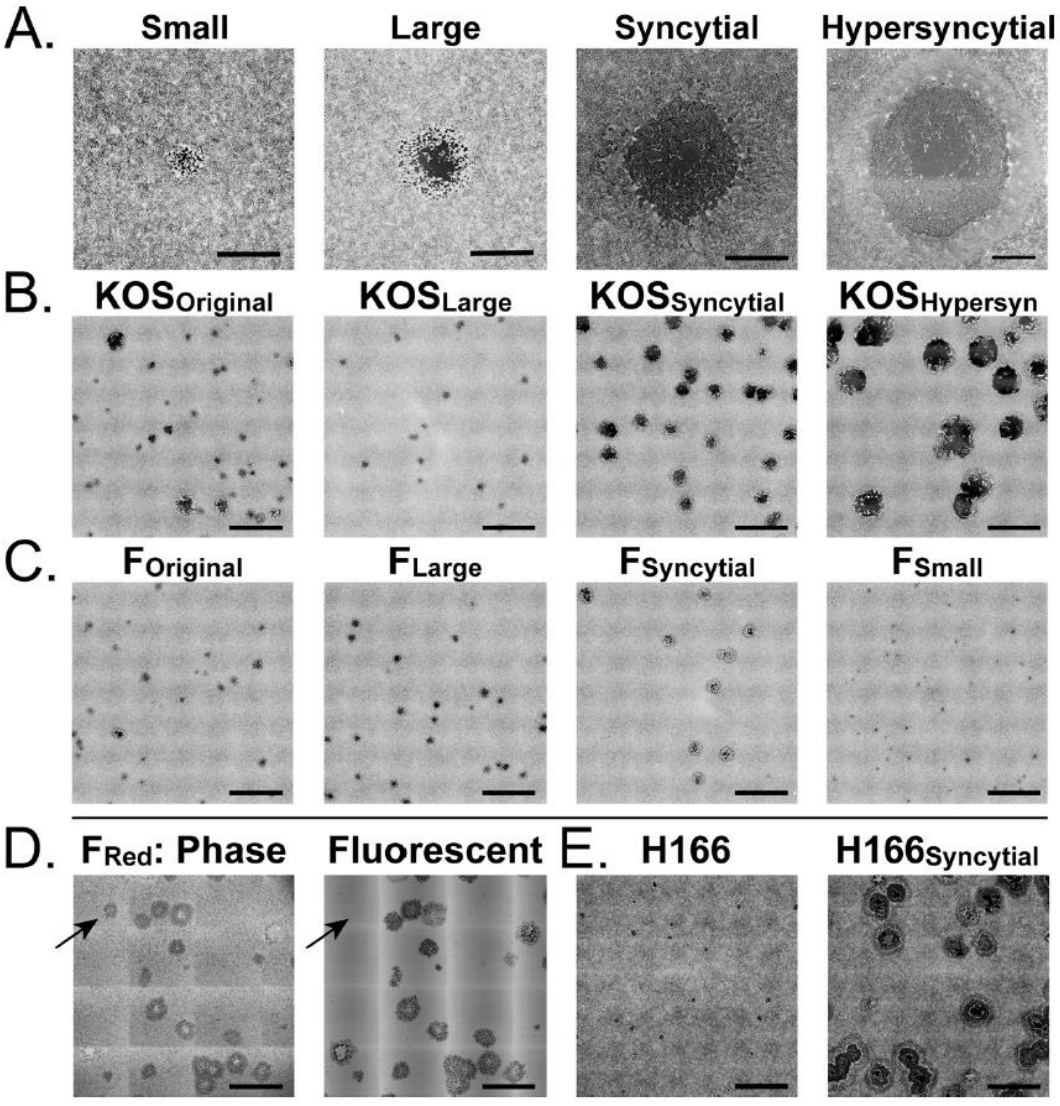


**Figure 7 - Viral plaque morphologies.**

Plaque morphologies in HSV-1 stocks, before and after plaque purification. Lab-passaged stocks of classical HSV-1 KOS and F strains contain multiple plaque morphologies. (A) With multiple rounds of limiting dilution, distinct plaque morphologies can be separated into populations that breed true and exhibit greatly reduced diversity (B) HSV-1 KOS can be separated into large, syncytial, and hypersyncytial variants. (C) HSV-1 F can be separated into large, syncytial, and small variants. (D) A previously described BAC-cloned HSV-1 F genome that has been

modified to encode an mRFP fusion to the viral capsid (Antinone and Smith 2010). Recovery of this cloned genome into mammalian cells regenerates the plaque morphology diversity observed in HSV-1 F stocks. (E) The low-passage-number clinical isolates HSV-1 H166 and $H166_{Syncytial}$ are distinct strains isolated from the cerebrospinal fluid of the same patient during an episode of viral meningitis. The size and appearance of cytopathic and syncytial plaques mirror those seen in the lab-isolated variants of HSV-1 KOS and F. Viruses were plated at limiting dilutions on monolayers of Vero cells and then fixed and stained with methylene blue at 3 dpi. Images were exported from Nikon NIS-Elements software. Contrast was inverted using Adobe Photoshop to show plaques more clearly. Bars: 1 mm (A), 5 mm (B, C, and E), and 2 mm (D). Reproduced with permission from Parsons *et al* (2015). Copyright © American Society for Microbiology, mBio (2015) 6(2):e02213-14. doi: 10.1128/mBio.02213-14.

#### 3.1.6.3. Temperature restriction

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

Virus production, viral gene transcription and viral genome replication are gradually shut off once cells are moved from permissive temperature to the non-permissive temperature (≥30 °C) (Dishon *et al.*, 2007; Ilouze *et al.*, 2012b; Imajoh *et al.*, 2015). Although most of the 110 ORFs, are still transcribed 24 h after the temperature shift, they are gradually turned off, few ORFs such as ORF114 and 115 were still expressed 18 days after temperature shift. However, infected cells could persist for 30 days at 30 °C preserving the potential to reinitiate a productive infection when returned to permissive temperatures (Dishon *et al.*, 2007). This status of unproductive infection with the potential to reactivate resembles latency as described for *Herpesviridae*. Putatively, ORF115, a viral membrane protein, may represent an Epstein-Barr virus-like membrane-bound antigen associated with latency (Ilouze *et al.*, 2012b).

## 3.2. CyHV-3 disease

### 3.2.1. Epidemiology

#### 3.2.1.1. Fish species susceptible to CyHV-3 infection

CyHV-3 can infect a wide range of species but it only causes 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 *et al.*, 2010b). Hybrids of common carp × goldfish have also been reported to show some susceptibility to CyHV-3 infection; however, the mortality rate observed was rather low (5%) (Hedrick *et al.*, 2006). PCR detection of CyHV-3 on cyprinid and non-cyprinid fish species, as well as on freshwater mussels and crustaceans, suggests that these species could act as vector of the virus (Table 4) (El-Matbouli *et al.*, 2007; El-Matbouli & Soliman, 2011; Fabian *et al.*, 2013; Kempter & Bergmann, 2007; Kempter *et al.*, 2012; Kempter *et al.*, 2009; 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 disseminate it to permissive carp (Bergmann *et al.*, 2010a; El-Matbouli & Soliman, 2011; Fabian *et al.*, 2013; Kempter *et al.*, 2012; Radosavljevic *et al.*, 2012). An increasing number of studies suggested that CyHV-3 can infect goldfish asymptomatically (Bergmann *et al.*, 2010a; El-Matbouli & Soliman, 2011; Sadler *et al.*, 2008), although some discrepancies existed 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 lists one KHVD susceptible species (*Cyprinus carpio* and its hybrids) and several suspected fish carrier species (goldfish, grass carp, ide, catfish, Russian sturgeon and Atlantic sturgeon) (OIE, 2016). Interestingly, a recent study reported the detection of CyHV-3 in migratory wild ducks in North America suggesting a potential role of aquatic birds in the spread of this virus (Torres-Meza *et al.*, 2020).

**Table 4 - Organisms tested for CyHV-3 infection (adapted with permission from Rakus *et al.* (2013); Original publisher BioMed Central).**

| 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,f,g,h,i] /No[j] | Yes[g] | Yes[h] | Yes[g,h,i] /No[j] |
| **Ide (*Leuciscus idus*)** | Yes[a,c] | nt | nt | nt |
| **Grass carp (*Ctenopharyngodon idella*)** | Yes[a,c,i] | nt | nt | Yes[c,i] |
| **Silver carp (*Hypophthalmichthys molitrix*)** | Yes[c,i] | nt | nt | Yes[c,i] |
| **Prussian carp (*Carassius gibelio*)** | Yes[c,i] / No[d] | nt | nt | Yes[i] /No[d] |
| **Crucian carp (*Carassius carassius*)** | Yes[c] | nt | nt | nt |
| **Tench (*Tinca tinca*)** | Yes[c,d,i] | nt | nt | Yes[c,d,i] |
| **Vimba (*Vimba vimba*)** | Yes[b,c] | nt | nt | Yes[c] |
| **Common bream (*Abramis brama*)** | Yes[c,d] | nt | nt | Yes[c] |
| **Common roach (*Rutilus rutilus*)** | Yes [c,d] | nt | nt | Yes[c] /No[d] |
| **Common dace (*Leuciscus leuciscus*)** | Yes [b,c,d] | nt | nt | No[d] |
| **Gudgeon (*Gobio gobio*)** | Yes [c,d] | nt | nt | Yes[d] |
| **Rudd (*Scardinius erythrophthalmus*)** | Yes[d] | nt | nt | Yes[d] |
| **European chub (*Squalius cephalus*)** | Yes[c]/No[d] | nt | nt | nt |
| **Common barbel (*Barbus barbus*)** | Yes[c] | nt | nt | nt |
| **Belica (*Leucaspius delineatus*)** | Yes[c] | nt | nt | nt |
| **Common nase (*Chondrostoma nasus*)** | Yes[c] | nt | nt | nt |
| ***Acipenseridae*** | | | | |
| **Russian sturgeon (*Acipenser gueldenstaedtii*)** | Yes[e] | nt | nt | nt |
| **Atlantic sturgeon (*Acipenser oxyrinchus*)** | Yes[e] | nt | nt | nt |
| ***Cobitidae*** | | | | |
| **Spined loach (*Cobitis taenia*)** | Yes[c] | nt | nt | nt |
| ***Cottidae*** | | | | |
| **European bullhead (*Cottus gobio*)** | Yes[c] | nt | nt | nt |
| ***Esocidae*** | | | | |
| **Northern pike (*Esox lucius*)** | Yes [c,d] | nt | nt | Yes[d] |
| ***Gasterosteidae*** | | | | |
| **Three-spined stickleback (*Gasterosteus aculeatus*)** | Yes[d] | nt | nt | No[d] |
| ***Ictaluridae*** | | | | |
| **Brown bullhead (*Ameiurus nebulosus*)** | Yes[d] | nt | nt | No[d] |
| ***Loricariidae*** | | | | |
| **Ornamental catfish (*Ancistrus sp.*)** | Yes[a] | nt | nt | nt |
| ***Percidae*** | | | | |
| **European perch (*Perca fluviatilis*)** | Yes [c,d] | nt | nt | Yes[c] /No[d] |
| **Ruffe (*Gymnocephalus cernua*)** | Yes[c] /No[d] | nt | nt | Yes[c,d] |
| Invertebrates | | | | |
| **Swan mussels (*Anodonta cygnea*)** | Yes[k] | nt | nt | nt |
| **Scud (*Gammarus pulex*)** | Yes[k] | nt | nt | nt |

nt - not tested

[a] Bergmann *et al.* (2009); [b] Kempter and Bergmann (2007); [c] Kempter *et al.* (2012); [d] Fabian *et al.* (2013) ; [e] Kempter *et al.* (2009); [f] El-Matbouli *et al.* (2007); [g] El-Matbouli and Soliman (2011); [h] Bergmann *et al.* (2010a); [i] Radosavljevic *et al.* (2012); [j] Yuasa *et al.* (2013); [k] Kielpinski *et al.* (2010).

3.2.1.2. Geographical distribution and prevalence

The geographical distribution of 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 generally contributes to the spread of the virus before methods of detection were available and implemented (OIE, 2016). The disease was known to occur in, or had been reported in fish imported into, at least 28 different countries (OIE, 2016). There were no reports of CyHV-3 detections in South America or Australia, and the only reports from the African continent were from South Africa (OIE, 2016).

In Europe, CyHV-3 has been detected from most countries. In recent years, three novel CyHV-3-like viruses were also identified by PCR in the Netherlands, UK, Austria and Italy, sharing only 95 to 98% nucleotide identity with the CyHV-3 J, CyHV-3 I and CyHV-3 U strains. Carp potentially infected by CyHV-3 variants did not show symptoms consistent with CyHV-3 infection and originated from locations with no actual CyHV-3 outbreaks. These strains might represent low- or non-pathogenic variants of CyHV-3 (Engelsma *et al*., 2013).

In Asia and the Middle East, the first outbreaks of CyHV-3 with mass mortalities were seen in Israel in 1998 and in the following 3 years, the virus had spread to 90% of the carp farms (Perelberg *et al.*, 2003). In south-eastern Asia, the first outbreaks of CyHV-3 with mass mortalities of cultured koi carp occurred in Indonesia in 2002 and were associated with the koi carp imported from Hong Kong (Haenen *et al.*, 2004; Sunarto *et al.*, 2011).

In North America, the first cases of KHVD were from koi dealers (Gray *et al.*, 2002; Hedrick *et al.*, 2000). Then, in 2004, CyHV-3 was leading to 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 detected for the first time during outbreaks in wild common carp in Ontario in 2007 and further outbreaks were reported in Ontario and Manitoba in 2008 (Garver *et al.*, 2010).

3.2.1.3. Persistence of CyHV-3 in the natural environment

CyHV-3 remains infectious in water for at least 4 hours, but not longer than 21 hours, at water temperatures between 23 °C and 25 °C (Perelberg *et al.*, 2003). Further studies revealed a significant reduction of CyHV-3 infectivity within 3 days in environmental water and 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 *et al.*, 2006). Supporting this hypothesis, a study showed that bacteria isolated from carp habitat water and carp intestine contents possessed some anti-CyHV-3 activity (Yoshida *et al.*, 2013). These studies

suggested that CyHV-3 can be rapidly inactivated in environmental water with the absence of susceptible hosts.

CyHV-3 has been quantified in environmental samples by cation-coated filter concentration of virus linked to quantitative PCR (qPCR) (Haramoto *et al.*, 2009; Honjo *et al.*, 2010). Average concentration of CyHV-3 in infected lake water showed an annual variation with a peak in summer but a reduction 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 *et al.*, 2009a). These areas are the main spawning sites of carp in Lake Biwa. This observation supports the hypothesis of increasing prevalence of CyHV-3 during spawning season (Uchii *et al.*, 2011). The researchers also suggested that, in highly turbid water, viruses may avoid degradation by adsorbing to organic or non-organic particles (Minamoto *et al.*, 2009a). Further studies of carp spawning areas in Lake Biwa reported the detection of CyHV-3 viral DNA in plankton samples and in particular *Rotifera* species (Minamoto *et al.*, 2011).

### 3.2.2. Clinical aspects

CyHV-3 disease is seasonal, occurring mainly at water temperatures between 18 °C 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 to 100% (Ilouze *et al.*, 2006b). The disease can be reproduced experimentally by immersion of the fish in water containing the virus, by ingestion of contaminated food, by cohabitation with infected fish and, more artificially, by injection of infectious materials (Fournier *et al.*, 2012; Perelberg *et al.*, 2003). Fish infected with CyHV-3 via these various routes and kept at permissive temperature will 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 *et al.*, 2009). Furthermore, CyHV-3 infected fish are more susceptible to secondary infections by other pathogens such as bacteria, parasites or fungi, which may contribute to the mortalities observed in the infected populations (McDermott & Palmeiro, 2013).

#### 3.2.2.1. Clinical signs

The first clinical signs usually appear at 2–3 dpi, while the first mortalities frequently occur at 6-8 dpi (McDermott & Palmeiro, 2013). The process of infection and the clinical signs observed are variable between individual fish, even after simultaneous and controlled experimental CyHV-3 inoculation. Fish express the following clinical signs such as folding of the dorsal fin, increased respiratory frequency, gathering near well-aerated areas, skin changes including gradual hyperaemia at the base of fins, increased (sometimes decreased) mucus secretion, hemorrhages and ulcers on the skin, sloughing of scales and fin erosion, sandpaper-like appearance of the skin, 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 occasionally 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 is pathognomonic of CyHV-3 disease.

#### 3.2.2.2. Anatomopathology

The external post-mortem symptoms that can be observed on the skin include pale and irregular patches, haemorrhages, fin erosions, ulcers and flaking of the epithelium. The main lesion in the gills is the necrosis with multifocal or diffuse discoloration, sometimes associated with extensive erosions of the primary lamellae. Some of these anatomopathological lesions are illustrated in Fig. 8a. Other inconsistent necropsy changes include enlarging, darkening and/or mottling of some internal organs associated with petechial haemorrhages, 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 is pathognomonic of CyHV-3 disease.

#### 3.2.2.3. Histopathology

Histopathological alterations are observed in the skin, gills, kidneys, heart, liver, spleen, gut and brain of CyHV-3 infected fish (Hedrick *et al.*, 2000; Miyazaki *et al.*, 2008). In the skin, the lesions can appear as early as 2 dpi and worsen with time (Fig. 8b) (Miwa *et al.*, 2015). The cells exhibiting degeneration and necrosis show various stages of nuclear degeneration (e.g. pale coloration, karyorrhexis, pycnosis), frequently associated with characteristic intranuclear inclusion bodies (Fig. 8b, panel 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 half 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.*, 2015). A recent report revealed that the damages caused to the skin of the body and fins were the most pronounced lesions (Miwa *et al.*, 2015).

During the process of CyHV-3 infection, typical histopathological changes are observed in the two compartments of the gills, the gill lamellae and the gill rakers (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, oedema and congestion (Miyazaki *et al.*, 2008; Ouyang *et al.*, 2013; Pikarsky *et al.*, 2004). As a consequence of the pronounced hyperplasia, the secondary lamellae inter-space is progressively filled by cells. At later stages, the gill

lamellae architecture can be completely destroyed by necrosis, erosion and fusion of the primary lamellae (Pikarsky *et al.*, 2004). These lesions can be visualized macroscopically and are frequently associated with secondary infections (Pikarsky *et al.*, 2004). In the gill rakers, the changes are even more recognizable, which 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.

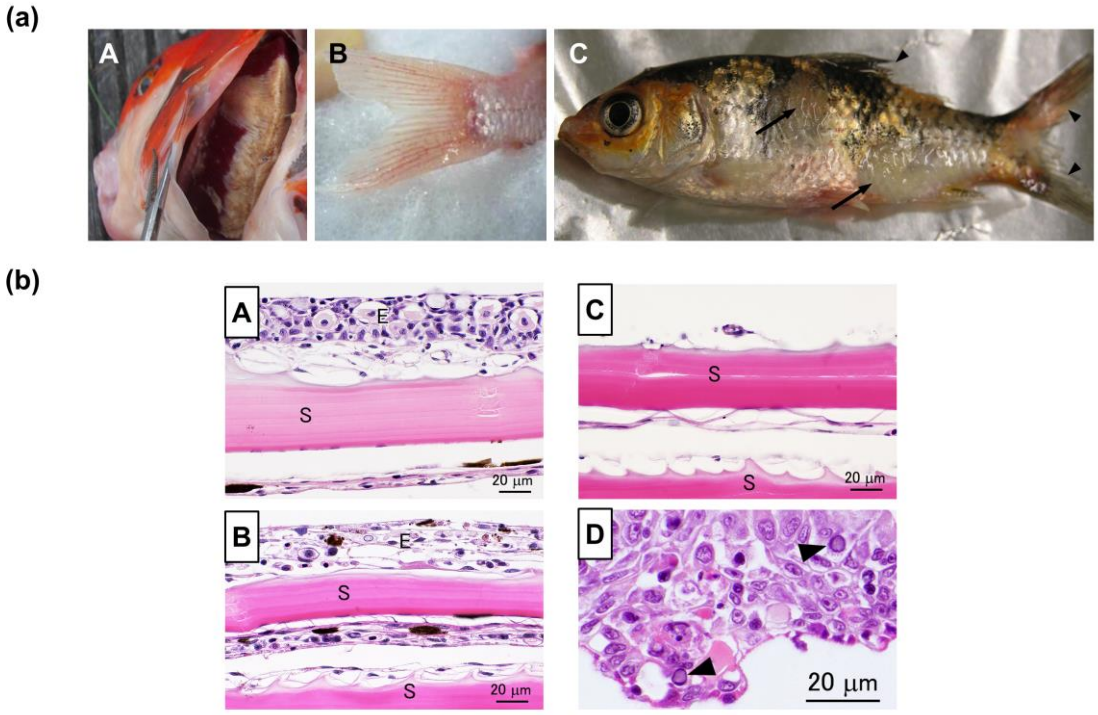


**Figure 8 - Illustration of anatomopathological and histopathological lesions induced by CyHV-3.**

(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). Adapted with permission from Michel *et al.* (2010a). (b) Histopathological lesions in the skin. Sections of the skin of carp stained with haematoxylin 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). Adapted with permission from Miwa *et al.* (2015). Copyright © Wiley & Sons, Inc.

In the kidney, a weak peritubular inflammatory infiltrate is visible 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 present in hematopoietic cells (Miwa *et al.*, 2015; Miyazaki *et al.*, 2008). In the spleen, splenocytes appeared necrotic (Miyazaki *et al.*, 2008). In the heart, myocardial cells can exhibit nuclear degeneration and alteration of the myofibril bundles with disappearance of the cross-striation (Miyazaki *et al.*, 2008). In the liver, hepatocytes are the most susceptible cell type (Miyazaki *et al.*, 2008). Inflammatory infiltrates can be

observed in the parenchyma (Pikarsky *et al.*, 2004). In the intestine and stomach, the clinical symptoms 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 brain, focal meningeal and parameningeal inflammation can be observed (Pikarsky *et al.*, 2004). Analysis of the brain of infected fish expressing 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 can be detected at 12 dpi in all compartments of the brain. These cells were mainly ependymal cells and, to a lesser extent, neurons (Fig. 9) (Miwa *et al.*, 2015). At late stage, the lesions were accompanied by perivascular lymphocyte infiltration and gliosis. The peak of nervous lesions coincided in time with the peak of neurological clinical signs (Miwa *et al.*, 2015).

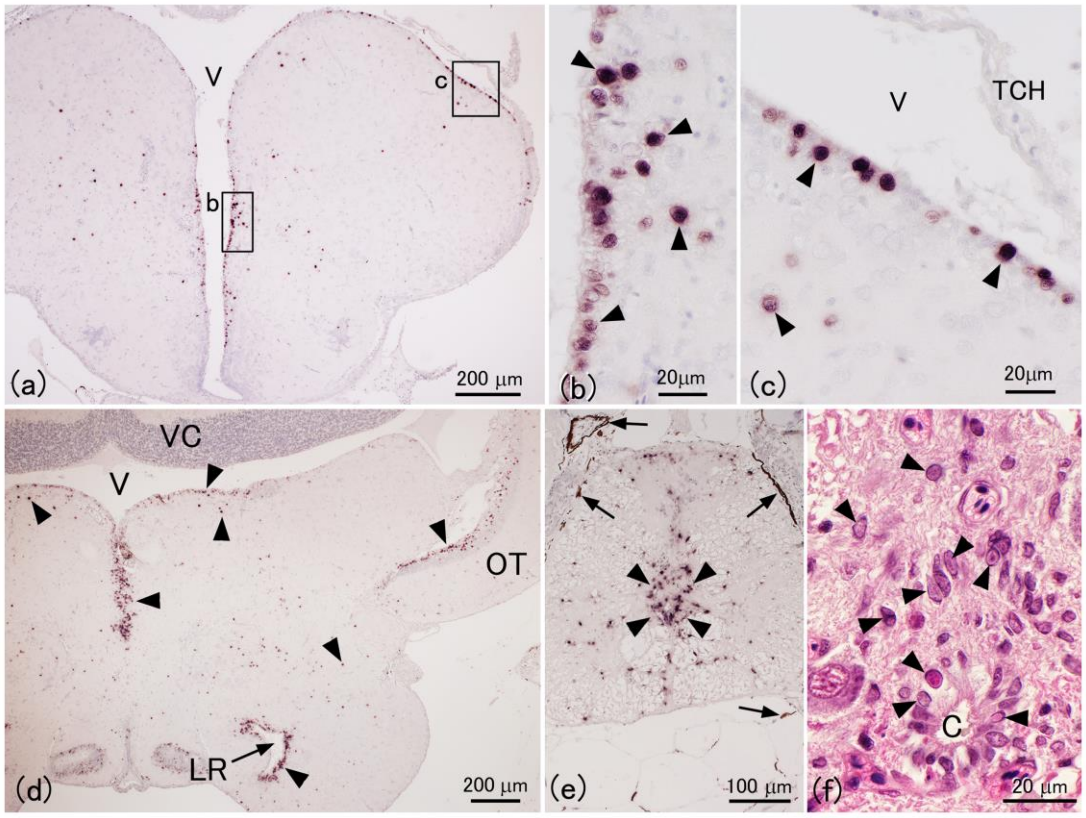


**Figure 9 - Illustration of histopathological lesions induced in the central nervous system of carp by CyHV-3.**

(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 panel (a) are shown enlarged in panels (b) and (c). Arrows indicate melanin. (f) A section of the spinal cord stained with haematoxylin 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.* (2015). Copyright © Wiley & Sons, Inc.

### 3.2.3. Pathogenesis

All members of the family *Herpesviridae* exhibit 2 distinct phases, lytic replication and latency, in their infection cycle. While lytic replication is associated with production of progeny virions, latency entails the maintenance of the viral genome as a non-integrated episome and the expression of limited viral genes and microRNAs. Upon reactivation, lytic replication ensues from the latent infection. Studies on several members of the family *Alloherpesviridae* suggested the existence of these two types of infection. Most of these studies are related to CyHV-3 and provide the evidence 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 non-permissive (Uchii *et al.*, 2014). The data related to these two phases of CyHV-3 pathogenesis are summarized below.

#### 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 *et al.*, 2006b; Miyazaki *et al.*, 2008; Miyazaki *et al.*, 2005; Pikarsky *et al.*, 2004; Pokorova *et al.*, 2005) and the intestine (Dishon *et al.*, 2005; Ilouze *et al.*, 2006b). The two hypotheses relied on several observations: (*i*) the gills present histopathological lesions earlier after virus inoculation by immersion in water (Hedrick *et al.*, 2000; Pikarsky *et al.*, 2004), (*ii*) viral DNA can be detected in gills and gut as early as 1 dpi (as in virtually all organs including skin mucus) (Gilad *et al.*, 2004), and (*iii*) gills are thought to be the main portal of entry for many fish pathogens. More recent studies using *in vivo* imaging system (IVIS) demonstrated that the skin is the major portal of entry for CyHV-3 after immersion in water containing virus (Fig. 10) (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 bioluminescent signal was detected as early as 12 hpi in most of the fish infected by the virus expressing luciferase, while all fish were clearly positive at 24 hpi with the positive signal preferentially localized on the fins (Costes *et al.*, 2009). This finding was supported by independent reports that showed 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 in the fin epithelium as early as 2 dpi (the earliest positive organ) (Miwa *et al.*, 2015). Fish epidermis has also been proved to support early infection of a Novirhabdovirus (IHNV; infectious hematopoietic necrosis virus) in trout, suggesting that the skin is an important portal of entry for viruses infecting fish (Harmache *et al.*, 2006).

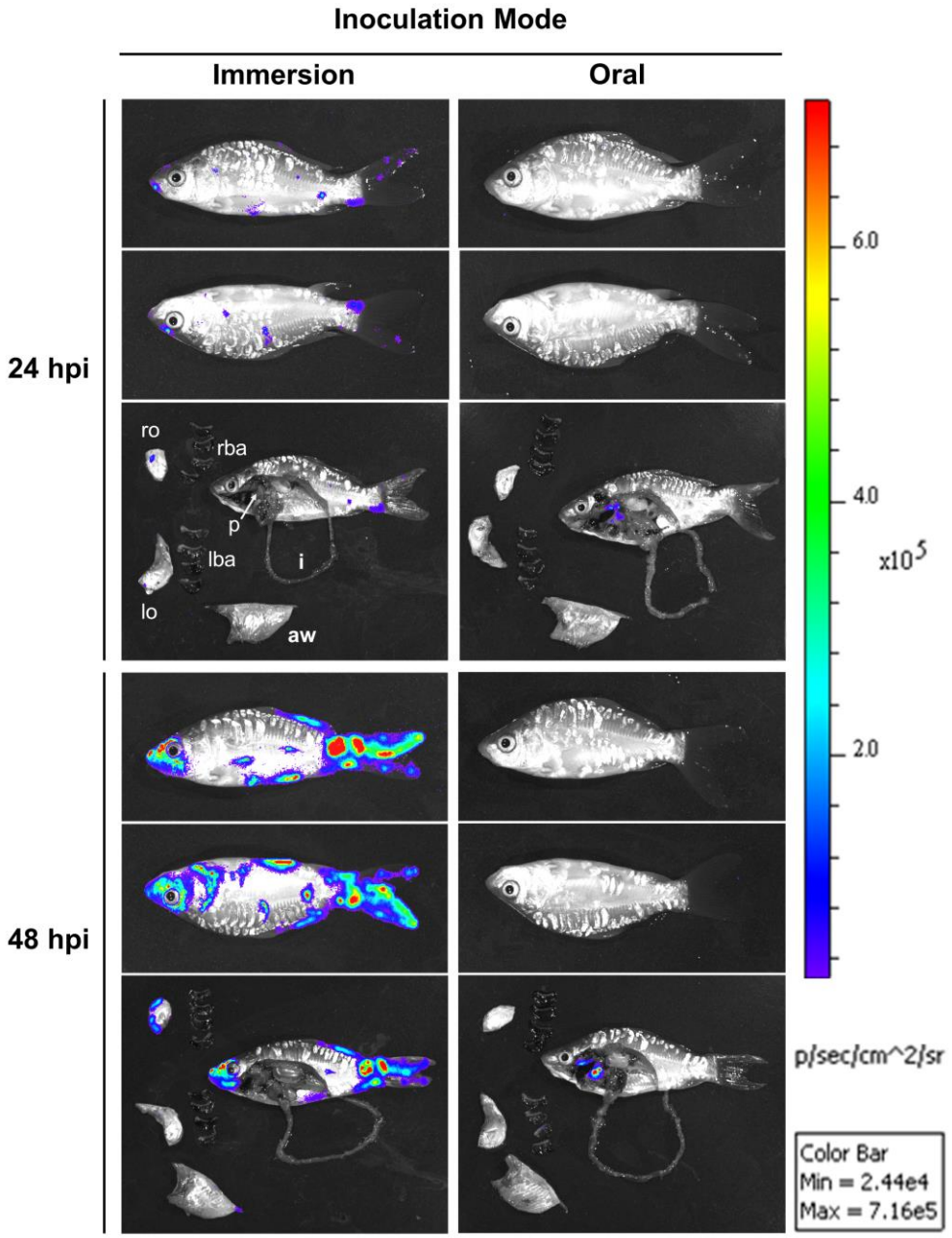


**Figure 10 - The portals of entry of CyHV-3 in carp analyzed by *in vivo* bioluminescent imaging.**

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 pseudo-color scale ranging from violet (least intense) to red (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 have 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 a natural route in which infection occurs, other epidemiological conditions could support entry of virus through the digestive tract. To test this hypothesis, carp were fed with material containing a CyHV-3 recombinant strain expressing luciferase as a reporter gene, and bioluminescence imaging analyses were performed at indicated time points (Fig. 10) (Fournier *et al.*, 2012). The data suggested that the pharyngeal periodontal mucosa is the major portal of entry through oral inoculation. This mode of inoculation led to the spread of the infection to the various organs tested, inducing clinical symptoms and mortality rates comparable to the infection through immersion (Fournier *et al.*, 2012).

3.2.3.1.2 Secondary sites of infection

After infection at the portal of entry, CyHV-3 rapidly spreads in the infected fish as demonstrated by the detection of CyHV-3 DNA in almost all tissues as early as 1-2 dpi (Gilad *et al.*, 2003; Ouyang *et al.*, 2013; Pikarsky *et al.*, 2004). The tropism of CyHV-3 for white blood cells could explain such a rapid spread of the virus within the body (Eide *et al.*, 2011a). Viral DNA can be isolated from blood as early as 1 dpi of CyHV-3 infection (Pikarsky *et al.*, 2004). During the early days post-infection, most of the organs (including those that act as portals of entry) express increasing viral charge according to time post-infection. The cause of death is still a matter of discussion. The severe CyHV-3 infection observed in gills and kidneys, together with the associated histopathological changes, could be responsible for acute death (Gilad *et al.*, 2004; Hedrick *et al.*, 2000). It has also been proposed that the severe skin lesions may lead to hypo-osmotic shock (Miwa *et al.*, 2015).

3.2.3.1.3 Excretion and transmission

Horizontal transmission of CyHV-3 occurs either by direct contact between fish or by indirect modes. Study of the CyHV-3 portals of entry illustrated that it can enter carp either through infection of the skin or infection of the pharyngeal periodontal mucosa (Fig. 10). Therefore, direct transmission could result from skin to skin contact between infected fish and naïve ones, or from cannibalistic and necrophagous behaviours of carp (Fournier *et al.*, 2012; Raj *et al.*, 2011). Interestingly, horizontal transmission in natural ponds seems exacerbated in hot spots when carp are mating (Uchii *et al.*, 2011), which could favour skin-to-skin 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 *et al.*, 2012), aquatic invertebrates feeding by water filtration (Kielpinski *et al.*, 2010), and finally the water as the major abiotic vector (Minamoto *et al.*, 2009b). Indeed, virus propagation in organs such as the skin, gills, 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).

#### 3.2.3.2. Latent infection

Although latency has not been demonstrated conclusively in members of the *Alloherpesviridae* family as it has been for members of the family *Herpesviridae*, increasing evidence supports the existence of a latent infection. The evidences supporting CyHV-3 latent infection are summarized below. Low amounts of CyHV-3 DNA have been detected two months post-infection in the gills, kidneys and brain of fish that survived from the primary infection and were no longer expressing 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.*, 2015; Yuasa & Sano, 2009). In addition, CyHV-3 DNA, but no virions, was detected in several organs of fish post infection (Eide *et al.*, 2011a). Finally, CyHV-3 DNA can be routinely detected in apparently healthy fish (Cho *et al.*, 2014).

CyHV-3 can persistently infect farmed (Baumer *et al.*, 2013) or wild carp populations (Uchii *et al.*, 2009; Uchii *et al.*, 2014). After at least 2 years after an initial outbreak, CyHV-3 DNA can still be detected in the brain of both large-sized seropositive fish and small-sized seronegative fish from wild population of common carp (Uchii *et al.*, 2009). The data suggest that transmission occurred between latently infected fish that survived from previous outbreaks and naïve generations (Uchii *et al.*, 2009). A more recent study implies that the seasonal reactivation enables CyHV-3 to persist in a wild population (Uchii *et al.*, 2014). Indeed, mRNA transcription of DNA replication related genes can be detected in the brain of seropositive fish, suggesting reactivation, while expression of presumed latency related genes can only be detected in some fish (Ilouze *et al.*, 2012a; Uchii *et al.*, 2014). St-Hilaire *et al.* (2005) described that fish can express clinical symptoms and die from CyHV-3 infection following a temperature stress several months after the initial exposure to the virus. Reactivation of infectious particles was demonstrated by contamination of naïve fish. Another report showed that a netting stress induced viral reactivation without symptoms 81 days after initial infection as detected by qPCR on gill samples (Bergmann & Kempter, 2011).

Few studies suggested that CyHV-3 could be latent in white blood cells (Eide *et al.*, 2011a; Eide *et al.*, 2011b; Reed *et al.*, 2014; Xu *et al.*, 2013a). Firstly, CyHV-3 DNA could be detected in white blood cells of koi carp in the absence of any clinical signs or detectable infectious viral particles with previous exposure to the virus (Eide *et al.*, 2011a). Similar results were confirmed in wild carp from private ponds in Oregon with no history of CyHV-3 outbreaks (Xu *et al.*, 2013a). Interestingly, the amounts of CyHV-3 DNA detected ranged from 2-60 copies per microgram of isolated DNA in white blood cells of previously infected koi, genome charges similar to those reported during latency of members of the family *Herpesviridae* (Eide *et al.*, 2011b). Notably, similar amount of viral DNA were

found in other tissues with no evidence of whether the distribution of these widespread tissues reflects detection of latently infected circulating white blood cells or latently infected resident cells (Eide *et al.*, 2011b). Amongst white blood cells, $IgM^+$ B cells expressed the highest viral charge suggesting that they represent the site of CyHV-3 latency (Reed *et al.*, 2014). Indeed, the number of CyHV-3 DNA copies was 20 times higher in purified $IgM^+$ B cells compared to the remaining white blood cells. However, due to lack of selectivity of the $IgM^+$ sorting method, 10% of $IgM^+$ B cells still present in the remaining white blood cells, which results in an unclear explanation that whether the low amount of CyHV-3 DNA detected in the remaining white blood cells is due to the existence of another cell type also supporting latent infection or the $IgM^+$ B cells contamination (Reed *et al.*, 2014). The CyHV-3 transcriptome study of latently infected $IgM^+$ B cells demonstrated that the transcription of ORF6 was associated with latent infection of $IgM^+$ B-cells (ORF1-5 and 7-8 were not transcribed) (Reed *et al.*, 2014). More recent studies showed that pORF6 was detected in $IgM^+$ B cells from latently infected koi suggesting its putative role in latency (Reed *et al.*, 2017). Interestingly, one domain of pORF6 (aa 342-472) was found to be homologous to the sequences of EBNA-3B (EBV nuclear antigen) and the N-terminal regulator domain of ICP4 (Infected-cell polypeptide 4). EBNA-3B plays a key role during the latency of the gammaherpesvirus EBV and is involved in regulation of cellular gene expression, while ICP4 is found in alphaherpesviruses and also acts as a transcriptional regulator (Reed *et al.*, 2014).

#### 3.2.3.3. Effect of water temperature

CyHV-3 disease occurs naturally when water temperature ranges from 18 °C and 28 °C (Gotesman *et al.*, 2013; Rakus *et al.*, 2013). Experimentally, KHVD has also been reproduced in temperatures ranging from 16 °C to 28 °C (Gilad *et al.*, 2003; Gilad *et al.*, 2004; Yuasa *et al.*, 2008). The lowest temperature reported during a CyHV-3 outbreak is 15.5 °C observed during a field survey in Japan (Hara *et al.*, 2006). Interestingly, CyHV-2 induces mortalities in goldfish at a slightly extended temperature range from 15-30 °C (Ito & Maeno, 2014) suggesting a similarly adaptable temperature range in cyprinid herpesviruses. In the process of CyHV-3 infection, the onset of mortality was influenced by the water temperature; the first mortality occurred between 5–8 and 14–21 dpi when the fish were kept at the temperatures between 23-28 °C and 16-18 °C, respectively (Gilad *et al.*, 2003; Yuasa *et al.*, 2008). Moreover, fluctuations of +/- 3 °C in daily temperature induce increasing stress in fish with releasing more cortisol in water and increasing susceptibility to CyHV-3 infection (higher mortality rate and viral excretion) (Takahara *et al.*, 2014).

Switching water temperature from permissive to non-permissive low (≤ 13 °C) or high (30 °C) for newly infected fish (between 1 and 5 dpi) can reduce the mortality significantly (St-Hilaire *et al.*, 2009; St-Hilaire *et al.*, 2005; Sunarto *et al.*, 2014; Ronen *et al.*, 2003). Indeed, CyHV-3 can replicate at low temperature without inducing mortalities, and relatively high amount of viral 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 non-structural 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; Sunarto *et al.*, 2014), while no virions could be isolated (Sunarto *et al.*, 2014). In addition, fish infected by CyHV-3 and maintained at low temperature (≤ 13 °C) then switched back to permissive temperature frequently express KHVD (Eide *et al.*, 2011b; Gilad *et al.*, 2003; St-Hilaire *et al.*, 2009; St-Hilaire *et al.*, 2005; Sunarto *et al.*, 2014) and were able to transmit the virus to naïve cohabitants (St-Hilaire *et al.*, 2005). All 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 non-permissive for the outbreak.

Recently, Rakus *et al.* (2017) showed that carp infected by CyHV-3 can express beneficial behavioural fever. Indeed, in environment that allows the expression of behavioural fever at 32 °C, no mortality was observed. In contrast, in conditions that incompatible with the expression of this behaviour, mortality reached around 100%. Interestingly, CyHV-3 encodes a gene (ORF12) that delays the expression of behavioural fever. This gene encodes a soluble viral receptor able to bind and neutralize TNF alpha, which showed to be a mediator of behavioural fever in this homologous infectious model (Rakus *et al.*, 2017). By delaying the expression of behavioural fever, ORF12 is likely to enhance viral transmission through the host population. Furthermore, by allowing fish finally to express behavioural fever in the natural environment, the virus could be latent in the surviving fish and spread the herpesvirus infection throughout their lives.

In general, the effect of temperature on the biological cycle of CyHV-3 *in vivo* can be twofold. Firstly, it could control the switch from latency to lytic infection and *vice versa*. Secondly, it clearly regulates the amplitude of viral replication during lytic infection. Further studies are required to demonstrate the relative importance of the two effects and their putative interactions.

### 3.2.4. Diagnosis and vaccination

#### 3.2.4.1. Diagnosis

Diagnosis of KHVD in clinically infected fish can be achieved by numerous methods. The manual of diagnosis for aquatic animals lists clinical signs, histopathological alterations and transmission electron microscopy as suitable for presumptive diagnosis of KHVD (OIE, 2016). Notably, none of these tests are fully validated and it is therefore suggested that diagnosis of CyHV-3 disease should not rely on just one test but a combination of two or three tests. Furthermore, final diagnosis must rely on direct detection of viral DNA or viral particle isolation and identification (OIE, 2016).

PCR-based techniques constitute rapid and sensitive diagnostic methods. Different conventional PCR assays have been shown to allow the detection of CyHV-3 DNA as well in cell culture supernatant as in fish tissues. They are based on the amplification of non-coding DNA (Gilad *et al.*, 2002; Gray *et al.*, 2002), the TK gene (Bercovier *et al.*, 2005) or the DNA polymerase gene (Ishioka *et al.*, 2005). In addition to these conventional PCR methods, qPCR assays were also developed for detection of CyHV-3. The most sensitive and commonly used PCR method is the Gilad Taqman real-time PCR assay (Gilad *et al.*, 2004; OIE, 2016). This technique can detect CyHV-3 viral DNA as early as 1 dpi in most of the tissues and low copy numbers of viral DNA in the gills, kidney and brain of survivor fish as long as 64 dpi. (Gilad *et al.*, 2004). A Loop-mediated isothermal amplification (LAMP) is a rapid single step assay and very convenient for pond-side diagnosis because it does not require a thermal cycler. LAMP of the TK gene has been developed for detection of CyHV-3 and shown to be comparably sensitive to conventional PCR assays (Gunimaladevi *et al.*, 2004; Yoshino *et al.*, 2006; 2009). An improved method combining LAMP with DNA hybridization technology and antigen-antibody reactions provided better sensitivity and specificity than classic PCR (Soliman & El-Matbouli, 2010).

Immunodiagnostic methods are not routinely used for the diagnosis of CyHV-3 disease. Due to insufficient knowledge of the serological responses of fish to virus infections, these techniques have not, so far, been accepted as valuable screening methods for assessing the viral status of fish populations (OIE, 2016). However, CyHV-3 could be detected in tissues and touch imprints of organs from infected fish by immunohistochemistry and immunofluorescence assays, respectively (Pikarsky *et al.*, 2004). Some ELISA-based tests have been shown to detect CyHV-3 specific antibodies at high serum dilution (Adkison *et al.*, 2005; St-Hilaire *et al.*, 2009). An ELISA method is also available to detect CyHV-3 antigens in fish droppings (Dishon *et al.*, 2005). A CyHV-3 detection kit (The FASTest Koi HV kit) adapted to field conditions has been developed and allowed a quick (only 15 minutes) on-site detection of CyHV-3 in gill swabs. This lateral flow device relies on the detection of the ORF65 expression product, a glycoprotein encoded by CyHV-3. This kit provides a sensitivity of 100% (Vrancken *et al.*, 2013). Recently, non-lethal diagnostic methods for CyHV-3 in koi *Cyprinus carpio* were recommended that combination of ELISA and gill qPCR should be used in the diagnosis of CyHV-3 exposure of suspected carrier-state fish (Soto *et al.*, 2020).

Cell culture isolation is described for CyHV-3 diagnosis (using CCB and KF-1 cell lines), but it is not as sensitive as the published PCR-based methods. Consequently, this is not considered to be a reliable diagnostic method for KHVD (OIE, 2016).

#### 3.2.4.2. Vaccination

The economic losses induced by KHVD stimulated the development of prophylactic measures. Various techniques have been tried, such as selection of carp genetically resistant to CyHV-3 (Shapira

*et al.*, 2005), passive immunization by administration of pooled sera from immunized fish (Adkison *et al.*, 2005) or by addition of anti-CyHV-3 IgY antibodies to fish food (Liu *et al.*, 2014), and natural immunization aimed to induce a protective adaptive immune response (Ronen *et al.*, 2003). In addition, several vaccine candidates conferring significant protection were also developed. They are summarized in this section.

With the identification of CyHV-3 as the causative agent of KHVD, and the disease explosion only when the water temperature ranges from 18 to 28 °C, 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 reduced at temperatures above 30 °C (Dishon *et al.*, 2007). Based on this protocol, healthy fingerlings were firstly contaminated with the virus by cohabitation with sick fish for 3–5 days at permissive temperature (22-23 °C). Then the fish were transferred to ponds for 25–30 days at non-permissive water temperature (≈30 °C). Despite its ingenuity, this protocol presents several disadvantages, including (*i*) creating a reservoir of latently infected fish with a virulent strain therefore likely to represent a potential source of CyHV-3 outbreaks if they later cohabit with naïve carp; (*ii*) increasing susceptibility of the fish to secondary infections; (*iii*) costly procedure caused by keeping water at non-permissive temperature; (*iv*) relatively low protection (only 60% ) from a lethal CyHV-3 challenge (Ronen *et al.*, 2003).

Due to the limited efficacy and the risks associated to the methods described above, vaccination turned out to be the most promising approach. 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. Firstly, the vaccine must be compatible with mass vaccination and administered via a single dose as early as possible in life. Secondly, the lowest cost for vaccine production and administration is necessary (Sommerset *et al.*, 2005). More practically, cost-effective mass vaccination of young fish by immersion vaccination is ideal, 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 low and their susceptibility to the disease is high (Brudeseth *et al.*, 2013). Immersion vaccination is particularly adapted to common carp culture which 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, such as, when the individual value is relatively high and when vaccination can be delayed until the size of the fish is compatible with their manipulation (Plant & Lapatra, 2011).

A recent review has summarized current knowledge and future prospects of vaccines against CyHV-3 (Boutier *et al.*, 2019). Various vaccine candidates against CyHV-3 have been developed including DNA vaccines, bacterial vector vaccines, inactivated vaccines and attenuated vaccines (Boutier *et al.*, 2019). Such as, injectable DNA vaccines consisting of plasmids encoding envelope

glycoproteins ORF25 and ORF81 were shown efficacious under experimental conditions (Zhou *et al.*, 2014a; Zhou *et al.*, 2014b); recombinant lactobacillus (*Lactobacillus plantarum*) expressing G protein of SVCV fused to the ORF81 protein of CyHV-3 showed partial protection following oral vaccination (Cui *et al.*, 2015); and an inactivated vaccine candidate was described which consists of formalin-inactivated CyHV-3 trapped within a liposomal compartment also showing partial protection through immunization by addition to fish food (Yasumoto *et al.*, 2006). Nevertheless, all these vaccine candidates could represent a solution for individual vaccination of carp, but they are unfortunately incompatible with most of the field constraints described above. 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 naïve subjects. A conventional anti-CyHV-3 attenuated vaccine has been developed by serial passages in cell culture and ultra violet (UV) irradiation (O'Connor *et al.*, 2014; Perelberg *et al.*, 2008; Perelberg *et al.*, 2005; Ronen *et al.*, 2003; Weber *et al.*, 2014). This vaccine is commercialized in Israel by the KoVax Company for the vaccination of koi and common carp by immersion in water containing the attenuated strain. This vaccine was launched in the US market under the name Cavoy, but was withdrawn from sale after just a year. This vaccine has three major disadvantages. Firstly, 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. Secondly, the determinism of the attenuation is unknown, and consequently, reversions to a pathogenic phenotype cannot be excluded (Meeusen *et al.*, 2007). Thirdly, death of fish from various sizes were reported after vaccination leading to court cases between owners and the company distributing this vaccine in the US. More recently, an attenuated vaccine based on the double deletion of ORF56 and ORF57 was produced by BAC mutagenesis (Boutier *et al.*, 2015b). Although the functions of these ORFs are still unknown, ORF57 was shown to be an essential virulence factor in CyHV-3 (Boutier *et al.*, 2017). This vaccine exhibits properties compatible with mass vaccination: (i) it was shown to induce efficient protection after administration via a single dose by immersion in water containing the vaccine strain. This low-cost route of vaccination is particularly adapted to carp culture (Boutier *et al.*, 2015a); (ii) it replicates efficiently *in vitro* (which is essential for vaccine production); (iii) the deletion performed following a rational design makes reversion impossible and it stays genetically stable for at least 5 passages; (iv) the absence of virulence for young subjects was demonstrated, allowing vaccination in early stage (Boutier *et al.*, 2015b).

### 3.2.5. Host-pathogen interactions

#### 3.2.5.1. Susceptibility of common carp according to the developmental stage

Carp of all ages can be affected by CyHV-3 infection, 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).

Researchers found that carp larvae are also 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 embryo and larval stages (<21 days post-hatching) is limited compared to the other older stages (juvenile and fingerling stages; >21 days post-hatching) (Ronsmans *et al.*, 2014).

#### 3.2.5.2. Susceptibility of common carp according to host genetic background

At least two subspecies *Cyprinus carpio carpio* (Europe) and *C. c. haematopterus* (East Asia) in Eurasian continent are at the origin of common carp (Chistiakov & Voronova, 2009). During the long history of domestication, common carp of various origins have been intensively submitted to selective breeding which caused a high variety of breeds, strains and hybrids (Chistiakov & Voronova, 2009). In addition, domesticated common carp were spread worldwide by human activities (Uchii *et al.*, 2013). Fish from genetically distant populations may present different resistance to diseases. Traditional selective breeding methods and marker-associated selection proved to be useful ways to reduce the economic losses induced by infectious diseases (Midtlyng *et al.*, 2002).

Differences in resistance to CyHV-3 have been described among different strains and crossbreeds in carp. Resistance to CyHV-3 infection can be significantly increased by cross-breeding domesticated carp strains with wild carp strains (Dixon *et al.*, 2009). Carp genetic resistance to CyHV-3 has also been demonstrated by using 96 carp families derived from bi-allelic cross-breeding of two wild carp strains (Amur and Duna, native of the Danube and Amur rivers) and two domesticated Hungarian strains (Tat, Szarvas 15) (Dixon *et al.*, 2009; Ødegård *et al.*, 2010). These studies suggested that 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). Recently, Tadmor-Levi *et al.* (2017) reported the successful transfer of partial resistance to CyHV-3 from feral to cultured common carp strains using introgression approach.

Common carp from two different genetic origins (an ancient Japanese indigenous type and an introduced domesticated Eurasian type) inhabit the Lake Biwa in Japan (Mabuchi *et al.*, 2005). Interestingly, during the CyHV-3 outbreak in the Lake Biwa in 2004, mortalities were mainly recorded in the Japanese indigenous type (Ito *et al.*, 2014; Uchii *et al.*, 2013).

#### 3.2.5.3. Common carp innate immune response against CyHV-3

CyHV-3 invades fish through infection of the skin and/or the pharyngeal periodontal mucosa (Fig. 10) (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 and various proteins, such as enzymes, mucins, immunoglobulins, antimicrobial peptides and lytic agents, capable of neutralizing microbes (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 that inhibits CyHV-3 binding to epidermal cells supporting by mucus neutralization of viral infectivity through *in vitro* assay. Ronsmans *et al.* (2014) demonstrated that 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 CyHV-3. 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 investigated in the skin and gut of common carp (Adamek *et al.*, 2013; Syakuri *et al.*, 2013). In the skin, CyHV-3 infection results in down-regulation of genes encoding several important components of the skin mucosal barrier, such as antimicrobial peptides (β-defensin 1 and 2), mucin 5B, and tight junction proteins (claudin 23 and 30). This probably leads to the disintegration of the skin (down-regulation of claudins), the decreased amount of mucus and the sandpaper-like surface of the skin (down-regulation of mucins), as well as changes in the cutaneous bacterial flora and subsequent development of secondary bacterial infections (Adamek *et al.*, 2013). The studies above also revealed up-regulation of pro-inflammatory cytokine IL-1β and the inducible nitric oxide synthase (iNOS), as well as the activation of interferon class I pathways (Adamek *et al.*, 2013; Syakuri *et al.*, 2013).

Interferons (IFNs) are secreted mediators that play crucial 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 block 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 reduces CyHV-3 spreading in the cell culture (Adamek *et al.*, 2012). *In vivo*, CyHV-3 infection 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 *et al.*, 2014a; Adamek *et al.*, 2013; Syakuri *et al.*, 2013). However, no significant differences in the IFN type I response could be detected between two carp lines with different susceptibility to CyHV-3 (i.e. R3 and K carp lines) (Adamek *et al.*, 2014a). Additional studies *in vitro* suggested that CyHV-3 does not induce apoptosis, in contrast to 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 *et al.*, 2012). Finally, stimulation of the apoptosis intrinsic pathway induced by CyHV-3 infection as detected by the expression of pro-apoptotic proteins (Apaf-1, p53, and Caspase 9), was delayed to 14 dpi (Miest *et al.*, 2015).

A further transcriptomic study uncovered the wide array of immune-related genes involved in the systemic anti-CyHV-3 immune response of carp (Rakus *et al.*, 2012). The response of two carp lines (R3 and K) with different resistance to CyHV-3 was studied by using DNA microarray and real-time

PCR. Significantly higher expression of several immune-related genes including those that are involved in pathogen recognition, complement activation, MHC-I restricted antigen presentation and development of adaptive mucosal immunity was detected in the more resistant carp line. Further real-time qPCR based analyses provided evidence for higher activation of $CD8^+$ T cells. Thereby, the differences in resistance to CyHV-3 can be correlated with differential expression of immune-related genes (Rakus *et al.*, 2012). Concerning the acute phase response induced by CyHV-3 infection, an up-regulation of complement-associated proteins and C-reactive proteins was also detected by 3 dpi, revealing a strong and quick innate immune response (Pionnier *et al.*, 2014).

#### 3.2.5.4. Common carp adaptive immune response against CyHV-3

The systemic immune response to CyHV-3 infection 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 revealed slight cross-reaction by ELISA and western blotting (WB) of anti-CyHV-3 antibodies to CyHV-1, probably due to shared epitopes in 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 at 7-14 dpi, rises till 20-40 dpi and finally progressively reduces with significant titres 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 challenge. Furthermore, at 280 dpi, although the titre of anti-CyHV-3 antibodies in previously infected fish is only slightly higher or comparable to that of naïve fish, they are still resistant to a lethal challenge, possibly due to the subsequent rapid response of B and T memory cells to antigen re-stimulation (Perelberg *et al.*, 2008).

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

Interestingly, a new immunoglobulin isotype, IgT (or IgZ) specialized in mucosal immunity in teleost fish was discovered, which enlarged the knowledge on mucosal immune response of teleost fish (Hansen *et al.*, 2005; Ryo *et al.*, 2010; Xu *et al.*, 2013b; Zhang *et al.*, 2010). This specific mucosal adaptive immune response further reveals the importance of antigen presentation at the pathogen portal

of entry to induce topologically adequate immune protection capable of blocking pathogen entry into the host (Gomez *et al.*, 2013; Rombout *et al.*, 2014).

3.2.5.5. CyHV-3 genes involved in immune evasion

Members of the family *Herpesviridae* have developed complex immune evasion mechanisms (Horst *et al.*, 2011). Bioinformatics analysis of the CyHV-3 genome revealed several genes encoding putative homologs of host or viral immune-related genes, such as 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 these genes in immune evasion mechanisms have been addressed partially. The main results are summarized below.

Ouyang *et al.* (2013) characterized the secretome of CyHV-3 and illustrated that ORF12 was the most abundant secreted viral protein in the supernatant of infected CCB cells. Recent studies have shown that pORF12 acts as a soluble decoy TNF-α receptor able to delay the expression of behavioural fever by infected carp (Rakus *et al.*, 2017). In addition, overexpression of the two vTNFRs (ORF4 and ORF12) leads to embryonic lethality, morphological defects and increasing apoptosis in zebrafish embryos (Yi *et al.*, 2015). CyHV-3 ORF16 was deleted using BAC mutagenesis, but 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 (Costes *et al.*, 2008).

Based on transcriptome analysis, CyHV-3 ORF112 is expressed as an immediate early gene (Ilouze *et al.*, 2012a) and its 278 amino acid expression product is incorporated into the virion (structural protein; Fig. 6) (Michel *et al.*, 2010b). No homology has been detected for the N-terminal part of the protein. In contrast, its C-terminal end encodes a functional Zalpha domain which contains 66 amino acids binding to left-handed dsDNA (Z-DNA) or left handed dsRNA (Z-RNA) (Athanasiadis, 2012). Zalpha domain has 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 *Chordopoxviridae* and ORF112 encoded by CyHV-3), acting as immune evasion factors. One of the three cellular proteins containing Zalpha domain is PKZ encoded by Cypriniformes and Salmoniformes (Rothenburg *et al.*, 2005), a paralog of the dsRNA-dependent protein kinase (PKR) expressed by all vertebrates. PKR and PKZ are interferon-induced proteins that play important roles in anti-viral innate immunity. It suggested that CyHV-3 encodes the protein containing Zalpha domain able to over-compete the binding of PKZ to Z-DNA (Tome *et al.*, 2013) and that the latter protein plays crucial roles in the innate immune response of carp against CyHV-3. Additional study revealed the crystal structure of ORF112-Zalpha domain in complex with Z-DNA and identified key interactions for the specificity of ORF112 (Kus *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 possessing both immunostimulatory 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 the host on various 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 antibodies secretion by $IgM^+$ B cells (Piazzon *et al.*, 2015a). Whether CyHV-3 ORF134 exhibits similar properties to carp IL-10 still needs to be investigated. The CyHV-3 pORF134 has 179 amino acids (Sunarto *et al.*, 2012) sharing 26.9% identity (67.3% similarity) with the common carp IL-10 over 156 amino acids (van Beurden *et al.*, 2011a). Proteomic analyses of the viral supernatant from CyHV-3 infected cells demonstrated that the ORF134 expression product is the second most abundant protein secreted (Ouyang *et al.*, 2013). It suggested that CyHV-3 ORF134 encodes a functional IL-10 homolog in an *in vivo* study using a zebrafish embryo model (Sunarto *et al.*, 2012). Indeed, injection of CyHV-3 IL-10 mRNA into zebrafish embryos increased the number of lysozyme-positive cells to a similar degree as observed with zebrafish IL-10 mRNA. Moreover, this effect was vanished when down-regulation of the IL-10 receptor long chain (IL-10R1) was performed using a specific morpholino. However, a recombinant CyHV-3 strain with the deletion of ORF134 presented a comparable replication *in vitro* and a similar mortality rate *in vivo* comparing with its parental and revertant strains, which implied that the IL-10 homolog encoded by CyHV-3 is essential neither for viral replication *in vitro* nor for virulence *in vivo* (Ouyang *et al.*, 2013).

# Objectives

Cyprinid herpesvirus 3 is the archetype of the family *Alloherpesviridae*. Complete genome sequences of 4 strains (KHV-U, KHV-J, KHV-I and KHV-GZ11) were available in the Genbank at the beginning of this PhD project, but no comparisons of the biologic properties of these strains were reported. Even if these strains represent the two existing lineages (Asian and European) described for CyHV-3, genome sequencing of additional strains was required to estimate the genetic diversity within this viral species. To reach this goal, we collected and sequenced a further seven strains from various geographical sources. Moreover, we compared their growth *in vitro* and virulence *in vivo*. The results of this first study revealed a negative correlation among strains between viral growth *in vitro* and virulence *in vivo*. This observation suggested the existence of alleles conferring adaptation to cell culture but reduced fitness *in vivo* and *vice versa*. The objectif of the second part of this thesis was to identify such alleles. A SNP of ORF131 encoding a structural glycoprotein was identified as a genetic trait discriminating "cell culture adapted" strains versus "*in vivo* adapted" strains. Two major goals of this second part of the thesis were to investigate both *in vitro* and *in vivo* the fitness of these genetic variants and to determine whether the presence of one variant could affect the fitness of the other.

# Experimental section

# Experimental section

## 1st study:

## Genomic and biologic comparisons of cyprinid herpesvirus 3 strains

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VETERINARY RESEARCH

RESEARCH ARTICLE Open Access

# Genomic and biologic comparisons of cyprinid herpesvirus 3 strains

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**Abstract**

Cyprinid herpesvirus 3 (CyHV-3) is the archetypal fish alloherpesvirus and the etiologic agent of a lethal disease in common and koi carp. To date, the genome sequences of only four CyHV-3 isolates have been published, but no comparisons of the biologic properties of these strains have been reported. We have sequenced the genomes of a further seven strains from various geographical sources, and have compared their growth in vitro and virulence in vivo. The major findings were: (i) the existence of the two genetic lineages previously described as European and Asian was confirmed, but inconsistencies between the geographic origin and genotype of some strains were revealed; (ii) potential inter-lineage recombination was detected in one strain, which also suggested the existence of a third, as yet unidentified lineage; (iii) analysis of genetic disruptions led to the identification of non-essential genes and their potential role in virulence; (iv) comparison of the in vitro and in vivo properties of strains belonging to the two lineages revealed that inter-lineage polymorphisms do not contribute to the differences in viral fitness observed; and (v) a negative correlation was observed among strains between viral growth in vitro and virulence in vivo. This study illustrates the importance of coupling genomic and biologic comparisons of viral strains in order to enhance understanding of viral evolution and pathogenesis.

## Introduction

Cyprinid herpesvirus 3 (CyHV-3; genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales*), also known as koi herpesvirus (KHV), is the etiologic agent of a lethal disease in common and koi carp (*Cyprinus carpio*) [1]. Since its emergence in the late 1990s, this highly contagious and dreadful disease has spread worldwide [2] and caused severe economic losses [3, 4]. CyHV-3 has been the subject of a growing number of studies and is considered to be the archetypal fish alloherpesvirus [1].

The CyHV-3 genome is 295 kbp in size and thus the largest described among all herpesviruses [5]. The complete DNA sequences of four CyHV-3 isolates derived from various geographical locations have been determined [6, 7]. Analyses of these sequences revealed a high level of genomic identity (> 99%), with inter-strain diversity comprising mainly single nucleotide polymorphisms (SNPs) and minor insertions or deletions (indels). They also indicated the existence of two main genotypic lineages initially termed European (based on one strain isolated in Israel and one strain originating from the USA) and Asian (based one strain isolated in Japan). Low inter-strain diversity and an association of these two lineages with geographic origin were further confirmed by PCR-based partial sequencing of a few variable markers for a substantial number of viral samples [8, 9]. However, preliminary data suggested that the situation might be more complex, as some strains originating from Europe resembled the Asian lineage, and vice versa [6, 8, 10]. Moreover, the coexistence in a single genome of loci belonging to both lineages suggested that recombination may have occurred [11]. However, definitive conclusions regarding these issues are difficult to make, since partial sequencing covering only a few loci was performed.

Many of the sequence differences between CyHV-3 strains concern indels caused by variable number of

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tandem repeats (VNTRs). VNTR polymorphism has shown some utility for differentiating strains of large DNA viruses, such as human herpesvirus 1 [12]. Analysis of multiple VNTR loci in CyHV-3 provided further support for the existence of the two main lineages described above, and, in addition, 87 haplotypes were identified [13, 14]. The discriminatory power of VNTRs is nonetheless more suited to fine tuning the tips of phylogenetic trees based on non-VNTR polymorphisms (SNPs and indels) [1]. Unfortunately, robust phylogenetic classification of this sort is lacking for CyHV-3 strains due to the low number of complete genome sequences available. This deficiency could be overcome using genome-wide analysis of multiple viral strains by high-throughput sequencing. This approach has been used successfully for several other herpesviruses, including human herpesvirus 1 [15], to give a much fuller picture of strain diversity [16]. Indeed, it was recently applied to sequencing CyHV-3 directly from infected fish tissues, without first isolating viral strains [17]. However, multiple strains were detected in each tissue, potentially causing the derived genome sequences to be artificial composites of the individual strains present.

In our study, we sequenced seven strains of CyHV-3 from various geographical origins, and compared their growth in vitro and virulence in vivo. We confirmed the existence of the two genetic lineages described previously as European and Asian, but identified inconsistencies between the geographic origin and the lineage of few strains. Potential inter-lineage recombinations were also demonstrated, which suggested the existence of a third, as yet unidentified lineage. Analyses of CyHV-3 genes that are disrupted, and therefore non-functional, led to the identification of several non-essential genes, some of which may affect virulence. Finally, a negative correlation was established between viral growth in vitro and virulence in vivo. Overall, our study illustrated the potential of comparing the genomic and biologic characteristics of viral strains in studying viral evolution and pathogenesis.

## Materials and methods

### Cells and viruses

Common carp brain (CCB) cells [18] were cultured in minimum essential medium (Sigma) containing 4.5 g/L glucose (D-glucose monohydrate; Merck) and 10% (v/v) fetal calf serum (FCS). The cells were cultured at 25 °C in a humid atmosphere containing 5% $CO_2$. A total of seven CyHV-3 strains from various geographic origins were used (Table 1). The Cavoy strain was amplified from a commercial aliquot of an attenuated vaccine produced by passage in cell culture of an isolate from Israel [19–21]. The T strain was subcloned from a large viral plaque obtained after 30 passages of a Taiwanese isolate on a koi fin cell line [22]. GZ11-SC is a subclone of the GZ11 strain from China [6]. It was generated by transfection of GZ11 DNA into CCB cells, followed by plaque purification of the regenerated virus.

**Table 1 CyHV-3 strains**

| Name[a] | Geographic origin | GenBank accession number | References |
|---|---|---|---|
| FL | Belgium | MG925487 | [45] |
| M3 | Belgium | MG925490 | [46] |
| I | Israel | MG925489 | [46] |
| Cavoy | Israel | MG925485 | [19–21] |
| E | United Kingdom | MG925486 | [47] |
| T | Taiwan | MG925491 | [47] |
| GZ11-SC | China | MG925488 | [6] |
| U | USA | DQ657948.1 | [7] |
| KHV-I | Israel | DQ177346.1 | [7] |
| J | Japan | AP008984.1 | [7] |
| GZ11 | China | KJ627438.1 | [6] |

[a] The first seven viral strains listed were used in this study.

### Genetic characterization of CyHV-3 strains

Viral DNA from CyHV-3 strains was characterized by restriction fragment length polymorphism using SacI digestion, and further characterized by full-length genome sequencing using methods described previously [23, 24]. Sequences have been deposited to the GenBank.

### Phylogenetic analysis

A multiple alignment of 11 full-length viral genome sequences was made using MAFFT online version 7 [25]. The sequences included the seven produced in this study and the four available in GenBank for the U, J, KHV-I and GZ11 isolates (Table 1) [6, 7]. The phylogenetic tree was generated using the UPGMA method (unweighted pair-group method with arithmetic means) implemented in the MEGA6 software [26], and evaluated by the interior branch test method with 1000 bootstraps.

### Recombination analysis

Detection of inter-strain recombination, identification of closest parental sequences and localization of possible recombination break points were carried out using the Recombination Detection Program 4 (RDP4) [27]. This software combines a variety of independent methods, including RDP [28], GENECONV [29], BOOTSCAN [30], MaxChi [31], CHIMAERA [32], SISCAN [33] and 3SEQ [34].

### Viral growth curve assay

Triplicate cultures of CCB cells were infected with each CyHV-3 strain at a multiplicity of infection (MOI) of 0.05 plaque-forming unit (pfu)/cell. After an incubation period of 2 h, the cells were washed with phosphate-buffered saline and overlaid with Dulbecco's modified essential medium (DMEM, Sigma) containing 4.5 g/L glucose and 10% (v/v) FCS. The supernatant was removed from infected cultures at successive intervals (0, 2, 4, 6 and 8 days post-infection (dpi)), and stored at −80 °C. Viral titration was carried out by duplicate plaque assays in CCB cells using methods described previously [35].

### Viral plaque size assay

CCB cells were cultured in six-well plates and inoculated with 200 pfu/well of CyHV-3 for 2 h and overlaid with DMEM containing 4.5 g/L glucose, 10% (v/v) FCS and 1.2% (w/v) carboxymethylcellulose (medium viscosity, Sigma). Viral plaques were treated for indirect immunofluorescent staining by using monoclonal antibody 8G12, which recognizes an unidentified CyHV-3 nuclear protein. Plaques were imaged by using a Nikon A1R confocal microscope, and plaque size was measured by using the ImageJ software [36].

### Fish

Common carp were kept in 60 L tanks at 24 °C. Water parameters were checked twice per week. Microbiological, parasitic and clinical examinations carried out immediately prior to the experiments demonstrated that the fish were healthy. All experiments were preceded by an acclimation period of at least 2 weeks.

### Inoculation of fish

Fish were infected by immersion in constantly aerated water containing 400 pfu/mL of virus for 2 h at room temperature, the volume of water being adjusted to a biomass of approximately 10% according to fish size and number. At the end of the incubation period, the fish were returned to the initial tank.

### Ethics statement

The experiments, maintenance and care of fish complied with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (CETS No. 123). The animal studies were approved by the local ethics committee of the University of Liège, Belgium (laboratory accreditation No. 1610008, protocol No. 1059). All efforts were made to minimize animal suffering.

### Statistical analysis

Viral plaque sizes and viral growth curves were compared by two-way ANOVA (analysis of variance) with interactions followed by Bonferroni post hoc test. Survival curves were compared by using Log-rank tests. Correlation analyses were done by Pearson two-tailed r test, and illustrated by linear regression, using Graphpad Prism 5. Statistical analyses of recombination events were generated with the RDP4 software. Calculation of $p$ values is based on binomial distribution (RDP), Blast-Like Karlin-Altschul & Permutation (GENECONV), Bootstrapping & binomial distribution & Chi squared test (BOOTSCAN), Chi squared test & permutation (MaxChi, CHIMAERA), Permutation & Z Test (SISCAN) and Exact test (3SEQ). Statistical significance was represented as follows: *ns* not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$.

## Results

The goal of our study was to gain insights into the evolution and pathogenesis of CyHV-3 by performing coupled genomic and biologic comparisons. With this goal in mind, seven viral strains were selected (Table 1). These strains originated from various countries and were supposed to represent the European and the Asian lineages of CyHV-3.

### Full-length genome analyses

Phylogenetic analysis of the seven new genome sequences and the four published sequences (Table 1) confirmed the high level of similarity (> 99% identity) reported previously. The existence of two major phylogenetic lineages was also confirmed (Figure 1), and a correlation was observed between geographic origin and viral lineage for most strains. However, strain M3 branched in the Asian lineage despite having been isolated in Europe, and strain GZ11 (and its subclone GZ11-SC) had a monophyletic origin with the European lineage despite having been isolated in China. The intermediate position of the GZ11 strain hinted that it may have been generated by recombination between the two lineages. This hypothesis was supported by examination of a genome sequence alignment (data not shown).

### Recombination

To explore potential inter-strain recombination, the eleven full-length genomes were analyzed using the RDP4 software. Potential recombination events were detected only in strain GZ11 and its subclone (Table 2 and Figure 2). The results suggested that strain GZ11 consists of a genome in the European lineage (Figures 2B, E and H) in which three recombination events have occurred. One (Figures 2A–C) represented the acquisition of 5.5 kb from the left end of the genome from a

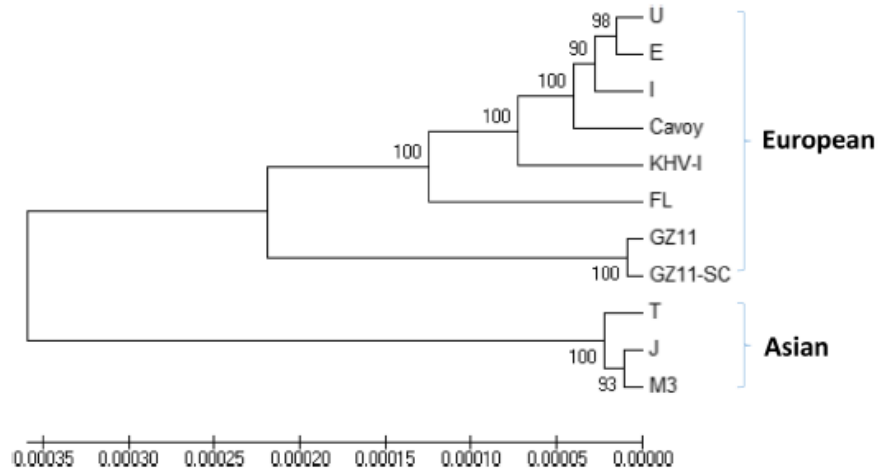


**Figure 1 Phylogenetic analysis of CyHV-3 genome sequences.** The analysis was based on full-length genome sequences excluding one of the terminal direct repeats. The two previously described lineages are indicated. The phylogenetic tree was built using UPGMA in MEGA6 with 1000 replicates. Values on internal branches refer to the percentage of bootstrap replicates in which the branch was found; only values greater than 50% are shown. The scale shows the number of substitutions per nucleotide.

**Table 2 Recombination events in strain GZ11**

| Event | Genome region[a] | Major parent[b] | Minor parent[c] | Detection method[d] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | R | G | B | M | C | S | T |
| 1 | 117-5598 | FL | Unknown | ** | * | * | ** | * | ns | * |
| 2 | 77366-94864 | Cavoy | T | *** | *** | *** | *** | *** | ns | *** |
| 3 | 269851-271724 | E | M3 | ** | ns | ns | ns | ns | ns | ns |

[a] Coordinates are listed in relation to the sequence of strain U (GenBank accession number DQ657948.1). The results for strain GZ11-SC were the same as for strain GZ11.

[b] Major parent strain was automatically predicted by the RDP software. It represents the closest relative of the recombinant strain taking into account the entire genome but excluding the recombination region.

[c] Minor parent was automatically predicted by the RDP software. It represents the closest relative of the recombinant strain taking into account the recombination region.

[d] Detection methods used in RDP4: R, RDP; G, GENECONV; B, BOOTSCAN; M, MaxChi; C, CHIMAERA; S, SISCAN; T, 3SEQ. Statistical significance is indicated according to the code described in "Materials and methods".

third, as yet unidentified lineage (Figure 2C). The other two represented the acquisition of 17.5 and 1.9 kb, respectively, from the Asian lineage (Figures 2F and I). Recombination events 1 and 2 were predicted by 6 detection methods suggesting a high probability of occurrence. In contrast, recombination event 3 was supported by only one detection method and should therefore be treated with caution.

### Viral growth in vitro

Viral fitness in vitro was assessed by viral growth assay and plaque size assay on CCB cells (Figure 3). All seven tested viral strains grew efficiently, reaching titers of at least $10^5$ pfu/mL at the peak of infection (Figure 3A). The kinetics of viral growth was similar for all strains. The numbers of infectious particles peaked at 4 dpi (strains T, Cavoy, FL and GZ11–SC) or 6 dpi (strains M3, I and E), and then declined due to virion inactivation. Despite these general similarities, statistical analyses at 4 dpi revealed quantitative differences between the strains. For example, strain T (the best-growing strain) reached a titer $> 10^7$ pfu/mL, which was > 100 times that of strain E (the worst-growing strain). These analyses revealed that strain T grew more efficiently than all other strains tested ($p < 0.001$), followed by strain Cavoy ($p < 0.001$ vs all other strains), strain FL ($p < 0.05$ vs strain GZ11-SC, $p < 0.01$ vs

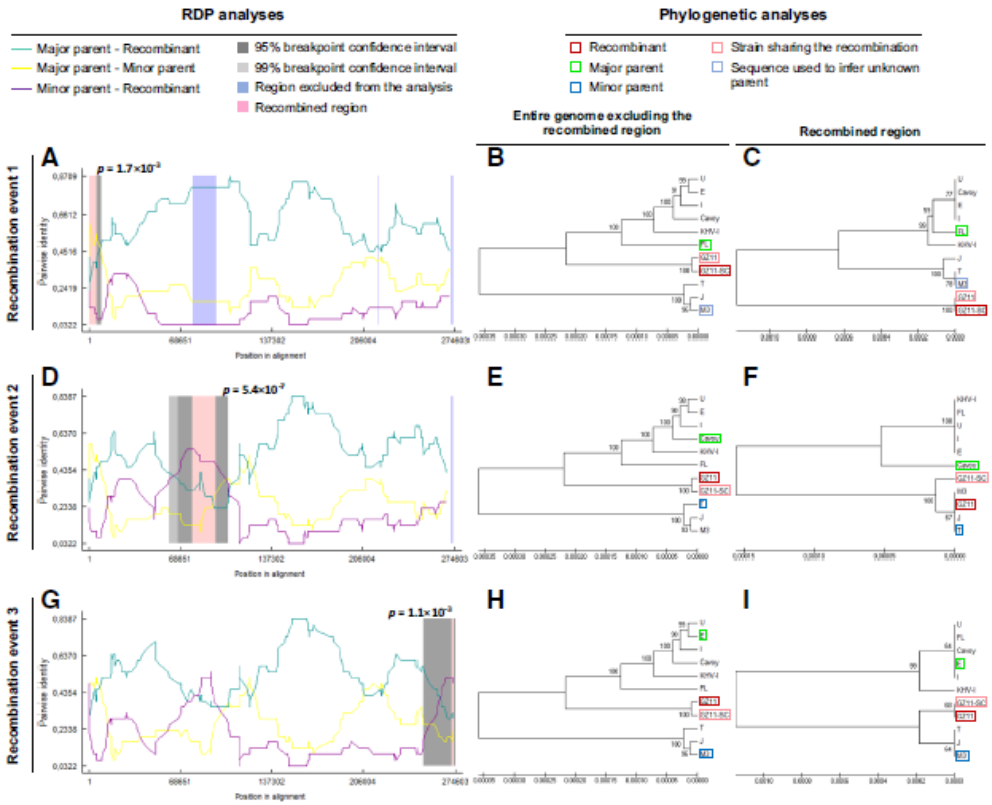


**Figure 2 Recombination between CyHV-3 lineages.** An alignment of the 11 CyHV-3 genomes listed in Table 1 was analysed using the RDP4 software. Three potential recombination events were identified for strains GZ11/GZ11-SC (illustrated by **A**–**I**, respectively). The left part illustrates the results of RDP analyses (**A**, **D** and **G**). The right part presents phylogenetic analyses based on the full-length genome (excluding one of the terminal direct repeats) excluding the region of recombination (**B**, **E** and **H**) or based on the recombination region only (**C**, **F** and **I**) using UPGMA in MEGA6 with 1000 replicates. Values on internal branches refer to the percentage of bootstrap replicates in which the branch was found; only values greater than 50% are shown. The scales illustrate the number of substitutions per nucleotide. The color code used is described at the top.

strain M3, and $p < 0.001$ vs strains I and E), strains M3, I and GZ11-SC (ns among each other, $p < 0.05$ strains I vs E, and $p < 0.001$ strains GZ11-SC and M3 vs strain E), and finally strain E. These results demonstrate that CyHV-3 strains exhibited different abilities to grow in vitro that are unrelated to the lineage to which they belong.

All viral strains tested were capable of forming plaques (Figure 3B). However, the results obtained at 10 dpi revealed significant differences in plaque size between strains. The largest plaques were produced by strain T, and these were about twice as big in area as those observed for strain GZ11-SC, which produced the smallest plaques. Statistical analyses of the results obtained at 10 dpi revealed that strain T produced the largest plaques ($p < 0.001$ vs all strains), followed by strain Cavoy ($p < 0.001$ vs all strains), strains FL, M3 and E (ns between each other, $p < 0.01$ vs strain I, $p < 0.001$ vs strain GZ11-SC), strain I ($p < 0.01$ vs strain GZ11-SC), and finally strain GZ11-SC. The two parameters used to assess viral replication in vitro (virion production in the extracellular medium and plaque size) were positively correlated ($p < 0.05$; Figure 3C).

### Virulence in vivo

The levels of virulence of the seven strains were compared after inoculating fish by immersion in water containing the virus, thereby mimicking a natural infection (Figure 4).

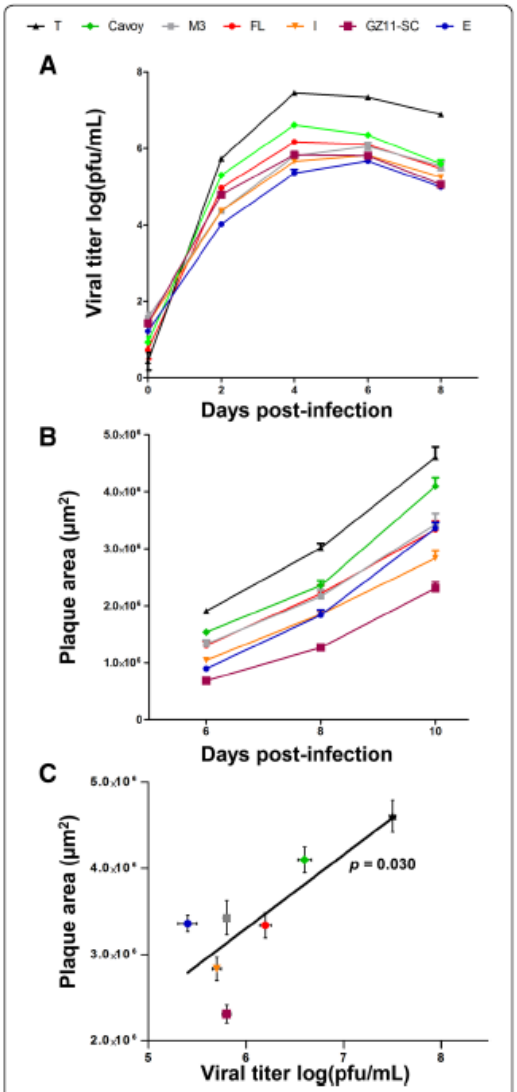


**Figure 3 Comparisons of viral growth in vitro. A** Viral growth assay. CCB cells were infected with the strains indicated (see top of the figure for the symbol code used) and the log10 value of the titer (pfu/mL) in the cell supernatant was determined at the indicated dpi. Data are presented as the mean + SEM (standard error of the mean) of triplicate measurements. **B** Viral plaque assay. CCB cells were infected with the strains indicated, and plaques areas were measured over time. Data presented are the mean + SEM for measurements of 20 randomly selected plaques. **C** Correlation between plaque size measured at 10 dpi (**B**) and viral titers measured at 4 dpi (**A**). Data presented are the mean ± SEM.

Regardless of the viral strain used, all infected fish developed CyHV-3 disease. However, the intensity and the kinetics of appearance of clinical signs varied between strains. Strains Cavoy and T induced the mildest disease, with infected fish expressing folding of the dorsal fin, hyperemia and apathy/anorexia. Around 20% of infected fish succumbed from this infection, including late mortalities induced by neurological symptoms observed in fish surviving the first peak of mortalities. Infection with strain FL led to typical disease including the symptoms described above and associated fin and skin lesions on most fish. Around 70% of infected fish died from this infection. Infection with strains M3, I, E and GZ11-SC led to very acute and highly virulent disease, with almost all fish dying by 10 dpi. None of the mock-infected fish expressed clinical signs or mortality.

Statistical analysis was conducted by two-by-two comparison using a Log-rank test, and the cut-off for significance was adjusted according to the number of comparisons made. Mock-infected groups had higher survival rates compared to all infected fish groups ($p < 0.0001$). Strains Cavoy and T induced higher survival rates than all others strains (ns between these strains, $p < 0.0001$ vs all other strains). Strain FL induced a higher survival rate than strains M3, I, E and GZ11-SC ($p < 0.0001$). Strain GZ11-SC was significantly more virulent than strains I and E ($p < 0.0001$), but not more virulent than strain M3. All other comparisons were not significant.

These results indicate that the strains tested can be classified as having low virulence (strains Cavoy and T), moderate virulence (strain FL) and high virulence (strains M3, E, I and GZ11-SC). As observed for viral growth in vitro, there was no clear association between virulence and genetic lineage. Correlation analysis between viral growth in vitro and virulence in vivo was performed as described above. Positive correlations were found between viral replication in vitro (viral growth assay and viral plaque assay) and survival rate in vivo (Figures 5A and B). These results indicate that the adaptation of a viral strain to cell culture is generally associated with its attenuation in vivo.

### Disabled genes

Coupled genomic and biologic comparison of CyHV-3 strains has the potential to indicate biologic roles for genes that are associated with polymorphisms and thus to link them to biological traits. CyHV-3 strains are known to carry mutations (generally frameshifts) in open reading frames (ORFs) that are likely to ablate gene functions, and these mutations may vary from strain to

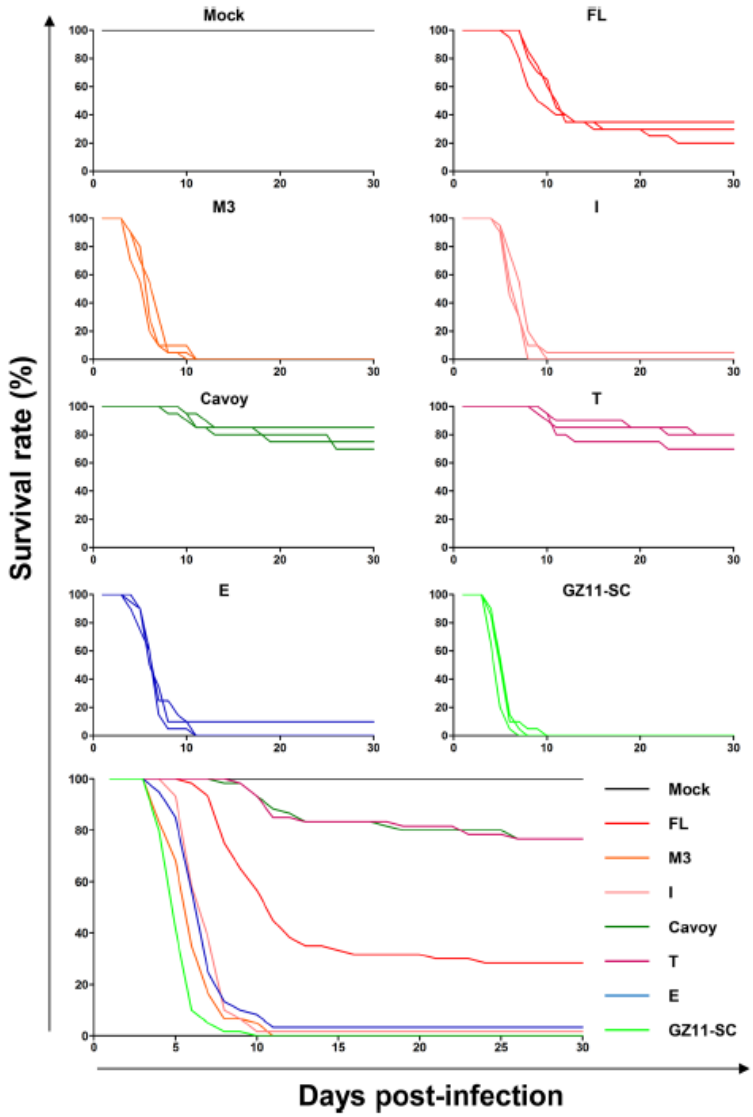


**Figure 4 Comparison of virulence in vivo.** The virulence of the indicated strains was tested in carp (triplicate groups each consisting of 20 subjects, average weight 5.03 ± 3.78 g, 7 months old). On day 0, fish were mock-infected or infected by immersion for 2 h in water containing 400 pfu/mL of virus. Survival rate was measured over a period of 30 dpi. The smaller upper panels show the survival curves observed for replicates. The larger lower panel shows the mean survival curves based on the three replicates.

strain. The genes disabled in the strains analysed in our study are summarized in Table 3. Two different situations were noted: ORFs having one or more frameshift near the beginning or in the central part of the ORF that is likely to ablate protein function, and ORFs having a frameshift near the end of the ORF that may not ablate protein function.

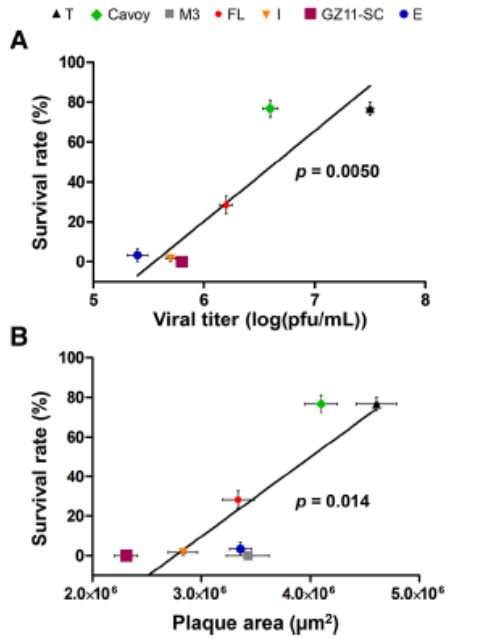


**Figure 5 Correlation of growth in vitro and virulence in vivo.** Data related to viral growth in vitro (viral titer observed 4 dpi and plaque size measured at 10 dpi; see Figure 3) and virulence in vivo (mean survival rates at 30 dpi; see Figure 4) were analysed. **A** Correlation between virulence and viral titer. **B** Correlation between virulence and plaque size. Data presented are the mean ± SEM.

Analysis of these results together with the biologic properties described above led to several conclusions. First, genes that exhibit mutations incompatible with the expression of a functional protein in any strain that is able to grow in cell culture can be classified as non–essential. These include ORF12, ORF26, ORF27, ORF30, ORF40, ORF52, ORF64, ORF105, ORF128 and ORF153. Second, genes that exhibit mutations incompatible with the expression of a functional protein in a highly virulent strain can be classified as non-essential to virulence. These include ORF12, ORF26, ORF30, ORF40, ORF64, ORF105 and ORF128. Third, mutated ORFs that are found exclusively in low or moderate virulence strains can be classified as potentially important virulence genes. These include ORF27, ORF52 and ORF153.

## Discussion

Comparisons of full-length genome sequences and biological properties in vitro and in vivo for seven viral strains allowed us to draw hypotheses about the evolution of CyHV-3 and the roles of some of its genes.

Analysis of full-length CyHV-3 genome sequences revealed a high level of similarity (> 99% identity), confirmed the existence of two major genetic lineages while also suggesting the existence of a third, as yet undetected lineage, and highlighted the occurrence of inter-lineage recombination. The level of similarity among CyHV-3 strains is by far greater than that reported for other herpesviruses [15, 16] and suggests that the pathogen has emerged relatively recently. This could have been facilitated by the selection for virulence of a viral strain that was already resident in common carp or that had transferred from another host species. However, the existence of identifiable genetic lineages with largely different panoplies of disabled genes, and the single example so far of inter-lineage recombination, suggest that these lineages have been evolving in different host populations. Moreover, the largely consistent association between genetic lineage and geographic origin indicates that the lineages have been spreading independently, with the few inconsistencies and the potential recombination in strain GZ11 (Figure 2 and Table 2) being potentially the consequence of recent geographic spread due to international trading of carp [2]. Importantly, the comparison of the in vitro and in vivo properties of strains belonging to the two lineages did not reveal differences between them, thus implying that inter-lineage polymorphisms do not contribute to the differences in viral fitness observed.

CyHV-3 strains are closely related to each other in sequence, but sufficient differences exist to indicate that their ancestors were infecting various common carp populations long before CyHV-3 disease was first described in the late 1990s. The evolutionary rate of CyHV-3 is unknown, but, if it is similar to that espoused for mammalian alphaherpesviruses ($3.5 \times 10^{-8}$ substitutions/nucleotide/year; [37]), the observation that the European and Asian CyHV-3 lineages differ by up to 6 substitutions in the 5130 bp ORF79 encoding the DNA polymerase catalytic subunit would suggest that the virus has been infecting common carp for some tens of thousands of years. On this basis, CyHV-3 disease is more likely to have emerged in the 1990s as an outcome of shifts in host or environmental co-factors rather than as the result of a virus jumping into common carp from another type of cyprinid fish [38]. The ancestral relationship between common carp and CyHV-3 has also potential implications in the Australian plan to use this virus as a biocontrol measure against invading carp [39]. The apparently long association between CyHV-3 and common carp, and the fact that introductions of common and koi carp into Australia have occurred on many occasions since the 1850s, including more recently than the 1990s [40], imply that the presence of CyHV-3 in Australia cannot be ruled out. CyHV-3-associated mass carp deaths in Australia

**Table 3 Disrupted genes**

| Genotype | Strain | ORF[a] | | | | | | | | | | | | | | | | | | Virulence[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| European | U | 16 | ~~26~~ | ~~27~~ | ~~30~~ | ~~40~~ | | 55 | | | 94 | | 108 | | | | | | | NT |
| | KHV | 16 | ~~26~~ | ~~27~~ | ~~30~~ | ~~40~~ | | 55 | | | | | 108 | 116 | ~~125~~ | ~~128~~ | ~~139~~ | | | NT |
| | GZ11 | 16 | ~~26~~ | ~~27~~ | | ~~40~~ | | 55 | 57 | | | | 108 | | | | | | | NT |
| | GZ11-5C | ~~12~~ 16 | ~~26~~ | | | ~~40~~ | | 55 | 57 | | | | 108 | | | | | | | High |
| | E | 16 | ~~26~~ | | ~~30~~ | ~~40~~ | | 55 | | | | | 108 | | | ~~128~~ | | | | High |
| | I | 16 | 20 ~~26~~ | | ~~30~~ | ~~40~~ | | 55 | | | | | 108 | | | | | | | High |
| | Cavoy | 16 | ~~26~~ | ~~27~~ | ~~30~~ | ~~40~~ | 48 | ~~52~~ | 55 | | | | ~~105~~ | 108 | | | | ~~153~~ | | Low |
| | FL | 16 | ~~26~~ | ~~27~~ | 28 ~~30~~ | ~~40~~ | | | 55 | | | | | 108 | | | | | | Moderate |
| Asian | T | | ~~26~~ | ~~27~~ | | ~~40~~ | | | | ~~64~~ | | | ~~105~~ | | | | | | | Low |
| | M3 | | ~~26~~ | | | ~~40~~ | | | | ~~64~~ | 87 | | ~~105~~ | | | | | | | High |
| | J | | ~~26~~ | | | ~~40~~ | | | | ~~64~~ | 87 | | ~~105~~ | | | | | | | NT |

[a] Numbers that are struck out identify ORFs having at least one frameshift near the beginning or in the central part of the ORF that is likely to ablate protein function. Numbers that are not struck out represent ORFs having a frameshift near the end of the ORF that may not ablate protein function.

[b] Virulence score based on the results presented in Figure 4. NT not tested.

have not been recorded, but this may simply reflect a lack of the environmental co-factors necessary for disease emergence. These considerations have prompted the advice that further assessments of efficacy (not to mention safety) should be carried out before the proposed release is attempted [39, 41].

Our results also identified non-essential viral genes and yielded information on their potential roles in virulence. Some genes are evidently not essential for viral growth in vitro and appear to play no essential role in virulence in vivo. However, the laboratory model of infection used would not have revealed genes that function in modification of host behavior, establishment of latency, reactivation from latency, and excretion of virus and transmission to naïve subjects [23, 38, 42]. Further experiments are needed to extend the in vivo studies.

All of the strains tested, including the Cavoy vaccine strain, were to some degree virulent (Figure 4). This observation implicates that none of the strains lacks the function of an essential virulence gene, such as ORF57 [24]. Interestingly, strain Cavoy expressed a level of virulence comparable to that of strain T, killing 20% of inoculated fish (Figure 4). This residual level of virulence is consistent with earlier observations [21, 43, 44] and could explain why this vaccine was removed from the market soon after its commercialization in the USA. Given that this strain is still used as a vaccine in some countries, its lack of safety must represent a serious source of concern for the aquaculture industry. Disrupted ORFs that are found exclusively in strains of low or moderate virulence are potentially important virulence genes. They include ORF27 (disrupted in strains FL, T and Cavoy), and ORF52 and ORF153 (both disrupted only in strain Cavoy) (Table 2). Notably, the strains with the highest fitness in cell culture were those associated with the longest cell passage history and the lowest virulence in vivo (Figures 3, 4, 5). Although these results suggest that disrupted genes may be potential virulence factors, they are also consistent with gene loss being fortuitously associated with viral adaptation to cell culture. The latter hypothesis is supported by the observation that viral passage in cell culture is frequently associated with the selection of mutations disabling ORF27 [7]. Further experiments are underway to address this issue.

Our study illustrates the power of coupling genomic and biologic comparisons of viral strains to study viral evolution and pathogenesis. It also provides a firm basis for further research on CyHV-3.

**Abbreviations**

CCB: common carp brain; CyHV-3: cyprinid herpesvirus 3; DMEM: Dulbecco's modified essential medium; dpi: days post-infection; FCS: fetal calf serum; indels: insertions or deletions; KHV: koi herpesvirus; ns: not significant; ORF: open reading frame; pfu: plaque-forming unit; RDP: recombination detection program; SEM: standard error of the mean; SNP: single nucleotide polymorphism; UPGMA: unweighted pair-group method with arithmetic means; VNTR: variable number of tandem repeats.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

MB and AV designed the study. YG did most of the experiments and performed statistical analyses. YG, MB, AD and AV contributed to the writing of the manuscript. YG, MB, and AV drafted the figures. CD, SB and PYL isolated some of the strains. NS, GW and AD determined the genome sequences of the CyHV-3 strains. All authors read and approved the final manuscript.

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**Availability of data and materials**

Genome sequences described in this manuscript are publicly available.

**Ethics approval and consent to participate**

The experiments, maintenance and care of fish complied with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (CETS no. 123). The animal studies were approved by the local ethics committee of the University of Liège, Belgium (laboratory accreditation no. 1610008, protocol no. 1059). All efforts were made to minimize animal suffering.

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# Experimental section

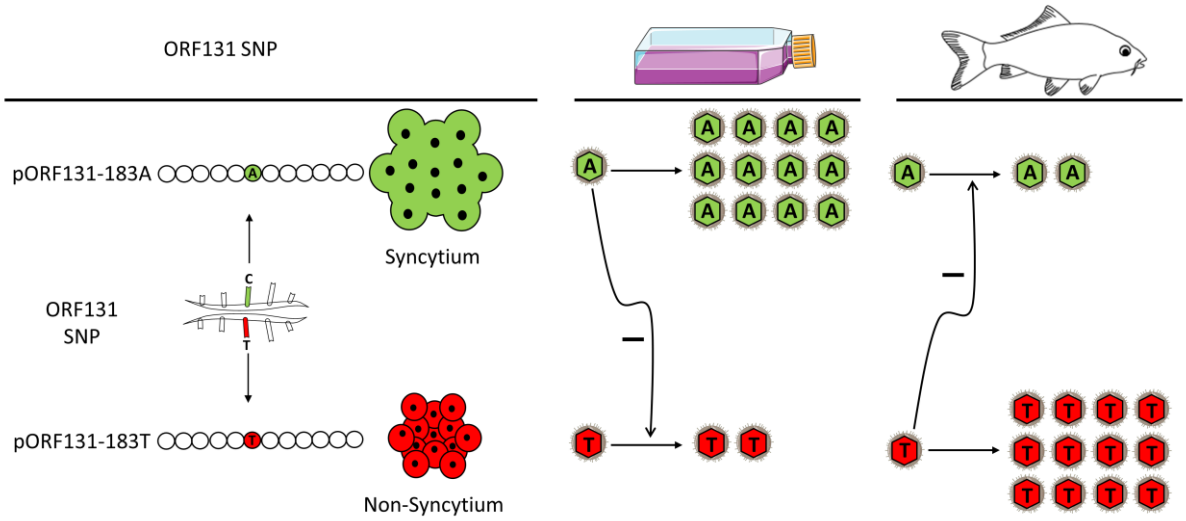
## 2nd study:

**Virus-induced inhibition of superinfection as a means for accelerating fitness-based selection of cyprinid herpesvirus 3 single nucleotide variants *in vitro* and *in vivo***

Under revision in Virus evolution

**Yuan Gao,** Noah Bernard, Bo He, Haiyan Zhang, Salomé Desmecht, Catherine Vancsok, Maxime Boutier, Nicolás M. Suárez, Andrew J. Davison, Owen Donohoe, and Alain F.C. Vanderplasschen

## *Graphical Abstract*



# Virus-induced inhibition of superinfection as a means for accelerating fitness-based selection of cyprinid herpesvirus 3 single nucleotide variants *in vitro* and *in vivo*

Running title: Cyprinid herpesvirus 3 ORF131 variants

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## ABSTRACT

Cyprinid herpesvirus 3 (CyHV-3) is the archetype of alloherpesviruses and is advantageous to research because, unlike many herpesviruses, it can be studied in the laboratory by infection of the natural host (carp). Previous studies have reported a negative correlation among CyHV-3 strains between viral growth *in vitro* (in cell culture) and virulence *in vivo* (in fish). This suggests the existence of alleles conferring enhanced fitness *in vitro* but reduced fitness *in vivo*, and *vice versa*. Here, we identified syncytial plaque formation *in vitro* as a common trait of CyHV-3 strains adapted to cell culture. Comparison of the sequences of virion transmembrane protein genes in CyHV-3 strains, and the use of various recombinant viruses, demonstrated that this trait is linked to a single nucleotide polymorphism (SNP) in ORF131 that results in codon 183 encoding either an alanine (A) or a threonine (T) residue. In experiments involving infections with recombinant viruses differing only by this SNP, the 183A allele associated with syncytial plaque formation was the more fit *in vitro* but the less fit *in vivo*. In experiments involving co-infection with both viruses, in addition to the more fit allele contributing to the purifying selection of the less fit allele by outcompeting the latter, we observed that this process may be accelerated by strong viral stimulation of superinfection inhibition at a cellular level, and stimulation of resistance to superinfection at a host level. Collectively, this study illustrates how the fundamental biological properties of some viruses and their hosts may have a profound impact on the degree of diversity that arises within viral populations.

**KEY WORDS**

Herpesvirus, alloherpesvirus, virus evolution, fitness-based selection, purifying selection

## 1. INTRODUCTION

The order *Herpesvirales* comprises large, double-stranded DNA viruses classified in three distantly related families: *Herpesviridae* (herpesviruses of reptiles, birds and mammals), *Malacoherpesviridae* (herpesviruses of molluscs) and *Alloherpesvirida*e (herpesviruses of amphibians and fish) (Hanson, Dishon, and Kotler 2011). Most members of the family *Alloherpesviridae* (referred to as alloherpesviruses) have been recognized because they cause disease outbreaks associated with mass mortalities that have a serious economic impact on the aquaculture sector.

Current knowledge of herpesvirus evolution relies almost entirely on the study of members of the family *Herpesviridae*. These studies suggest that, despite the proof-reading activity of herpesvirus DNA polymerases, the large size of herpesvirus genomes facilitates a degree of tolerance towards genetic drift (e.g. accumulation of SNPs and insertions/deletions (indels)) in successive rounds of viral replication (Renner and Szpara 2017). Larger scale changes due to genetic shift also occur, with inter-strain recombination resulting in individual strains acquiring combinations of advantageous genes or alleles initially present in different parental genomes (Kolb et al. 2017; Law et al. 2018; Umene 1999). This process requires co-infection of the same host cell by two parental viruses, and can result either from simultaneous infection or from infection by one strain followed by superinfection by another. The diversity generated by genetic drift and shift is subject to constant fitness-based selection dictated by environmental pressures, which may differ markedly *in vitro* (in cell culture) and *in vivo* (in the natural host). Understanding the key factors that determine how purifying (negative) selection operates on herpesvirus genomes would provide useful insights into the evolution of these viruses.

Compared to the family *Herpesviridae* there are relatively few studies regarding evolution within the family *Alloherpesviridae* (Waltzek et al. 2009; 2005; Aoki et al. 2007; Davison et al. 2013). Recently, we investigated the diversity of core gene sequences within species clades of the genus *Cyprinivirus* (referred to as cypriniviruses and comprising alloherpesviruses infecting cyprinids and eels) (Donohoe et al. 2021). This revealed significantly less genetic diversity within cyprinivirus species clades than within species in the family *Herpesviridae*, which may be linked, at least in part, to different environmental pressures. CyHV-3 is the archetypal cyprinivirus (Boutier, Ronsmans, Rakus, et al. 2015). This virus can be studied in the laboratory by infection of the natural host, the common and koi carp (*Cyprinus carpio*). Since its emergence in the 1990s, CyHV-3 has caused severe economic loss within the carp culture industry worldwide (Bondad-Reantaso et al. 2005; Perelberg et al. 2003). In addition, it has had an ecological impact by inducing carp mortalities in natural habitats (Rakus et al. 2013; Adamek et al. 2014). Genomic and biological comparisons of CyHV-3 strains have revealed a negative correlation among strains between viral growth *in vitro* and virulence *in vivo* (Gao et al. 2018), suggesting the existence of alleles - outside of the most conserved core genes, that confer advantages *in vitro* but reduced fitness *in vivo,* and *vice versa*. The identification of such alleles would provide a valuable opportunity to study competition between variants and also the process of directional selection under controlled conditions.

In the present study, we first identified syncytial plaque formation *in vitro* as a common trait of CyHV-3 strains exhibiting greater replicative fitness in cell culture. Next, we demonstrated by mutagenesis that the genetic determinant of this trait depends on a SNP in ORF131, which encodes an essential type 1 membrane glycoprotein present in the virion. Strains adapted to cell culture encode an alanine residue at codon 183 (the 183A allele),

whereas field strains encode a threonine residue at this position (the 183T allele). Pairs of viruses differing only by this SNP were generated and compared for fitness *in vitro* and *in vivo* by infection with single viruses or co-infection with both viruses. These experiments demonstrated the higher fitness of the 183A allele *in vitro* and the 183T allele *in vivo*. Interestingly, we found that purifying selection of the least fit variant occurs very rapidly during short lytic infections both *in vitro* and *in vivo*. Further investigations revealed that purifying selection of the least fit variant could, at least in part, be facilitated by the onset of the inhibition of superinfection (sometimes referred to as superinfection exclusion), at a cellular level *in vitro* and increases resistance to superinfection at a host level *in vivo*. We also propose that for CyHV-3 in particular, its intrinsic ability to rapidly and robustly stimulate superinfection inhibition may also act as a means to accelerate purifying selection of less fit variants in the field, which may contribute low diversity. Importantly it indicates that the fundamental biology of some viruses and their hosts may have a profound impact on the level of diversity that arises within viral populations.

## 2. MATERIALS AND METHODS

### 2.1 Cells and viruses

Common carp brain (CCB) cells (NEUKIRCH 1999) were cultured as described previously (Rakus et al. 2017). The seven CyHV-3 strains used had various geographic origins and have been described previously (see Table 1 in (Gao et al. 2018)).

### 2.2 Indirect immunofluorescence staining

Cells grown on glass coverslips were fixed in phosphate buffered saline (PBS) containing 4% (wt/vol) PAF at 4 °C for 15 min and then 20 °C for 10 min. After washing with PBS, samples

were permeabilized in PBS containing 0.1% (v/v) Nonidet P40 at 37 °C for 15 min. Immunofluorescence staining (incubation and washes) was performed in PBS containing 10% (v/v) fetal calf serum (FCS). Monoclonal antibody 4B5 against the CyHV-3 ORF65 protein (pORF65) was used as primary antibody (dilution 1:1000). Alexa 488 goat-anti-mouse immunoglobulin G (H+L) (Invitrogen) was used as secondary antibody (dilution 1:1000). After washing, the cells were mounted using Prolong Gold antifade reagent with 4',6'-diamidino-2-phenylindole (DAPI; Invitrogen).

### 2.3 Confocal microscopy and image analysis

Specimens were analyzed by confocal microscopy using a Leica SP5 or Nikon A1R instrument, and the results were compiled using ImageJ software (Abramoff, Magalhães, and Ram 2004).

### 2.4 DNA sequence alignment

Multiple DNA sequence alignments were made using MAFFT online version 7 (Katoh, Rozewicki, and Yamada 2019), and then processed using MEGA X software (S et al. 2018) (https://www.megasoftware.net/).

### 2.5 Production of CyHV-3 FL BAC plasmids and viruses

CyHV-3 strain FL was isolated in Belgium from a fish that died from CyHV-3 infection and used to produce the FL BAC plasmid (Costes et al. 2008), in which the BAC vector is inserted at the 3' end of ORF55, which encodes Thymidine Kinase (TK). The BAC vector includes an EGFP expression cassette. Consequently, viruses reconstituted from FL BAC plasmids without provision of a means for reverting the ORF55 locus to wild type (WT) express a

truncated TK from the viral sequence and EGFP from the BAC vector (which is retained in the viral genome). TK truncation was previously shown to have no effect on CyHV-3 growth *in vitro* but reduces virulence slightly *in vivo* (Costes et al. 2008). The FL BAC plasmid also encodes a truncated ORF27 and the ORF131 183A allele. FL BAC plasmid derivatives were produced using two-step positive/negative selection of the galactokinase gene (*galK*) in bacteria, and recombinant viruses were generated by co-transfection of these plasmids and, where necessary, recombination cassettes into CCB cells (Boutier, Ronsmans, Ouyang, et al. 2015). The strategy is outlined in Figs. 2A and 5A, and the primers used are listed in Table 1.

*Production of the FL rev and FL rev WT ORF27 viruses*. As shown in Fig. 2A, the truncated ORF27 encoded by the FL BAC plasmid was replaced by the WT ORF27 encoded by CyHV-3 strain M3 to generate the FL BAC ORF27 WT BAC plasmid. The FL BAC and FL BAC ORF27 WT BAC plasmids were then co-transfected into CCB cells together with the pGEMT-TK vector, as described previously (Costes et al. 2008), to generate the FL rev and FL rev WT ORF27 viruses encoding WT ORF55.

*Production of the FL EGFP rec ORF131-A and FL mCherry rec ORF131-A viruses*. As shown in Fig. 2A, the FL EGFP rec ORF131-A virus was produced by transfecting the FL BAC plasmid into CCB cells. The FL mCherry rec ORF131-A virus was produced by co-transfecting the FL BAC plasmid together with a recombination cassette to replace the EGFP coding region by a mCherry coding region. The resulting FL mCherry rec ORF131-A virus was plaque-purified on the basis of red fluorescence, as described previously (Boutier, Ronsmans, Ouyang, et al. 2015).

*Production of the FL EGFP rec ORF131-T and FL mCherry rec ORF131-T viruses*. As shown in Fig. 2A, these viruses were produced by co-transfection of the FL BAC ORF131 Del *galK* plasmid, which is deleted for ORF131 and thus unable to reconstitute virus

(Vancsok et al. 2017), together with recombination cassettes. Thus, the FL EGFP rec ORF131-T virus was generated using the ORF131-183T cassette, which consisted of the ORF131 183T allele (amplified from CyHV-3 strain E) flanked by 500 bp upstream and downstream of ORF131 in CyHV-3 strain FL. This virus was modified further to produce the FL mCherry rec ORF131-T virus by using a mCherry cassette to replace the EGFP ORF. The mCherry rec ORF131-T virus was plaque-purified on the basis of red fluorescence, as described previously (Boutier, Ronsmans, Ouyang, et al. 2015).

*Production of the FL rev ORF131-A and FL rev ORF131-T viruses*. As shown in Fig. 5A, these viruses were produced by co-transfection of the FL BAC or FL BAC ORF131 Del *galK* plasmid and the recombination cassettes described above targeting ORF131 and ORF55.

### 2.6 Genetic characterization of CyHV-3 recombinants

The molecular structures of all recombinant strains were confirmed by monitoring SacI restriction fragment length polymorphism (RFLP) by agarose gel electrophoresis and full-length genome sequencing as described previously (Boutier, Ronsmans, Ouyang, et al. 2015).

### 2.7 Multistep growth curves

Triplicate cultures of CCB cells were infected at a multiplicity of infection (MOI) of 0.05 plaque forming unit (PFU)/cell. After an incubation period of 2 h, the cells were washed with PBS and overlaid with Dulbecco's modified essential medium (DMEM, Sigma) containing 4.5 g glucose/L and 10% (v/v) FCS. The supernatant was removed from the infected cultures at successive intervals and stored at -80 °C. Infectious virions were titrated by duplicate plaque assay in CCB cells as described previously (Ouyang et al. 2013).

### 2.8 Plaque size assay and syncytial plaque assay

CCB cells grown in 6-well plates were infected with CyHV-3 strains at 200 PFU/well. After an incubation period of 2 h, the cells were overlaid with DMEM containing 10% FCS (v/v) and 1.2% (w/v) carboxymethylcellulose (CMC; medium viscosity; Sigma) in order to obtain isolated plaques (Vanderplasschen et al. 1993). At various times postinfection (PI), the cells were fixed in PBS containing 4% PAF at 4 °C for 15 min and then 20 °C for 10 min. Images of the monolayers were captured using a Nikon A1R epifluorescence microscope. Plaque size was determined by measuring the area of 20 independent plaques in duplicate wells using ImageJ software (Abramoff, Magalhães, and Ram 2004). Syncytium formation was quantified by examining 100 plaques in triplicate wells.

### 2.9 Live-cell imaging and analysis

CCB cells cultured in 24-well plates were imaged hourly or every 3 h for 7 d using an IncuCyte Zoom HD/2CLR time-lapse microscopy system (Sartorius) equipped with an IncuCyte Zoom 20× Plan Fluor objective (Sartorius). Images were collected in phase contrast and in the green (EGFP) and red (mCherry) channels. Stacks of images were exported in tagged image file format (TIF) using the time plot function. Fluorescence analysis was adapted to set the basic analytical parameters with adaptive segmentation and a threshold adjustment (GCU) of 20,000 (https://ki.se/en/media/111654/download).

### 2.10 Fish

Common carp (*Cyprinus carpio carpio*) and koi carp (*Cyprinus carpio koi*) were kept in 60 L tanks at 24 °C. Water parameters were checked twice per week. Microbiological, parasitic and clinical examinations were conducted immediately prior to the experiments to ensure that the

fish were healthy. All experiments were preceded by an acclimatization period of at least 2 weeks.

### 2.11 Infection of fish with CyHV-3

Infection was carried out either by immersion of uninfected fish in water containing virus or by cohabitation of uninfected fish with infected fish. Fish were inoculated by immersion by placing them in water containing virus for 2 h under constant aeration, with the volume of water adjusted to the number and size of fish to give a biomass of around 10%. At the end of the incubation period, the fish were returned to 60 L tanks. For inoculation by cohabitation, infected fish were released into a tank of uninfected fish at a ratio of 4 infected fish per 20 uninfected fish (Fig. 5C). After a co-habitation period of 2 d, the primary infected fish were removed from the tank.

### 2.12 Ethics statement

The experiments, maintenance and care of fish complied with the guidelines of the European Convention CETS 123. The animal studies were approved by the local ethics committee of the University of Liège, Belgium (laboratory accreditation no. 1610008, protocol no. 2130). All efforts were made to minimize suffering.

### 2.13 Quantification of viral genome copies in fish organs by real-time PCR

Viral genome copies were quantified by real-time TaqMan qPCR, as described previously (Boutier, Ronsmans, Ouyang, et al. 2015), by amplifying fragments of CyHV-3 ORF89 and the carp glucokinase gene. The limit of detection was defined as the mean Cq for no template control (NTC) samples minus one standard deviation (SD). Reactions were performed using a

CFX96 Touch real-time PCR detection system (Bio-Rad) with detection in the FAM channel, and analysis was performed using CFX Manager 3.0 software (Bio-Rad). The primers and probes used are listed in Table 1.

### 2.14 RNase H-dependent PCR (rhPCR) for SNP genotyping

The rhAmp SNP genotyping system (Integrated DNA Technologies) was used with plasmids encoding the ORF131-A or ORF131-T allele as references. Primers used for construction of these plasmids and for rhPCR are listed in Table 1. The standard protocol (www.idtdna.com/rhAmp-SNP-protocol) was used for rhPCR in a 5 µL volume, with modifications to improve allele specificity (Fig. S1). A target enrichment step was used prior to rhPCR to aid the relative quantification of alleles in samples with low viral content (Fig. S1). The limit of detection was defined as the mean Cq for NTC samples minus one SD. The rhPCR assays were performed using a CFX96 Touch real-time PCR detection system with detection in the FAM (for ORF131-A) and HEX (for ORF131-T) channels, and the data were analysed using CFX Manager 3.0 software. The primers used are listed in Table 1.

### 2.15 *In vivo* bioluminescence imaging

Firefly luciferase was imaged using an IVIS Spectrum *in vivo* imaging system (PerkinElmer), as described previously (Boutier, Ronsmans, Ouyang, et al. 2015; Costes et al. 2009). Fish were analyzed *in vivo* lying on their right and left sides and *ex vivo* after euthanasia. Dissected organs were analyzed independently of the body. Images were acquired using a field view of C, a maximum auto-exposure time of 1 min, a binning factor of 4, and an f/stop of 1. The relative intensities of transmitted light from bioluminescence and scales were determined automatically and represented as a pseudo-color image ranging from violet (least intense) to

red (most intense) using Living Image 4.7.3 software. Regions of interest (ROIs) were drawn manually by surrounding the organs or body outline, and the average radiance (p/sec/cm²/sr) was taken as the final measure of the bioluminescence emitted over the ROI.

### 2.16 Statistical analysis

Syncytium quantification results (Fig. 1B and 2B) were analyzed using one-way ANOVA, and multiple comparisons between groups of interest were made using a post-hoc pairwise Tukey test. Results for viral growth (Fig. 2D), plaque size (Fig. 2E), *in vitro* competition (Fig. 2F), virion infectivity (Fig. 4B-G), viral load as measured by qPCR (Fig. 5D) and bioluminescence measurements (Fig. 6B) were analyzed using two-way ANOVA, and multiple comparisons between groups of interest were made using a post-hoc pairwise Tukey test. Survival curves (Fig. 5B) were compared using Logrank tests. These analyses were done using Graphpad Prism 8. The number of fish positive for each ORF131 allele (Fig. 5D) was compared by the Durbin rank sum test (Durbin 1951), and comparison between groups of interest was made using a post-hoc pairwise Wilcoxon test (with p-values adjusted using the Benjamini-Hochberg method) implemented in R using the R core stats package ("R Core Team (2020). — European Environment Agency" n.d.) and the PMCMR package (Pohlert 2015). The variables used for each omnibus test and multiple comparisons selected for statistical illustrations are described in the figures, in which statistical significance for all tests is represented by the following symbols: *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$. Non-significant results are represented by the ns symbol, with the exception of results from post-hoc multiple comparisons, where, for the purposes of visual clarity, they are represented by the absence of any symbol.

## Table 1 Oligonucleotide primers.

| | Primer name | Sequence (5'- 3') | Coordinates*/ GenBank accession no. |
|---|---|---|---|
| **Synthesis of recombination cassettes** | | | |
| **Cassette name** | | | |
| ORF 27 Del *galK* | ORF27 *galK* F | CAGTGTCAAAGAATCATTTTTC TTGACCTGGTAGACTTTTTGTC TGTGTGTACCCCGGTGAATCGT GGTCATATC*CCTGTTGACAATT AATCATCGGCA* | 48459-48533 |
| | ORF27 *galK* R | GGTCCTCACCCGAGTACAAGAC CAAGCTGTACCCGGGATGGAAG GCGCCGCACAGCTGGGCCTCGG CCGTCGAGA*TCAGCACTGTCCT GCTCCTT* | 50155-50229 |
| ORF 131 Del *galK* | ORF131 *galK* F | GTGAGGGAGTGATATGGAGTGA ACGTAAATGGAGGGGCGCTGCG GAGGTT*CCTGTTGACAATTAAT CATCGGCA* | 225410–225484 |
| | ORF131 *galK* R | TCGAGACGCCCGAACTGGTCGA GGCCTACGTGAACGACGTCAAG GTCCGCTCAGCACTGTCCTGCT CCTT | 226426–226352 |
| mCherry | mCherry F | CTTGTACAGCTCGTCCATGC | |
| | mCherry R | ATGGTGAGCAAGGGCGAGGA | |
| ORF 131-183T | ORF131-ex-F | CAGCCTGAAGACGATGTCC | 225010-225028 |
| | ORF131-ex-R | AGAGTTTCATGAGACGCCAAA | 226851-226871 |
| **qPCR analysis** | | | |
| **Gene amplified** | | | |
| CyHV-3 ORF89 | KHV-86F | GACGCCGGAGACCTTGTG | AF411803 |
| | KHV-163R | CGGGTTCTTATTTTTGTCCTTG TT | |
| | KHV-109P | (6FAM) CTTCCTCTGCTCGGCGAGCACG (BHQ1) | |
| Carp glucokinase | CgGluc-162F | ACTGCGAGTGGAGACACATGAT | AF053332 |
| | CgGluc-230R | TCAGGTGTGGAGCGGACAT | |
| | CgGluc-185P | (6FAM) AAGCCAGTGTCAAAATGCTGCC CACT (BHQ1) | |
| **Allele genotyping** | | | |
| CyHV-3 ORF131 | ORF131-5F | GTCGGGAGTGGAGTGGTG | 225986-226004 |
| | ORF131-5R | CGCAATTTACTGCCATGTGT | 226451-226470 |
| CyHV-3 ORF131 SNP | ORF131-A-Rev | /rhAmp-F/AGGAAGAGTTCCTGATACAG GrCCGCC/GT2/# | 226229-226248 |
| | ORF131-T-Rev | /rhAmp-Y/AGGAAGAGTTCCTGATACAG ArCCGCC/GT2/# | 226229-226248 |
| | ORF131-Fw | GCCGACGTTGATGGTGATGATr GGGTG/GT2/# | 226141-226159 |

*Coordinates based on the reference CyHV-3 genome (GenBank accession no. DQ657948.1)

Underlined: sequence not corresponding to the CyHV-3 genome

Italic: sequence corresponding to *galK*

#Allele-specific primers: The extended 5' sequence of ORF131-A-Rev primer (/rhAmp-F/) is complementary to a universal forward primer and FAM-labelled universal probe for detection of the ORF131A allele. The extended 5' sequence of the ORF131-T-Rev primer (/rhAmp-Y/) is complementary to a universal forward primer and a Yakima Yellow-labelled universal probe (detected in HEX channel) for detection of the ORF131T allele. GT2 acts a blocking moiety, r denotes an RNA base.

## 3. RESULTS

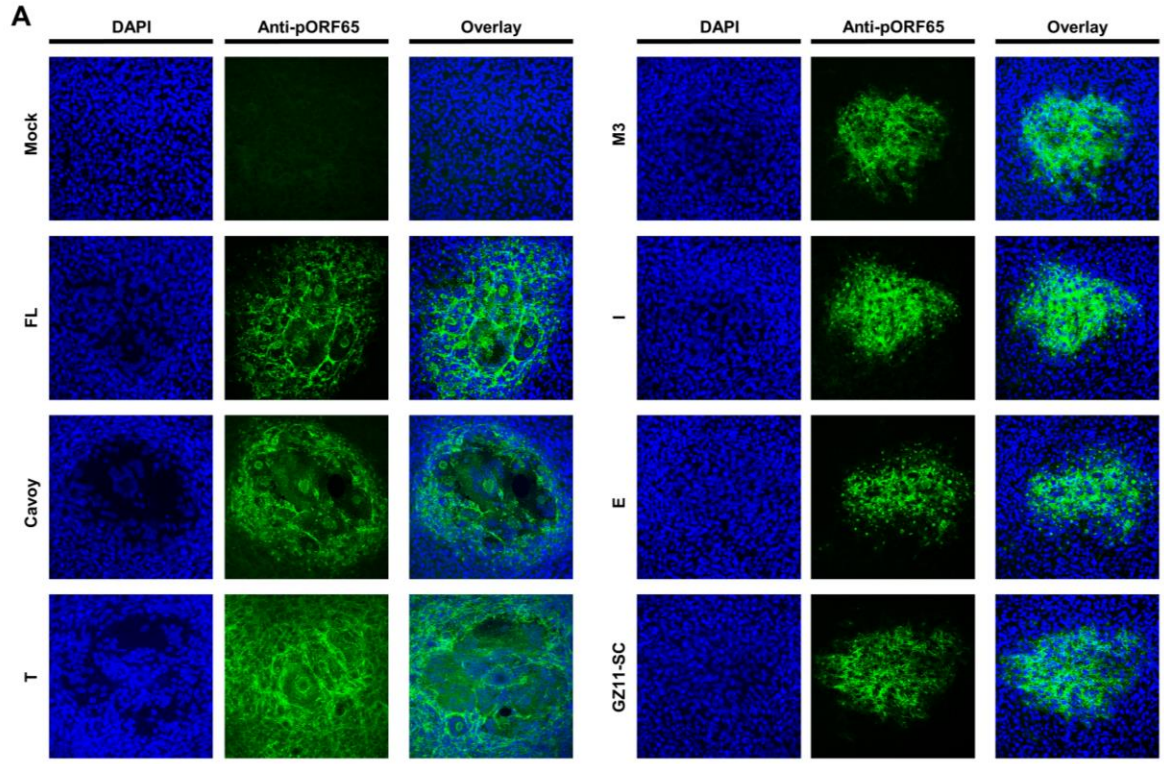
### 3.1 CyHV-3 strains can be classified into two groups on the basis of syncytial plaque formation

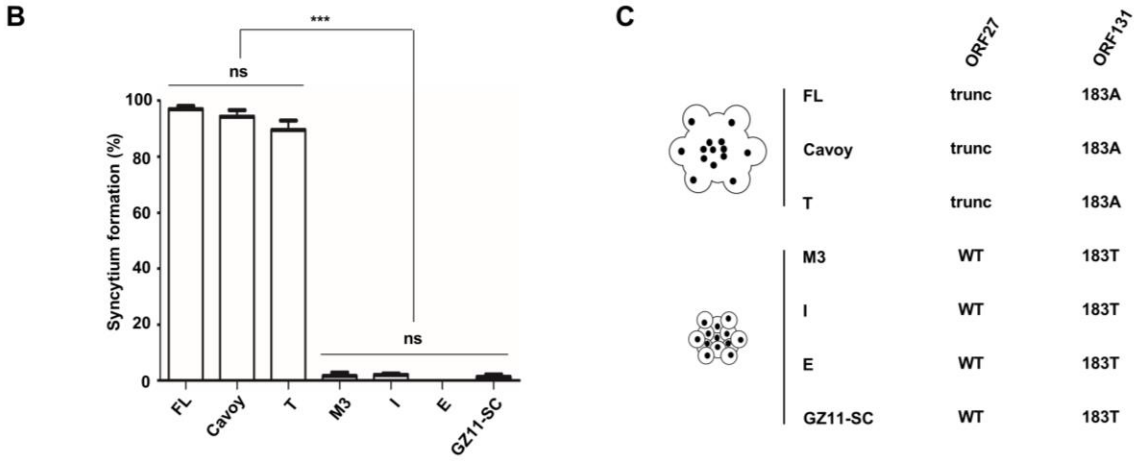
Recently, we performed genomic and biologic comparisons of seven CyHV-3 strains that represent all known CyHV-3 clades (Gao et al. 2018). Comparison of the properties of these strains *in vitro* and *in vivo* revealed a negative correlation between viral growth *in vitro* and virulence *in vivo*. The FL, Cavoy and T strains were the most fit in cell culture but the least virulent *in vivo*. The opposite was the case for the M3, I, E and GZ11-SC strains. In the present study, we compared the viral plaque phenotypes of these strains (Fig. 1A and B). In contrast to the strains that were more virulent *in vivo*, the strains adapted to cell culture formed syncytial plaques. Typically, syncytial plaque formation involves fusogenic activity resulting directly or indirectly from cell surface expression of viral glycoproteins (Kim et al. 2017; Compton and Schwartz 2017; Kuny et al. 2020). Therefore, as a means of identifying genetic traits linked to syncytial plaque formation, we compared the sequences of the predicted transmembrane proteins encoded by each strain. This led to the identification of two candidate loci (Fig. 1C). Each syncytial strain encodes a unique, mutated form of ORF27 resulting in a truncated protein (Fig. 1D). In addition, each syncytial strain has the same SNP in ORF131 (183A), whereas the non-syncytial strains have 183T (Fig. 1C and E). Sequence alignments indicated that 183A in the syncytial strains did not have a monophyletic origin and did not emerge by inter-strain recombination. Since we had demonstrated previously that the FL, Cavoy and T strains were the most fit in cell culture (Gao et al. 2018), we reasoned that syncytial plaque formation may represent a form of adaptation to this environment, as has been reported recently for herpes simplex virus type 1 (HSV-1), a member of the family

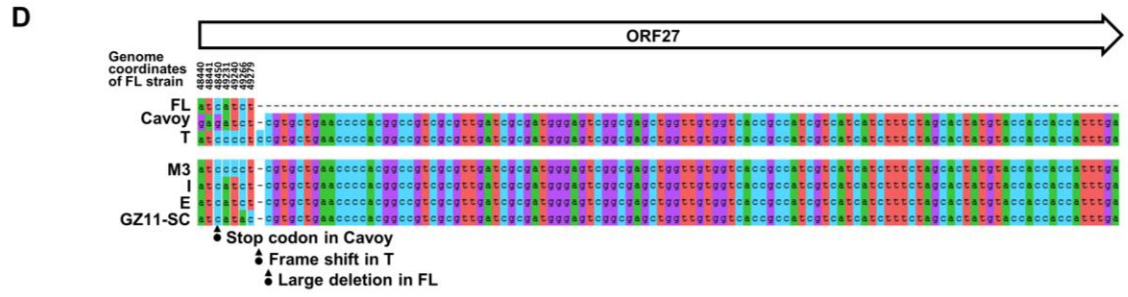
*Herpesviridae* (Kuny et al. 2020). The results presented above demonstrate that CyHV-3 strains can be classified into two groups on the basis of their ability to form syncytial plaques in cell culture. Sequence analysis suggested that the genetic determinants of syncytial plaque formation are located in ORF27 or ORF131 or both, and that they arose independently in each strain.

### 3.2 The ORF131 183A allele determines syncytial plaque formation

Next, we used recombination technologies with the FL BAC plasmid of the CyHV-3 genome to investigate the contribution of the ORF27 and ORF131 mutations to the syncytial phenotype. This plasmid and the virus (FL rev; Fig. 2A) produced from it by transfection encode a truncated ORF27 and the ORF131 183A allele. Using this plasmid, we produced a second virus (FL rev WT ORF27; Fig. 2A), in which ORF27 had been reverted to WT. Like FL rev, this virus produced syncytia in cell culture (Fig. 2B), thus demonstrating that the truncation in ORF27 was not essential for syncytial plaque formation. We then produced four more viruses (FL EGFP rec ORF131-A, FL mCherry rec ORF131-A, FL EGFP rec ORF131-T and FL mCherry rec ORF131-T; Fig. 2A) to investigate the role of the ORF131 SNP in syncytial plaque formation. These viruses encoded a truncated ORF27, a fluorescent reporter cassette (EGFP or mCherry, exhibiting green or red fluorescence, respectively; Fig. 2C) and the ORF131 183A or 183T allele. The two strains with the 183A allele formed syncytia, whereas the two strains with the 183T allele did not (Fig. 2B). These results demonstrated that the ORF131 183A allele is responsible for syncytial plaque formation.


A
DAPI
Anti-pORF65
Overlay
Mock
FL
Cavoy
T
M3
I
E
GZ11-SC


B
***
ns
ns
Syncytium formation (%)
100
80
60
40
20
0
FL
Cavoy
T
M3
I
E
GZ11-SC
C
ORF27
ORF131
FL trunc 183A
Cavoy trunc 183A
T trunc 183A
M3 WT 183T
I WT 183T
E WT 183T
GZ11-SC WT 183T


D
ORF27
Genome coordinates of FL strain
FL
Cavoy
T
M3
I
E
GZ11-SC
Stop codon in Cavoy
Frame shift in T
Large deletion in FL

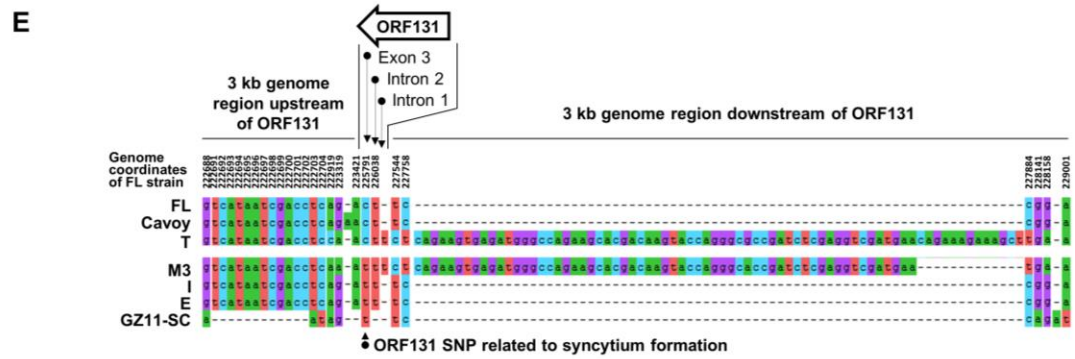
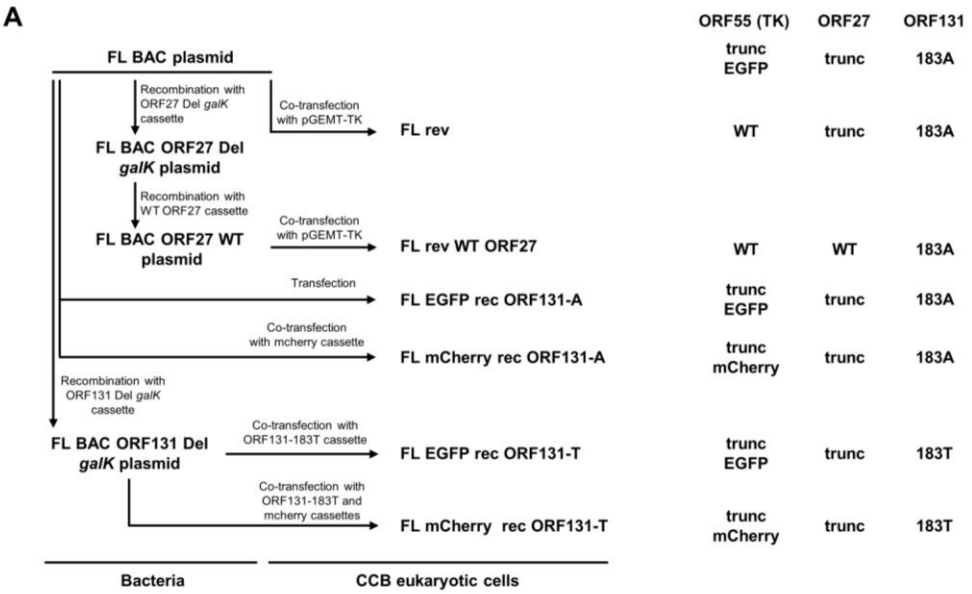

E
ORF131
Exon 3
Intron 2
Intron 1
3 kb genome region upstream of ORF131
3 kb genome region downstream of ORF131
Genome coordinates of FL strain
FL
Cavoy
T
M3
I
E
GZ11-SC
ORF131 SNP related to syncytium formation

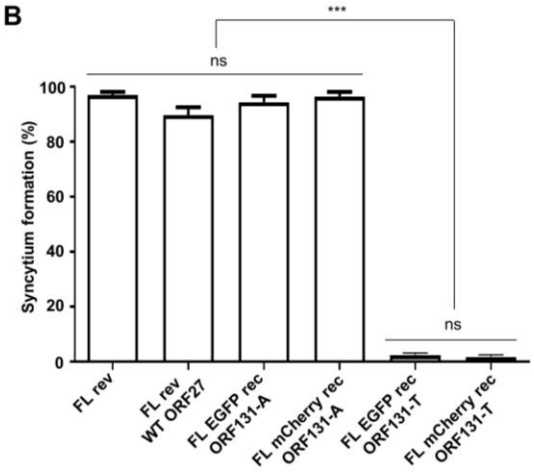
**FIG 1 Viral plaque phenotypes and potential genetic determinisms**.

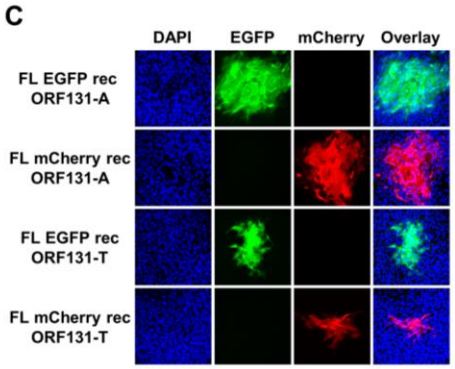
(A) Viral plaque phenotyping. CCB cells were mock-infected or infected with the CyHV-3 strains indicated and overlaid with CMC in order to obtain isolated plaques. At 3 d PI, the cell monolayers were stained with DAPI and immunostained with an anti-pORF65 monoclonal antibody. Representative plaques are shown. Each panel corresponds to an area of 387.5 $\mu m^2$ from each specimen. (B) Quantification of syncytial plaque formation. The percentage of syncytial plaques was quantified at 3 d PI. The data represent the mean + SEM of triplicate measurements of 100 plaques. One-way ANOVA analysis indicated that the strain (***) had a significant impact on phenotypic observations. (C) ORF27 and ORF131 genotypes of the strains tested. trunc: truncation. (D) Multiple sequence alignment of ORF27 encoded by CyHV-3 strains. The genome of the FL strain was used as reference (MG925487.1). (E) Multiple sequence alignment of ORF131 and flanking regions encoded by CyHV-3 strains. The SNP correlated with syncytial plaque formation is indicated.

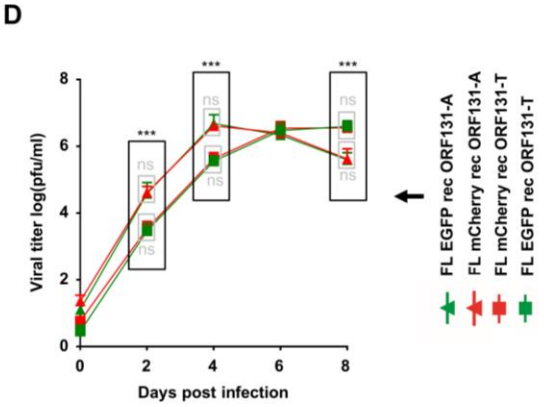
### 3.3 The ORF131 183A allele confers higher fitness *in vitro* than the 183T allele

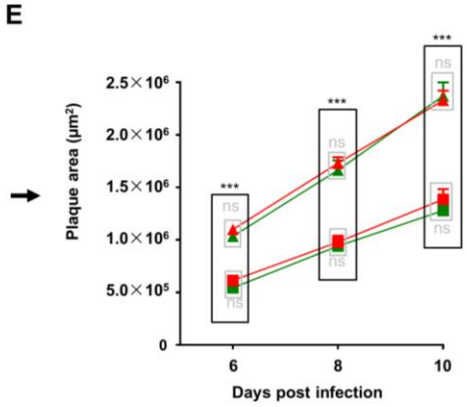
The differences in growth between CyHV-3 strains in cell culture (Fig. 1C of the present study and Fig. 3 of our previous study (Gao et al. 2018)), and the further genetic characterization of these strains in the present study, suggested that the ORF131 183A allele confers higher fitness in cell culture relative to the 183T allele. To test this hypothesis, the four viruses mentioned above were assessed by multistep viral growth assay and plaque size assay (Fig. 2D and E). Independent of the fluorescent reporter gene, viruses encoding the 183A allele exhibited more efficient replication (Fig. 2D) and formed larger plaques (Fig. 2E). Moreover, viruses expressing the same ORF131 allele but encoding different fluorescent reporter genes exhibited similar viral growth properties and plaque sizes. These results suggest that the ORF131 183A allele present in CyHV-3 strains adapted to cell culture represents a mutant that is selected *in vitro* and outcompetes the parental virus with the 183T allele. To test this hypothesis, FL mCherry rec ORF131-A and FL EGFP rec ORF131-T were used as a mixture to co-infect CCB cells at MOIs of 0.01 and 0.09, respectively (Fig. 2F). Despite the initial bias in favor of the virus encoding the ORF131 183T allele, the relative proportion of the virus encoding the ORF131 183A allele increased progressively during


A
ORF55 (TK)
ORF27
ORF131
FL BAC plasmid
trunc EGFP
trunc
183A
Recombination with ORF27 Del galK cassette
Co-transfection with pGEMT-TK
FL rev
WT
trunc
183A
FL BAC ORF27 Del galK plasmid
Recombination with WT ORF27 cassette
FL BAC ORF27 WT plasmid
Co-transfection with pGEMT-TK
FL rev WT ORF27
WT
WT
183A
Transfection
FL EGFP rec ORF131-A
trunc EGFP
trunc
183A
Co-transfection with mcherry cassette
FL mCherry rec ORF131-A
trunc mCherry
trunc
183A
Recombination with ORF131 Del galK cassette
FL BAC ORF131 Del galK plasmid
Co-transfection with ORF131-183T cassette
FL EGFP rec ORF131-T
trunc EGFP
trunc
183T
Co-transfection with ORF131-183T and mcherry cassettes
FL mCherry rec ORF131-T
trunc mCherry
trunc
183T
Bacteria
CCB eukaryotic cells


B
***
ns
ns
Syncytium formation (%)
100
80
60
40
20
0
FL rev
FL rev WT ORF27
FL EGFP rec ORF131-A
FL mCherry rec ORF131-A
FL EGFP rec ORF131-T
FL mCherry rec ORF131-T


C
DAPI
EGFP
mCherry
Overlay
FL EGFP rec ORF131-A
FL mCherry rec ORF131-A
FL EGFP rec ORF131-T
FL mCherry rec ORF131-T


D
Viral titer log(pfu/ml)
8
6
4
2
0
0
2
4
6
8
Days post infection
***
ns
FL EGFP rec ORF131-A
FL mCherry rec ORF131-A
FL mCherry rec ORF131-T
FL EGFP rec ORF131-T


E
Plaque area (μm²)
2.5×10⁶
2.0×10⁶
1.5×10⁶
1.0×10⁶
5.0×10⁵
0
6
8
10
Days post infection
***
ns

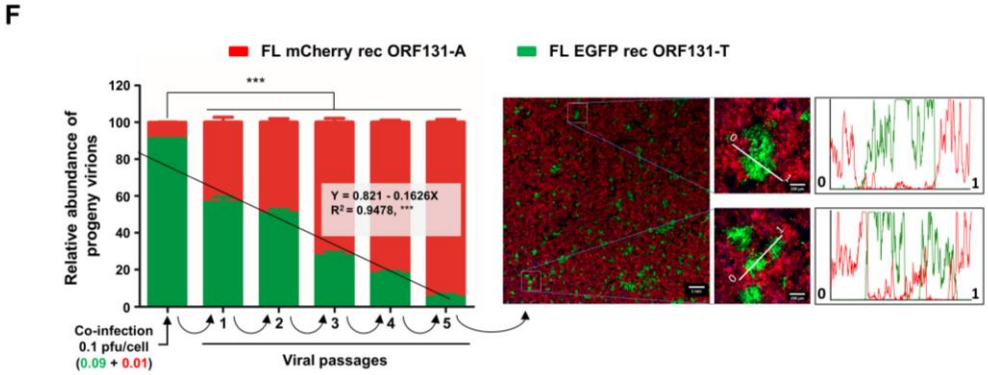

F
FL mCherry rec ORF131-A
FL EGFP rec ORF131-T
Relative abundance of progeny virions
120
100
80
60
40
20
0
***
Y = 0.821 - 0.1626X
R² = 0.9478, ***
Co-infection
0.1 pfu/cell
(0.09 + 0.01)
1
2
3
4
5
Viral passages

**FIG 2 Genetic determinism of syncytial plaque formation.**

(A) Flow chart of the production CyHV-3 recombinant viruses. Each virus was assigned the name indicated in the middle part of the panel. The right part of the panel summarizes the genotypes of ORF55, ORF27 and ORF131. WT, wild type; Del, deleted; trunc, truncated. (B) Quantification of syncytial plaque formation. The percentage of syncytial plaques was quantified at 3 d PI. The data represent the mean + SEM of triplicate measures of 100 plaques. One-way ANOVA analysis indicated that the virus (***) had a significant impact on phenotypic observations. (C) Plaque phenotyping. CCB cells were infected with the viruses indicated. At 3 d PI, the cell monolayers were stained with DAPI and observed by epifluorescence microscopy. Representative plaques are shown in image panels corresponding to an area of 387.5 µm$^2$. (D) Multistep viral growth assay. CCB cells were infected with the strains indicated at a MOI of 0.05, and virus in the cell supernatant was titrated at the time points PI indicated. Data are presented as the mean + SEM of triplicate measurements. Two-way ANOVA analysis indicated that the virus (***) and the time PI (***) had significant impacts on titer. (E) Plaque size assay. CCB cells were infected as described in Materials and Methods, and plaque areas were measured at the indicated time points PI. Data presented are the mean + SEM for duplicate measurements of 20 randomly selected isolated plaques. Two-way ANOVA analysis indicated that genotype (***) and time PI (***) had significant impacts on plaque size. (F) Growth competition in cell culture. CCB cells were co-infected in triplicate with the two viruses indicated (MOIs of 0.01 and 0.09 for FL mCherry rec ORF131-A and FL EGFP rec ORF131-T, respectively). At 48 h PI, the cell supernatants were used to inoculate fresh CCB monolayers. Virus was thus passaged five times. The relative abundance of the two viruses in each sample was quantified by plaque assay as described in Materials and Methods. The results are presented as the mean relative proportion + SEM determined for each virus. Two-way ANOVA analysis indicated that virus (***) and passage number (***) had significant impacts on the proportion of each virus. Linear regression and correlation analysis for the reduction of FL EGFP rec ORF131-T according to passage number is shown in the left part of the panel. The right part of the panel presents an epifluorescence image with the overlay of DAPI, mCherry and EGFP channels from one of the monolayers at the fifth passage. Scale bar, 1 mm. Two randomly selected ROIs were magnified, and intensity plots for the two channels were determined along the lines indicated. Scale bar, 200 µm.

passage, representing the vast majority of virus at passage 5. We also observed that the infected cell monolayer at passage 5 essentially lacked cells co-infected with both viruses (i.e. yellow cells, expressing both reporters), even at the borders between areas of cells infected with the viruses expressing either mCherry or EGFP (Fig. 2F).

### 3.4 CyHV-3 inhibits superinfection *in vitro*

If superinfection (one virus entering a cell already infected with another) is not inhibited, most co-infected cells should arise by this means, simply because such events should occur more frequently than the simultaneous entry of two different viruses into the same cell. However, the results in Fig. 2F suggested that inhibition of superinfection by CyHV-3 occurs and that this is independent of the ORF131 allele encoded. To test this hypothesis, we performed the experiment described in Fig. 3. Cells were first co-infected by spinoculation with a pair of viruses expressing EGFP or mCherry and representing all possible combinations of the two ORF131 alleles (183A/183A, 183A/183T, 183T/183A and 183T/183T; Fig. 3A). In order to monitor the progeny originating from individual cells, infected monolayers were trypsinized at 24 h PI and used to seed uninfected cell cultures. The monolayers were then overlaid with CMC to induce the formation of plaques, and placed in a time-lapse microscopy system for long-term observation of epifluorescence due to expression of EGFP (green) or mCherry (red). Regardless of the combination of ORF131 alleles, the spread of virions from cells co-infected with both viruses (expressing both EGFP and mCherry and therefore yellow) did not lead to the formation of plaques consisting mainly of co-infected cells. On the contrary, it led to plaques consisting predominantly of a mosaic of singly infected cells (expressing either EGFP or mCherry and therefore green or red). A few co-infected cells were observed, and these were isolated within the mosaic plaques rather than concentrated where the zones expressing either EGFP or mCherry met (Movie S1). Moreover, the frequency of co-infected cells was systematically lower than the expected frequency under simulations of lack of inhibition of superinfection (Fig. 3C). These observations indicate that CyHV-3 infection *in vitro* is rapidly followed by inhibition of superinfection. This conclusion suggests that the small number of co-infected cells observed are the result of simultaneous infection, rather than superinfection. Inhibition of superinfection may contribute to the selection of alleles

conferring higher fitness in a population of genetically heterogeneous viruses. We also observed that, again regardless of the viruses involved, infected cells migrated within the monolayer during the early stage of infection (see the relative position of numbered cells in Fig. 3B in relation to time PI).

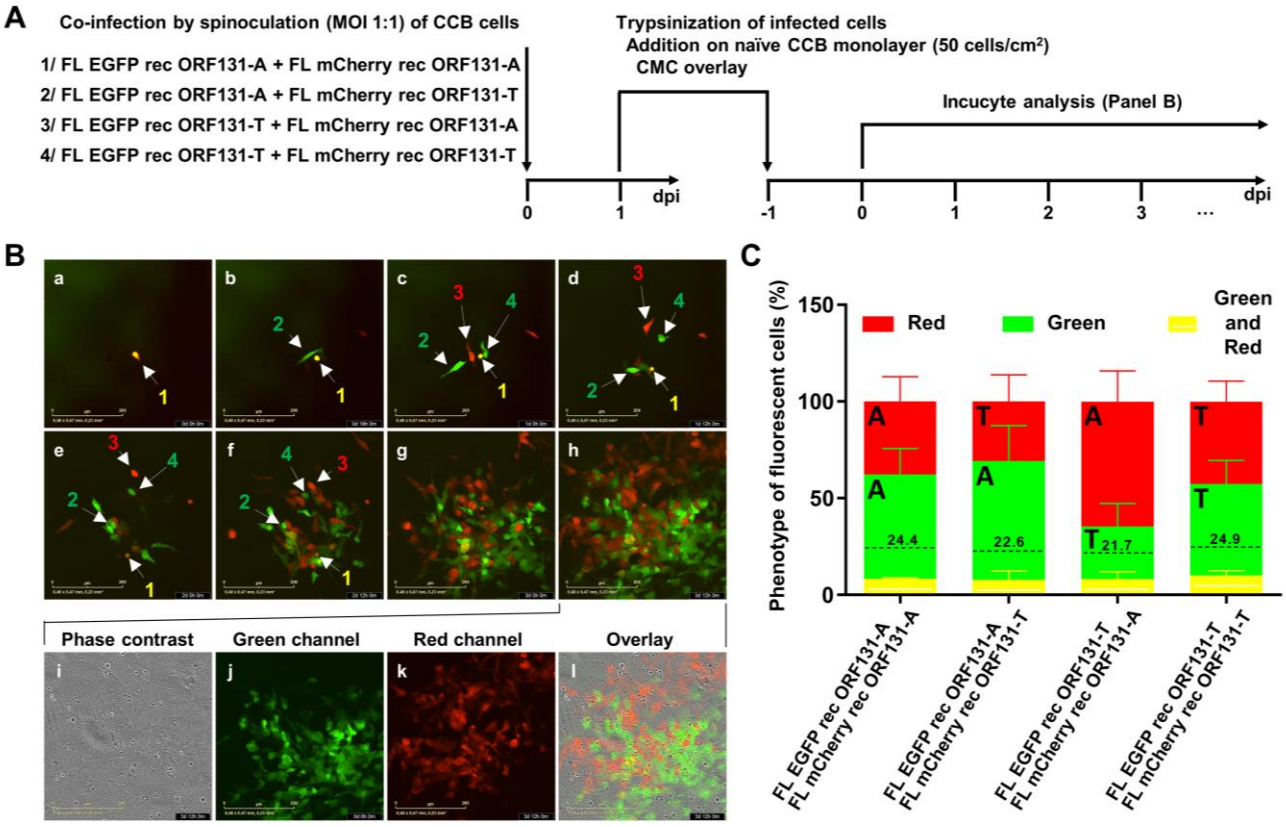


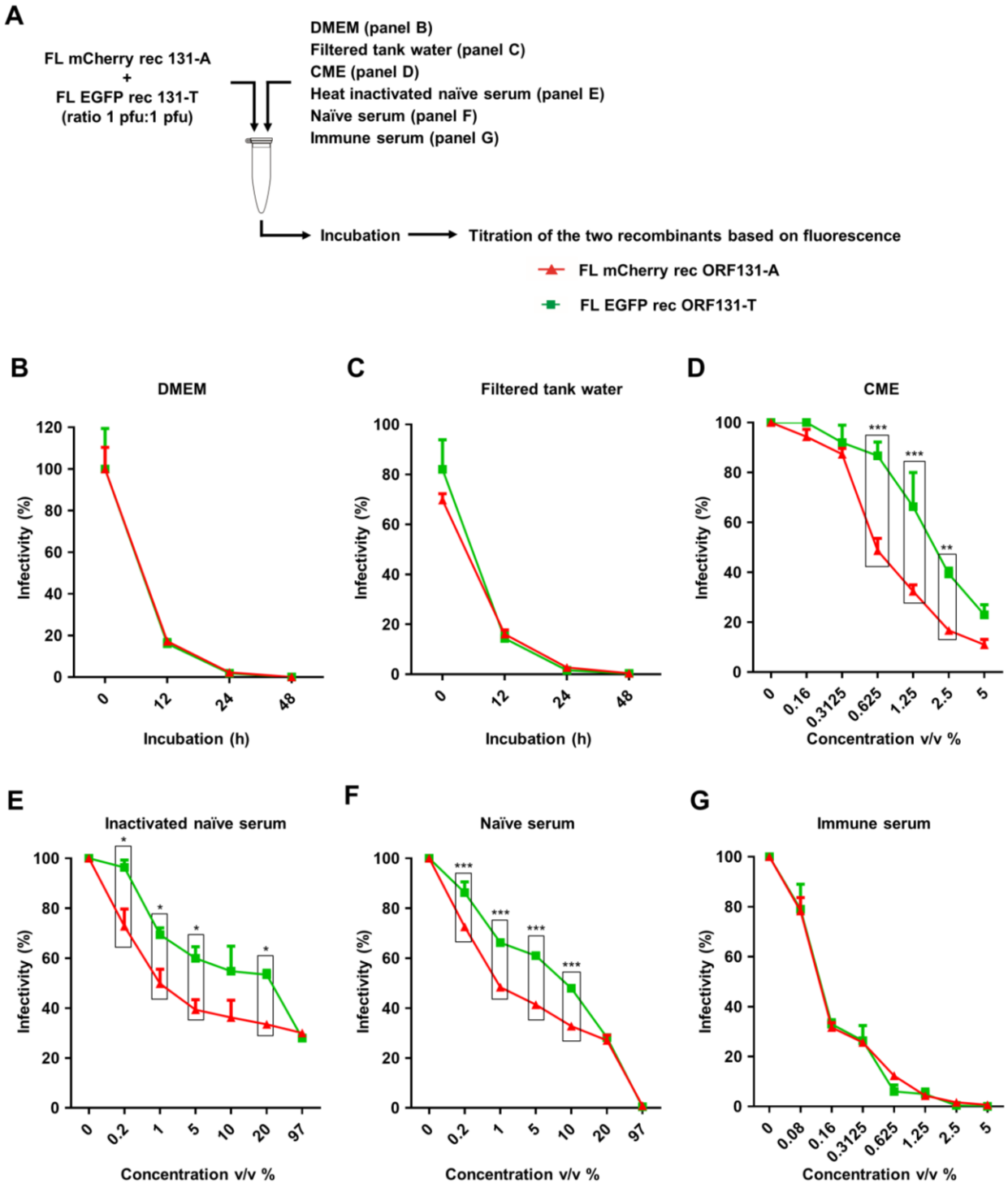

**FIG 3 Inhibition of superinfection *in vitro*.**

(A) Flow chart of the experiment. CCB cells were co-infected by spinoculation (1,000×g for 2 h at 25 °C) with the pairs of recombinants indicated (MOI of 1 for each recombinant). At 1 d PI, the cells were trypsinized and added to fresh CCB monolayers at a density of 50 cells/cm2, and then overlaid with CMC. Doubly infected cells (identified on the basis of expression of both mCherry and EGFP) and subsequent spread of viral infection were monitored every hour for 7 d by time-lapse microscopy. (B) Images of co-infection with FL EGFP rec ORF131-A and FL mCherry rec ORF131-A, which were typical of all four pairs of viruses. Images a-h illustrate the same area of the monolayer at the time points PI indicated. The numbers represent the order of appearance of cells expressing fluorescence, and the colour of the numbers correspond to the marker responsible (red, mCherry; green, EGFP; and yellow, both). Images i-l illustrate the monolayer in panel h presented in phase contrast, green

fluorescence, red fluorescence and an overlay. A movie covering the entire observation period is provided as supplemental material (Movie S1). (C) Relative proportions of singly and doubly infected cells in plaques derived from isolated cells infected with both viruses, according to the marker responsible (see above). A, ORF131-A allele; T, ORF131-T allele. The dotted lines represent the percentage of doubly infected cells expected in the absence of inhibition of superinfection. These values were calculated by multiplying the observed relative proportions of singly infected cells expressing EGFP or mCherry. The data represents the mean + SEM based on the analysis of three plaques, each containing 300 - 2,000 cells.

### 3.5 Effects of ORF131 alleles on virion stability

The observation that strains of CyHV-3 adapted to cell culture encode the ORF131 183A allele, whereas field strains encode the 183T allele, suggested that the former confers a higher fitness in cell culture and the latter confers a higher fitness *in vivo*. To further test the latter hypothesis and to understand the basis of the fitness, we investigated the effects of the alleles on virion stability and on virion resistance to neutralization by epidermal mucus and serum immune factors, using FL mCherry rec ORF131-A and FL EGFP rec ORF131-T. In order to avoid potential artefacts resulting from variations between virus preparations (e.g. relative abundance of cell debris or non-infectious particles), we used the approach depicted in Fig. 4. This had the benefit of facilitating the comparison of viruses encoding the two alleles after mixing them (Fig. 4A). Tests of virion stability in culture medium (DMEM) and filtered tank water revealed no significant difference between the viruses, and most of the infectivity was lost from both viruses by 12 h of incubation (Fig. 4B and C). In contrast, neutralization assays performed with clarified mucus extract (CME), heat-inactivated naïve serum or naïve serum revealed a higher resistance of virions encoding the 183T allele (Fig. 4D-F). However, a difference was not observed in neutralization assays performed using immune serum (Fig. 4G).




## FIG 4 Effects of ORF131 alleles on virion stability.

(A) Flow chart of the experiment. Viruses expressing red (ORF131-A) or green (ORF131-T) fluorescence were mixed at equivalent infectivity levels in a single tube and incubated at 25 °C in the indicated medium

compositions (panels B-G). Stability was measured with respect to negative controls i.e. 0 h incubation (panels B and C) or 0% v/v of the agent tested (panels D–G, 2 h incubation period). (C) Water taken from a mock-infected fish tank and sterilized by filtration through a 0.22 µm filter. (D) CME produced from uninfected fish, as described previously (Raj et al. 2011). The CME used contained 5.0 µg/µL protein. (E) Inactivated naïve carp serum prepared by heating naïve carp serum at 56 °C for 30 min. (F) Naïve carp serum. (G) CyHV-3 immune carp serum collected from fish vaccinated using an attenuated recombinant vaccine (Boutier, Ronsmans, Ouyang, et al. 2015). Data presented are the mean + SEM for triplicate measurements. Two-way ANOVA was used for statistical analysis, taking (B-C) ORF131 allele and incubation time or (D-G) ORF131 allele and agent concentration as variables. With the exception of genotype in panel B, C and G, all variables (***) were found to have a significant impact on virion stability, as measured by infectivity (%).

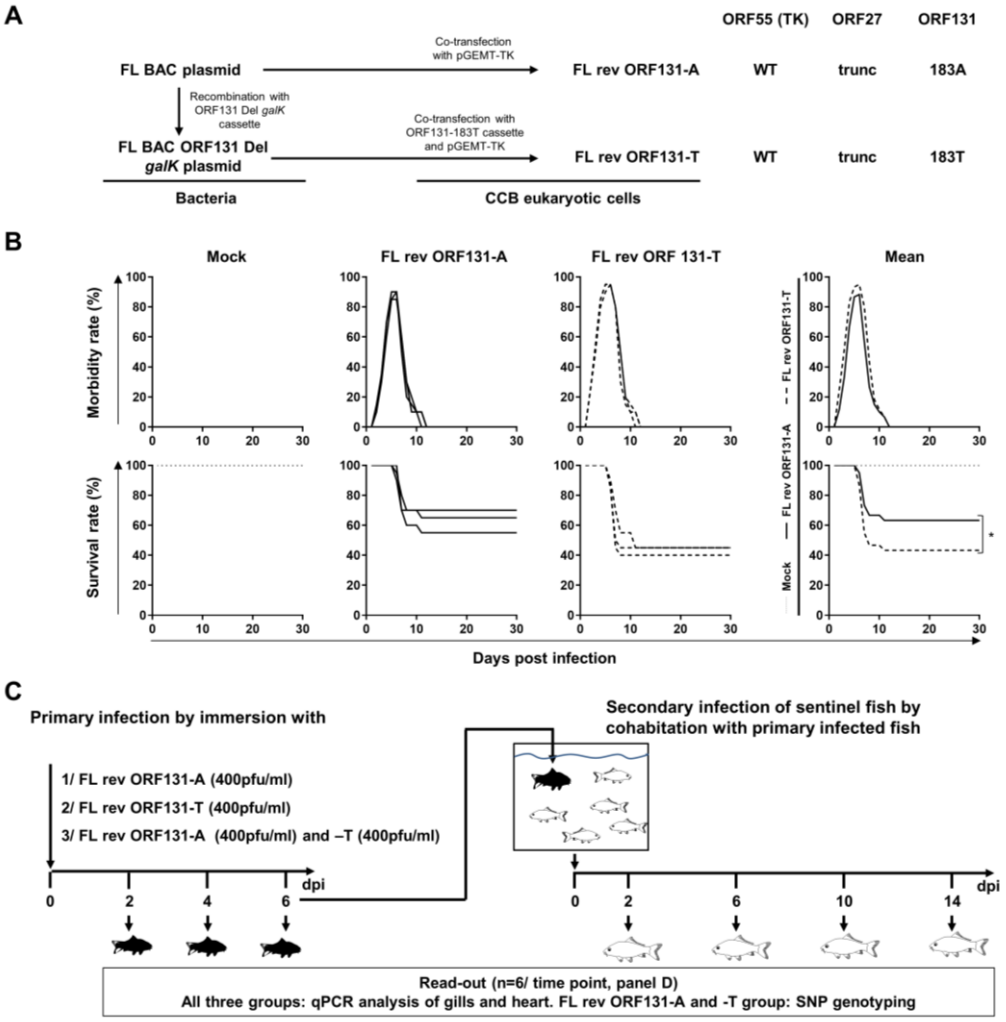
### 3.6 The ORF131 183T allele confers higher fitness *in vivo* than the 183A allele

Having investigated the relative fitness of each ORF131 allele *in vitro,* we subsequently also compared the relative fitness *in vivo*. All CyHV-3 recombinants used up to this point in the study encoded a truncated version of the TK gene. While this was previously shown to have no effect on the growth of CyHV-3 *in vitro,* it does reduce virulence slightly *in vivo* (Costes et al. 2008). Consequently, using the strategy described in Fig. 5A, we produced two viruses encoding WT ORF55 (encoding TK), each with a different version of the ORF131 allele (A or T), referred to as FL rev ORF131-T and FL rev ORF131-A respectively. The structures of the recombinants were confirmed by combined SacI RFLP and full-length genome sequencing (data not shown). The virulence of these two viruses was compared *in vivo* through inoculation of fish by immersion in water containing virus, thereby mimicking natural infection (Fig. 5B). In both infected groups, fish developed CyHV-3 disease, with the intensity and kinetics of appearance of clinical signs being similar for the two viruses. However, FL rev ORF131-T induced significantly higher mortality than FL rev ORF131-A (Fig. 5B). This suggested that the SNP in ORF131 that differentiates the two viruses also affects virulence *in vivo*. To test this hypothesis, we studied the virulence of the two viruses further by comparing viral loads in the fish by qPCR. Furthermore, transmission of the viruses

from infected fish to uninfected cohabitant sentinels was also investigated (Fig. 5C and D). In addition to comparing the viruses in separate infections conducted in parallel, we also investigated co-infections where fish were inoculated with equal amounts of both viruses, thus allowing the relative fitness of each virus *in vivo*, to be established. This necessitated the development of a method capable of specifically, sensitively and accurately quantifying the relative abundance of the viruses (which differ by a single nucleotide) in biological samples. To reach this goal, we developed an rhPCR assay that was capable of measuring the relative proportions of the viruses in a dynamic range of 10-90% (Fig. S1).

Fish infected by immersion with FL rev ORF131-T exhibited higher viral loads than fish infected with FL rev ORF131-A. Fish co-infected with both viruses expressed higher viral loads than fish infected with FL rev ORF131-A alone, and the loads were similar to those in fish infected with ORF131 183T alone (Fig. 5D, upper panel). The rhPCR analysis of co-infected fish for both organs tested (gills and heart) revealed that, for all but one fish, the vast majority, if not all, of the viral load consisted of FL rev ORF131-T (Fig. 5D, right column, two upper panels). No significant differences were observed between the primary infected groups in terms of the number of positive fish (Fig. 5D). These results indicate a higher fitness of the ORF131 183T allele *in vivo*.

Given the differences in stability that were observed in previous experiments (Fig. 4 D, E and F), we also investigated the ability of the two viruses to spread from infected fish to co-habitant uninfected fish. The groups of fish initially infected with FL rev ORF131-T or co-infected with a mixture of this virus and FL rev ORF131-A induced a higher number of positive fish than those infected with FL rev ORF131-A alone (Fig. 5D, lower panels). Analysis of infected co-habitant fish by rhPCR did not result in detection of the 183A allele. These results suggest that, although FL rev ORF131-A is able to replicate and transmit in the


A
ORF55 (TK)
ORF27
ORF131
FL BAC plasmid
Co-transfection with pGEMT-TK
FL rev ORF131-A
WT
trunc
183A
Recombination with ORF131 Del galK cassette
FL BAC ORF131 Del galK plasmid
Co-transfection with ORF131-183T cassette and pGEMT-TK
FL rev ORF131-T
WT
trunc
183T
Bacteria
CCB eukaryotic cells
B
Mock
FL rev ORF131-A
FL rev ORF 131-T
Mean
Morbidity rate (%)
Survival rate (%)
FL rev ORF131-T
FL rev ORF131-A
Mock
Days post infection
C
Primary infection by immersion with
1/ FL rev ORF131-A (400pfu/ml)
2/ FL rev ORF131-T (400pfu/ml)
3/ FL rev ORF131-A (400pfu/ml) and –T (400pfu/ml)
dpi
Secondary infection of sentinel fish by cohabitation with primary infected fish
dpi
Read-out (n=6/ time point, panel D)
All three groups: qPCR analysis of gills and heart. FL rev ORF131-A and -T group: SNP genotyping
D
FL rev ORF131-A
FL rev ORF131-T
FL rev ORF131-A + FL rev ORF131-T
A versus T: p(cop) ***; p(no) ns
A versus A+T: p(cop) ***; p(no) ns
T versus A+T: p(cop) ns; p(no) ns
A versus T: p(cop) **; p(no) *
A versus A+T: p(cop) ***; p(no) *
T versus A+T: p(cop) *; p(no) ns
Primary infection
Secondary infection
Gills
Heart
Viral genome copies/10⁶ carp glucokinase gene copies (log10)
Number of positive fish
SNP genotyping - percentage of T (%)
Days post infection

## FIG 5 Effects of ORF131 alleles on virulence.

(A) Flow chart illustrating the production of FL rev ORF131-A and FL rev ORF131-T. The right part summarizes the viral genotypes with respect to ORF55, ORF27, and ORF131. (B) Effect of ORF131 alleles on virulence. Viral virulence was tested in carp in triplicate groups, with each group containing 20 subjects (average weight 15.1g ± 3.8g). At day 0, the fish were infected for 2 h by immersion in water containing no (mock-infected) or 400 PFU/ml virus. The morbidity rate (top part) and survival rates (bottom part) were measured over a period of 30 d PI. Each condition (Mock, FL rev ORF131-A and FL rev ORF131-T) is presented separately in the first three columns by individual graphs. The last column summarizes the combined data from each condition by mean curves based on three replicates. Survival curves were compared using log-rank tests. (C) Flow chart illustrating the experiment to investigate ORF131 allele competition *in vivo*. This experiment consisted of duplicate groups per condition, with each group containing 20 subjects. The fish were infected by immersion for 2 h in water containing 400 PFU/ml FL rev ORF131-A or FL rev ORF131-T or both (black fish). At 6 d PI, the infected fish were used to infect sentinel naïve fish (white fish; duplicate groups containing 20 subjects). (D) Primary infected fish (top panel) and sentinel fish (secondary infection, bottom panel) were sampled (n=6) at the indicated times PI and analyzed by qPCR (three columns in the left part). Fish sampled from the co-infected groups were also analyzed by ORF131 allele genotyping (right column). For both qPCR and allele genotyping assays, the number of viral genome copies is expressed as $\log_{10}$ copies per $10^6$ carp glucokinase gene copies. Individual values represent the mean of duplicate measurements for each fish. Mock-infected fish were used as a negative control, and no viral genome copies were detected in these fish. The number of positive subjects among the six fish analyzed is represented by the grey bars. Given the limit of sensitivity of the allele assay, the dotted lines in the graphs in the third column represent the minimum detectable viral load ($4\times10^4$ initial copies per reaction) without the need for further target enrichment. Samples with viral loads lower than this threshold, were submitted to amplification of the region of ORF131 containing the SNP region by conventional PCR prior to allele genotyping (see Fig. S1 and Table S1). Statistical analysis was performed by combining data from both organs sampled (heart and gills). Combining data from both organs, two-way ANOVA analysis indicated that viruses (***) used and time PI (***) had significant impacts on viral load. Combining data from both organs and all times PI, post-hoc pairwise Tukey tests indicated there were significant differences in viral load based on which viruses were used to infect fish (p(cop)). Data on the number of positive fish per group (p(no)) were analyzed in a similar manner using non-parametric tests. Combining data from both organs, the Durbin rank sum test indicated that time PI (*) had a significant impact on the number of positive fish, but the virus used for each infection only had a significant impact on the number of positive fish during the secondary infection (*). Post-hoc pairwise Wilcoxon tests, combining data from both organs and all times PI, indicated that the numbers of positive fish were significantly lower in the FL rev ORF131-A group, compared to other groups but only during the secondary infection (p(no)).

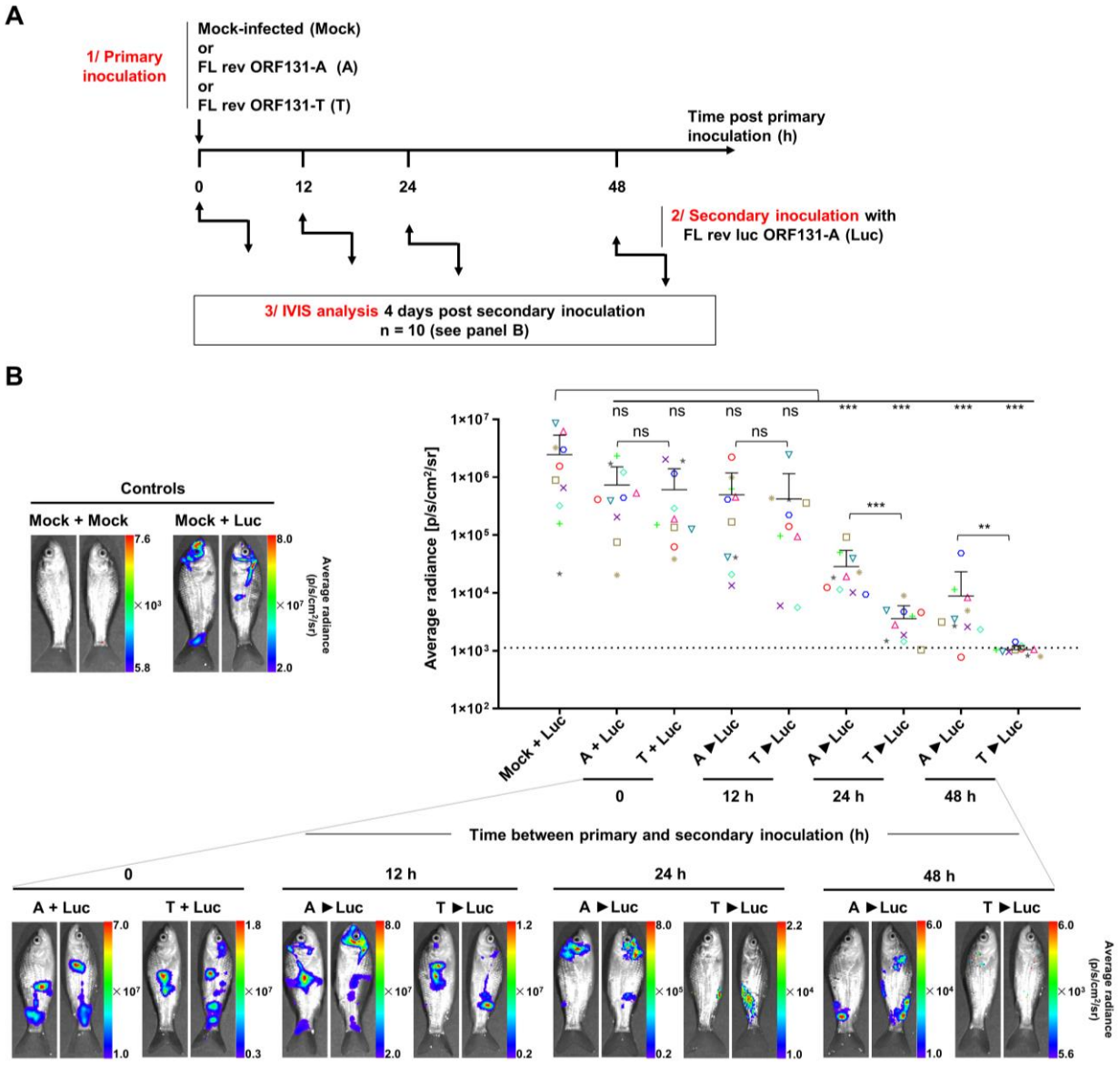
context of infection *in vivo*, it is less fit than FL rev ORF131-T in this environment. Furthermore, during co-infection *in vivo*, the replication and transmission of FL rev

ORF131-A is impaired in the presence of FL rev ORF131-T, with that later, having greater fitness *in vivo*, rapidly out-competing the former.

### 3.7 Fish infected by CyHV-3 rapidly become resistant to superinfection, and viruses encoding the ORF131 183T allele induce resistance more rapidly than viruses encoding the ORF131 183A allele

The results described above indicated that FL rev ORF131-T reduces the fitness of FL rev ORF131-A. One hypothesis is that the faster replication of FL rev ORF131-T in infected fish, combined with more efficient spread to uninfected fish, induces an innate immune response and impairs entry and replication of FL rev ORF131-A. To test this hypothesis, fish were first mock-infected or infected with one of the two viruses, the mock-infected fish forming the control group. The fish were then superinfected at various time points after primary infection with FL rev luc ORF131-A, which encodes firefly luciferase as a reporter (WT Luc in a previous study (Boutier, Ronsmans, Ouyang, et al. 2015)). Infection by this virus was monitored by bioluminescent imaging 4 d after superinfection (Fig. 6A). Regardless of the ORF131 allele used for the primary inoculation, fish in which superinfection was carried out at the same time as primary infection expressed comparable levels of bioluminescence (Fig. 6B). The levels in these fish were lower than those in the control fish, which were infected only with FL rev luc ORF131-A (Fig. 5D, Mock + Luc group), but these differences were not significant (Fig. 6B). In contrast, fish superinfected 12 h or more after primary inoculation expressed decreasing levels of bioluminescence, revealing a progressive and significant reduction of replication of the superinfecting virus relative to the control. Moreover, when superinfection was performed 24 or 48 h after primary inoculation, the inhibition was more

pronounced for the recombinant expressing ORF131 183T allele compared to the 183A allele recombinant.




## FIG 6 Inhibition of superinfection *in vivo*.

(A) Flow chart of the experiment. At the time of infection, koi carp (mean weight ± SD 25.1 ± 10.3 g) were mock-infected or infected for 2 h by immersion in water containing 400 PFU/ml FL rev ORF131-A (A) or FL rev ORF131–T (T). At the indicated times post primary infection, fish were infected by immersion in water containing 400 PFU/ml FL rev luc ORF131-A (Luc), which expresses luciferase as a reporter. At 4 d post

inoculation of Luc, fish (n=10, originating as 2 x 5 fish from duplicate tanks) were imaged for bioluminescent expression on the skin. (B) Average radiance (individual values, mean + SD) measured on the entire body surface of fish (individual values representing the mean values obtained for the left and right sides of each fish) was used as an indicator of inhibition of superinfection. The dotted line represents the threshold of positivity, which is the mean + 3 SD of the values obtained for mock-infected fish (data not presented). Two-way ANOVA analysis indicated that the ORF131 allele of the virus (***) used in primary infection and the time between primary and secondary infection (***) had significant impacts on average bioluminescence expression levels (p-values are represented).

## 4. DISCUSSION

In the present study, we identified syncytial plaque formation *in vitro* as a common trait of CyHV-3 strains that exhibited higher fitness in cell culture, and that this likely represents separate and independent adaptions to cell culture. The genetic determinism of this trait was linked to a SNP in ORF131, which encodes an essential type 1 virion membrane glycoprotein. Viruses differing only by this SNP (183A and 183T) were compared for their fitness *in vitro* and *in vivo*. The ORF131 183A allele associated with syncytial plaque formation was more fit *in vitro* but the less fit *in vivo* and *vice versa*. The identification of such variants, distinguishable by phenotype and genotype and lying on the opposite ends of the fitness spectrum in each environment, created a unique and valuable opportunity to study competition between variants in these different environments under controlled conditions. Importantly, for the first time, this allowed us to gain an insight into the process of directional selection between CyHV-3 variants.

Experiments involving co-infection by both viruses *in vitro* and *in vivo* showed that the more fit virus contributed to the purifying selection of the less fit virus by outcompeting the later, and that an intrinsic ability to stimulate strong superinfection inhibition (at a cellular and/or host level) may act as a means to accelerate this process.

Syncytial plaque formation induced by the ORF131 183A allele was associated with

higher fitness in cell culture (Fig. 2D and E). The association between these two phenotypic traits has been demonstrated previously for members of the family *Herpesviridae*, such as HSV-1, for which point mutations in virion fusion glycoprotein B (gB) induce both traits (Kuny et al. 2020; Parsons et al. 2015). There is no detectable orthologue of gB in alloherpesviruses, and the CyHV-3 ORF131 protein shows no sequence similarity to gB. In the case of HSV-1, it has been proposed that the enhanced replication of syncytial viruses in cell culture is a consequence of syncytium formation promoting the spread of infection in the cell monolayer. In the case of CyHV-3, we observed that syncytium formation occurred at the late stages of infection and involved infected cells only. Consequently, we hypothesised that the higher titer of syncytial CyHV-3 strains likely reflected more efficient virion production or higher infectivity of virions in cell culture. The observed purifying selection of the ORF131 183A allele *in vivo* demonstrated that this allele confers less fitness than the ORF131 183T allele. The latter also exhibited higher resistance against the innate immune components of epidermal mucus and serum (Fig. 4D-F), which may also contribute to greater fitness observed *in vivo*. Collectively, these observations provide scope for further investigation into the role of ORF131 and the impact of the SNP on its functions.

Extensive passage of viruses in cell culture has been used as a simple approach for producing live attenuated vaccine candidates. It allows the amplification and selection of variants encoding randomly acquired mutations in genes essential for virulence *in vivo* but dispensable for replication *in vitro*. The CyHV-3 ORF131 SNPs exposed a more complex situation in which the 183A allele conferred adaptation to cell culture but reduced virulence *in vivo* (Figs. 2 and 5). Recently, we described the rational development of an attenuated recombinant CyHV-3 vaccine using the FL strain as parent (Boutier, Ronsmans, Ouyang, et al. 2015). The ORF131 183A allele encoded by this strain could explain, at least in part, the

limited spread of this virus *in vivo* (Boutier, Ronsmans, Ouyang, et al. 2015). This observation also has implications for the future rational design of vaccines against cyprinid herpesvirus 1 and cyprinid herpesvirus 2, both of which encode ORF131 orthologs. SNPs within these orthologs may produce phenotypes similar to those described in the present study for CyHV-3.

Our recent evidence supporting a general low degree of diversity among cyprinivirus species clades compared to species clades within the family *Herpesviridae* suggests that a high degree of purifying selection has occurred after species clade divergence (Donohoe et al. 2021). We speculated that this difference could be linked, at least in part, to fundamental differences in biology between members of the two distantly related virus families. The present study on CyHV-3 provides further insights into this previous observation. Co-infection experiments using two viruses encoding different ORF131 alleles showed both *in vitro* and *in vivo* that the more fit virus rapidly contributes to the purifying selection of the less fit virus during short-term lytic infections, and that inhibition of superinfection may act as a means of accelerating this process. Both ORF131 alleles induced rapid inhibition of superinfection *in vitro* (Fig. 2F and Fig. 3, Movie S1)). Indeed, inhibition of superinfection was sufficiently strong and rapid to necessitate the use of spinoculation to establish co-infections (Fig. 3). Inhibition of superinfection *in vitro* at a cellular level has been reported for many viral families (Berngruber, Weissing, and Gandon 2010; Webster, Ott, and Greene 2013; Beperet et al. 2014; Biryukov and Meyers 2018), including members of the family *Herpesviridae* (Provost et al. 2017; Criddle et al. 2016; Koyuncu, Enquist, and Engel 2020; Chase et al. 1990), and can occur as early as 2-6 h PI (Meurens et al. 2004, 1; Banfield et al. 2003). To the best of our knowledge, such rapid and extreme inhibition of superinfection that we observed with CyHV-3 has not been described for other viruses, and thus opens up

interesting avenues for further investigation into the underlying mechanisms. In contrast to the situation *in vitro*, inhibition of superinfection *in vivo* at the host level took much longer to develop and revealed differences between the ORF131 alleles, in that the 183T allele induced inhibition more rapidly and efficiently. Furthermore, our recent experiments have revealed that such inhibition relies on the development of an innate mucosal immune response blocking the entry of superinfecting virus (Noah Bernard, unpublished data).

Notably, inhibition of superinfection could contribute, at least in part, to the lower diversity observed within the CyHV-3 species clade (Donohoe et al. 2021). This may occur through a reduction in the occurrence of co-infected cells, thus inevitably reducing genetic shift (inter-strain recombination). This reduction in the generation of diversity may be compounded by the inhibition of superinfection by variants with higher fitness. Thus, in addition to simply outcompeting less-fit variants, those with higher fitness may also rapidly deplete the repertoire of cells or highly permissive hosts available to less fit variants. The effects of this depletion may be more pronounced in highly competitive biological niches, for example in a situation where competition exists for potentially rare sub-populations of cells that support CyHV-3 latency. Furthermore, superinfection inhibition may also be important factor in tipping the balance and dictating the outcome of directional selection where the difference in replicative fitness between competing variants is minimal.

Given that the viruses we used to investigate this phenomenon *in vivo* differed by a single SNP, the application of rhPCR proved to be a crucial tool for monitoring the progress and nature of purifying selection *in vivo* (Fig. 5D). This method is an important innovation in SNP genotyping (Dobosy et al. 2011). Although rhPCR has been applied recently to qualitative viral SNP genotyping (Nakauchi et al. 2020), to best of our knowledge, until now, it has not been applied to measuring the proportions of SNP-based alleles in viral populations.

Despite the greater specificity of rhPCR than conventional PCR, we still observed non-specific amplification in the presence of non-target alleles (Fig. S1A). This may be due to mismatches that reduce, rather than eliminate, *Pyrococcus abyssi* RNase H2 activity (Dobosy et al. 2011), and the fact that Taq polymerase can, albeit inefficiently, extend the mismatched 3' ends of primers (Huang, Arnheim, and Goodman 1992). Given that the relative proportions of alleles within viral populations can differ over a much greater range than alleles within a single organism (in which the range is determined by zygosity and ploidy for the later), non-specific amplification may hinder robust relative quantification of the former. Modifications to reduce non-specificity (Fig. S1B) resulted in a useful dynamic range (Fig. S1C) but also in reduced sensitivity. However, the development of a simple and rational target enrichment process, which preserved the relative proportions of the alleles present (Fig. S1D), extended the usefulness of the rhPCR assay to samples with low viral loads. The approach developed in the present study can, in principle, be applied to any virus population and therefore may be of wider interest beyond this current study.

In conclusion, the present study indicates that CyHV-3 may have an intrinsic ability to actively contribute to the purifying selection of less fit variants by stimulation of superinfection inhibition at both the cellular and the host level. Many herpesviruses (or variants thereof) and other viruses also have the ability to inhibit superinfection. However, more widely, our observations demonstrate how the fundamental biology of some (perhaps many) viruses and their hosts may have a profound impact on the degree of diversity that arises within viral populations.

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## SUPPLEMENTARY MATERIALS

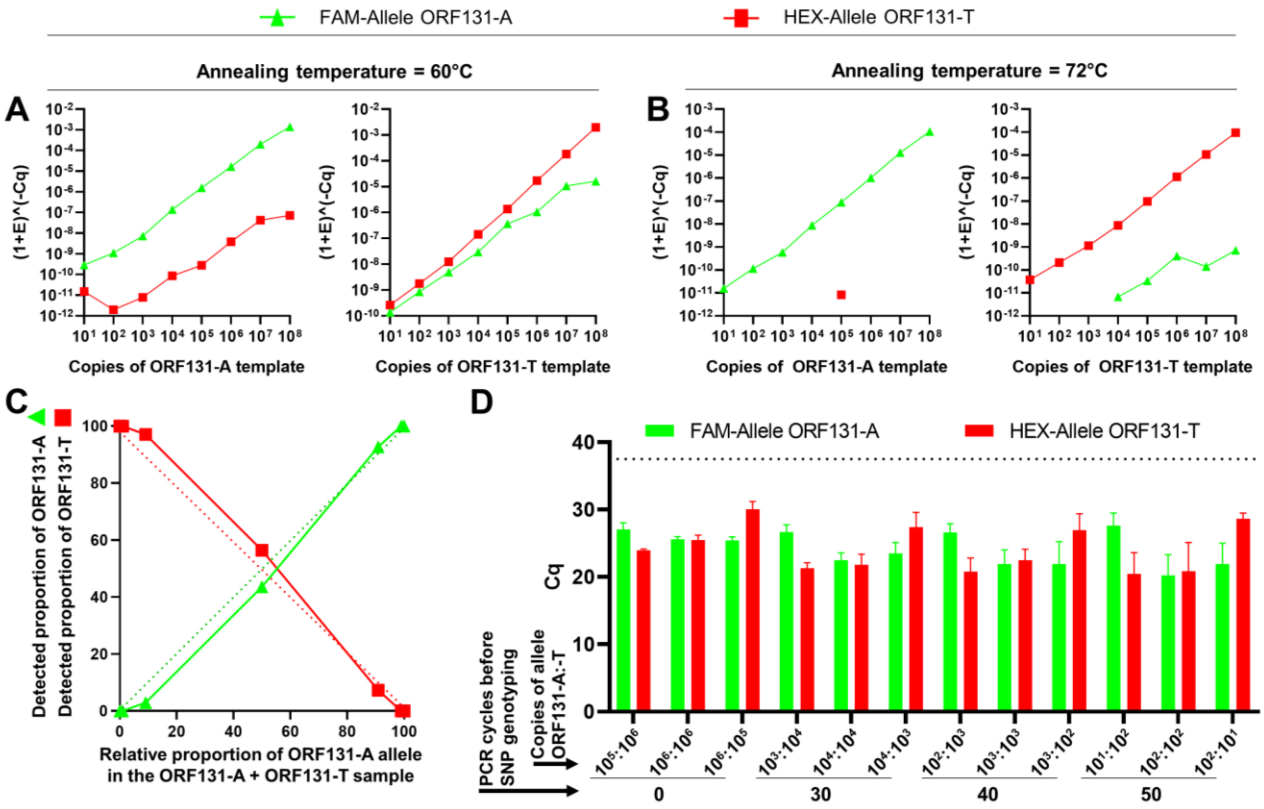



**Fig S1 Optimization of ORF131 SNP genotyping using rhPCR.**

(A) and (B) Standard curves based on reactions performed with increasing number of ORF131-A (left graph) or ORF131-T (right graph) allele copies. (A) Standard protocol performed with annealing temperature of 60 °C. Tests with pure ORF131-A template result in correct detection of ORF131-A allele in the FAM channel, but non-specific signal in for the ORF131-T allele in the HEX channel and *vice versa* when pure ORF131-T template is used. (B) Optimized protocol with annealing temperature of 72 °C improved the sub-optimum specificity observed in (B) with non-specific signal dramatically reduced. In both assays, non-specific signal was observed when target sequence was present at $>10^4$-$10^5$ copies. However, this non-specific signal contributed to only 0.1-1% of the signal in the other channel. Thus, with the modified protocol both assays were adequate for estimating relative levels of each target in the same samples (where up to $10^8$ copies of the target are present). (C) Analysis of spiked samples with optimized protocol to determine dynamic range. Spiked samples containing different ratios (0:1, 1:1, 1:10, 1:100, 1:1000, 1:0, 10:1, 100:1 and 1000:1) of ORF131-A and ORF131-T allele (using $10^5$-$10^8$ copies across different samples) were prepared and tested using the optimized protocol from (B). Dotted lines show expected results. Observed results were in reasonable agreement with expected results, with signals providing an adequate reflection of the relative proportions of each target present in the sample (useful dynamic range of ~10-90%) (D) Combining conventional PCR enrichment and SNP genotyping using rhPCR for

analysis of samples with low viral genome load. Increasing the annealing temperature to 72 °C as part of the optimized protocol in (B) reduced the sensitivity of the assay resulting in higher Cq values relative to a 60 °C annealing temperature. This resulted in reduced agreement between replicates, and thus reduced accuracy in comparison of relative abundance of each target at low viral loads. To solve this problem, we tested a target enrichment strategy involving the amplification of the ORF131-A and –T allele targets by conventional PCR using primers flanking the SNP locus (ORF131-5F and ORF131-5R Table 1) prior to SNP genotyping by rhPCR. To test this, samples were spiked with either equal amounts of each target or 10-fold difference in relative abundance of each target. Samples with sufficiently high amounts of each target (not requiring enrichment, $10^5$-$10^6$ initial copies per reaction) returned rhPCR Cq values that reflected the relative amounts of each target present in the samples (as per (C)). Samples with lower amounts of target ($10^3$-$10^4$ initial copies per reaction), requiring prior enrichment (30 cycles of PCR) prior to rhPCR, also returned rhPCR Cq values that reflected the relative amounts of each target initially present in the sample. The same was observed for samples spiked with progressively lower amounts of target which were subjected to greater amounts of PCR cycles during the enrichment process. These results indicate that reduced sensitivity of the modified method could be overcome by a target enrichment process that preserves the relative abundance of each target in the initial sample. In order to ensure the assay was tested under conditions represented of real experimental conditions, in (D) all samples were prepared by diluting in 50ng/µL of DNA extracted from carp tissue. This concentration was selected based on separate experiments which indicated that concentrations between 5ng/µL-50ng/µL carp tissue DNA could be used without inhibiting rhPCR. (data not shown). The dotted line shows the cut-off point for detection which was defined as the mean Cq for NTC samples minus the one SD.

**Movie S1** Inhibition of superinfection observed in cell culture. This movie is relates to the experiment described in Fig. 3B, and shows the progression of infection in a CCB cell monolayer from a single cell (yellow) infected by two viruses, one expressing mCherry (red) and the other expressing EGFP (green). Images were acquired every hour or 3 hours for 5 d PI. Movie S1 available at this link (https://www.dropbox.com/s/wzx344w1jytxdha/Movie%20S1.mp4?dl=0).

## Table S1 Raw data for Fig. 5D.

| Total (84) | Sample number | Sample name | Cq from Taqman qPCR | Viral copies | Number of PCR cycles used in target enrichment process | SNP genotyping (Amp rhPCR) | | | Abundance of 183-T allele relative to 183-A allele | Percentage of CyHV-3 population with 183-T allele |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cq from FAM-183-A | Cq from HEX-183-T | ΔCq | | |
| 10 | 71 | 4dpi 183A&T fish 6 gills | 25.51 | 4.05E+04 | N/A | 36.40 | 32.15 | 4.25 | 19.03 | 95.01 |
| | 97 | 6dpi 183A&T fish 1 gills | 23.53 | 7.75E+04 | N/A | 37.36 | 30.07 | 7.29 | 156.50 | 99.37 |
| | 99 | 6dpi 183A&T fish 2 gills | 24.68 | 3.84E+04 | N/A | 36.75 | 30.72 | 6.03 | 65.34 | 98.49 |
| | 101 | 6dpi 183A&T fish 3 gills | 17.63 | 2.77E+06 | N/A | 32.26 | 24.09 | 8.17 | 288.01 | 99.65 |
| | 103 | 6dpi 183A&T fish 4 gills | 20.58 | 4.62E+05 | N/A | 34.42 | 27.25 | 7.17 | 144.01 | 99.31 |
| | 105 | 6dpi 183A&T fish 5 gills | 22.61 | 1.35E+05 | N/A | 37.31 | 29.37 | 7.94 | 245.57 | 99.59 |
| | 107 | 6dpi 183A&T fish 6 gills | 19.8 | 7.42E+05 | N/A | 37.48 | 26.04 | 11.44 | 2778.33 | 99.96 |
| | II-99 | 10dpc 183A&Tfish 2 gills | 19.13 | 1.35E+06 | N/A | 36.46 | 25.33 | 11.13 | 2241.11 | 99.96 |
| | II-101 | 10dpc 183A&T fish 3 gills | 18.43 | 2.06E+06 | N/A | 35.3 | 24.97 | 10.33 | 1287.18 | 99.92 |
| | II-137 | 14dpc 183A&T fish 3 gills | 22.75 | 1.47E+05 | N/A | 36.94 | 29.13 | 7.81 | 224.41 | 99.56 |
| 20 | 63 | 4dpi 183A&T fish 2 gills | 27.65 | 1.06E+04 | 30 | 33.91 | 21.58 | 12.33 | 5148.73 | 99.98 |
| | 64 | 4dpi 183A&T fish 2 heart | 27.78 | 9.81E+03 | 30 | 37.86 | 21.24 | 16.62 | 100720.65 | 100.00 |
| | 67 | 4dpi 183A&T fish 4 gills | 26.25 | 2.56E+04 | 30 | 32.68 | 20.26 | 12.42 | 5480.15 | 99.98 |
| | 69 | 4dpi 183A&T fish 5 gills | 27.98 | 8.65E+03 | 30 | 31.24 | 22.15 | 9.09 | 544.96 | 99.82 |
| | 72 | 4dpi 183A&T fish 6 heart | 29.77 | 2.81E+03 | 30 | 37.84 | 22.04 | 15.8 | 57052.40 | 100.00 |
| | 98 | 6dpi183A&T fish 1 heart | 27.24 | 8.17E+03 | 30 | 33.79 | 21.39 | 12.4 | 5404.70 | 99.98 |
| | 100 | 6dpi 183A&T fish 2 heart | 28.31 | 4.25E+03 | 30 | 32.13 | 21.3 | 10.83 | 1820.35 | 99.95 |
| | 102 | 6dpi 183A&T fish 3 heart | 28.44 | 3.94E+03 | 30 | 38.1 | 23.13 | 14.97 | 32093.64 | 100.00 |
| | 104 | 6dpi 183A&T fish 4 heart | 29.68 | 1.86E+03 | 30 | 43.25 | 21.61 | 21.64 | 3268053.11 | 100.00 |
| | 108 | 6dpi 183A&T fish 6 heart | 26.39 | 1.37E+04 | 30 | 32.35 | 18.38 | 13.97 | 16046.82 | 99.99 |
| | II-62 | 6dpc 183A&T fish 1 heart | 28.92 | 3.36E+03 | 30 | 32.12 | 20.6 | 11.52 | 2936.74 | 99.97 |
| | II-97 | 10dpc 183A&T fish 1 gills | 25.7 | 2.42E+04 | 30 | 37.73 | 17.91 | 19.82 | 925581.18 | 100.00 |
| | II-98 | 10dpc 183A&T fish 1 heart | 26.16 | 1.82E+04 | 30 | 43.94 | 18.72 | 25.22 | 39081973.93 | 100.00 |
| | II-100 | 10dpc 183A&T fish 2 heart | 29.31 | 2.65E+03 | 30 | 42.96 | 23.26 | 19.7 | 851708.37 | 100.00 |
| | II-102 | 10dpc 183A&T fish 3 heart | 26.16 | 1.82E+04 | 30 | 38.74 | 19.05 | 19.69 | 845825.19 | 100.00 |
| | II-103 | 10dpc 183A&T fish 4 gills | 27.98 | 6.00E+03 | 30 | 39.05 | 20.25 | 18.8 | 456419.21 | 100.00 |
| | II-105 | 10dpc 183A&T fish 5 gills | 29.55 | 2.29E+03 | 30 | 32.47 | 20.83 | 11.64 | 3191.46 | 99.97 |
| | II-134 | 14dpc 183A&T fish 1 heart | 28.39 | 4.66E+03 | 30 | 38.21 | 18.69 | 19.52 | 751805.53 | 100.00 |
| | II-135 | 14dpc 183A&Tfish 2 gills | 28.26 | 5.05E+03 | 30 | 39.72 | 23.38 | 16.34 | 82952.62 | 100.00 |
| | II-138 | 14dpc 183A&T fish 3 heart | 29.25 | 2.75E+03 | 30 | 33.1 | 20.01 | 13.09 | 8719.32 | 99.99 |
| 17 | 25 | 2dpi 183A&T fish 1 gills | 33.2 | 3.26E+02 | 40 | 26.6 | 14.25 | 12.35 | 5220.60 | 99.98 |
| | 33 | 2dpi 183A&T fish 5 gills | 30.93 | 1.36E+03 | 40 | 35.19 | 17.12 | 18.07 | 275176.93 | 100.00 |
| | 61 | 4dpi 183A&T fish 1 gills | 34.61 | 1.35E+02 | 40 | 25.54 | 17.44 | 8.1 | 274.37 | 99.64 |
| | 65 | 4dpi 183A&T fish 3 gills | 31.14 | 1.19E+03 | 40 | 22.36 | 28.01 | -5.65 | 0.02 | 1.95 |
| | 66 | 4dpi 183A&T fish 3 heart | 34.51 | 1.44E+02 | 40 | 27.01 | 16.9 | 10.11 | 1105.13 | 99.91 |
| | 68 | 4dpi 183A&T fish 4 heart | 33.93 | 2.07E+02 | 40 | 32.01 | 16.32 | 15.69 | 52864.07 | 100.00 |
| | 70 | 4dpi 183A&T fish 5 heart | 35.02 | 1.04E+02 | 40 | 42.73 | 21.62 | 21.11 | 2263305.65 | 100.00 |
| | 106 | 6dpi 183A&T fish 5 heart | 31.37 | 6.63E+02 | 40 | 37.02 | 23.58 | 13.44 | 11113.30 | 99.99 |
| | II-61 | 6dpc 183A&T fish 1 gills | 30.66 | 1.16E+03 | 40 | 36.24 | 17.7 | 18.54 | 381150.17 | 100.00 |
| | II-67 | 6dpc 183A&T fish 4 gills | 32.47 | 3.84E+02 | 40 | 40.93 | 19.31 | 21.62 | 3223060.86 | 100.00 |
| | II-70 | 6dpc 183A&T fish 5 heart | 33.38 | 2.20E+02 | 40 | 33.39 | 22.71 | 10.68 | 1640.59 | 99.94 |
| | II-71 | 6dpc 183A&T fish 6 gills | 32.89 | 2.98E+02 | 40 | 44.63 | 19.92 | 24.71 | 27444239.01 | 100.00 |
| | II-72 | 6dpc 183A&T fish 6 heart | 31.81 | 5.74E+02 | 40 | 31.67 | 20.02 | 11.65 | 3213.66 | 99.97 |
| | II-104 | 10dpc 183A&T fish 4 heart | 32.5 | 3.77E+02 | 40 | 42.53 | 21.91 | 20.62 | 1611530.43 | 100.00 |
| | II-133 | 14dpc 183A&T fish 1 gills | 31.42 | 7.31E+02 | 40 | 30.05 | 18.09 | 11.96 | 3983.99 | 99.97 |
| | II-136 | 14dpc 183A&T fish 2 heart | 32.52 | 3.73E+02 | 40 | 30.11 | 21.26 | 8.85 | 461.44 | 99.78 |
| | II-139 | 14dpc 183A&T fish 4 gills | 33.35 | 2.24E+02 | 40 | 32.28 | 24.81 | 7.47 | 177.29 | 99.44 |
| 20 | 34 | 2dpi 183A&T fish 5 heart | 36.23 | 4.88E+01 | 50 | 27.91 | 19.42 | 8.49 | 359.54 | 99.72 |
| | 35 | 2dpi 183A&T fish 6 gills | 39.15 | 7.82E+00 | 50 | 37.6 | 18.74 | 18.86 | 475801.40 | 100.00 |
| | II-25 | 2dpc 183A&T fish 1 gills | 36.12 | 3.73E+01 | 50 | 45.1 | 17.6 | 27.5 | 189812531.25 | 100.00 |
| | II-28 | 2dpc 183A&T fish 2 heart | 39.14 | 5.97E+00 | 50 | 42.45 | 33.21 | 9.24 | 604.67 | 99.83 |
| | II-31 | 2dpc 183A&T fish 4 gills | 36.03 | 3.94E+01 | 50 | 40.33 | 20.86 | 19.47 | 726196.27 | 100.00 |
| | II-33 | 2dpc 183A&T fish 5 gills | 36 | 4.01E+01 | 50 | 48.37 | 18.14 | 30.23 | 1259321937.64 | 100.00 |
| | II-35 | 2dpc 183A&T fish 6 gills | 36.76 | 2.52E+01 | 50 | 32.23 | 22.23 | 10 | 1024.00 | 99.90 |
| | II-36 | 2dpc 183A&T fish 6 heart | 37.83 | 1.32E+01 | 50 | 39.82 | 20.61 | 19.21 | 606437.73 | 100.00 |
| | II-63 | 6dpc 183A&T fish 2 gills | 35.52 | 5.97E+01 | 50 | 30.47 | 19.86 | 10.61 | 1562.89 | 99.94 |
| | II-64 | 6dpc 183A&T fish 2 heart | 38.64 | 8.86E+00 | 50 | 42.48 | 22.17 | 20.31 | 1299927.74 | 100.00 |
| | II-65 | 6dpc 183A&T fish 3 gills | 37.56 | 1.72E+01 | 50 | 31.43 | 22.13 | 9.3 | 630.35 | 99.84 |
| | II-66 | 6dpc 183A&T fish 3 heart | 38.01 | 1.30E+01 | 50 | 29.16 | 22.18 | 6.98 | 126.24 | 99.21 |
| | II-69 | 6dpc 183A&T fish 5 gills | 36.22 | 3.88E+01 | 50 | 42.16 | 22.43 | 19.73 | 869604.57 | 100.00 |
| | II-106 | 10dpc 183A&T fish 5 heart | 34.9 | 8.73E+01 | 50 | 36.12 | 23.22 | 12.9 | 7643.41 | 99.99 |
| | II-107 | 10dpc 183A&T fish 6 gills | 35.38 | 6.47E+01 | 50 | 34.39 | 21.26 | 13.13 | 8964.45 | 99.99 |
| | II-108 | 10dpc 183A&T fish 6 heart | 35.04 | 7.98E+01 | 50 | N/A | 26.42 | N/A | N/A | 100.00 |
| | II-140 | 14dpc 183A&T fish 4 heart | 37.91 | 1.38E+01 | 50 | 35.59 | 23.86 | 11.73 | 3396.89 | 99.97 |
| | II-141 | 14dpc 183A&T fish 5 gills | 36.24 | 3.83E+01 | 50 | 36.41 | 25.61 | 10.8 | 1782.89 | 99.94 |
| | II-143 | 14dpc 183A&T fish 6 gills | 35.92 | 4.66E+01 | 50 | 35.13 | 23.5 | 11.63 | 3169.41 | 99.97 |
| | II-144 | 14dpc 183A&T fish 6 heart | 36.22 | 3.88E+01 | 50 | 50.61 | 30 | 20.61 | 1600398.78 | 100.00 |
| 17 | 26 | 2dpi183A&T fish 1 heart | N/A | N/A | | | | | | |
| | 27 | 2dpi 183A&T fish 2 gills | N/A | N/A | | | | | | |
| | 28 | 2dpi 183A&T fish 2 heart | N/A | N/A | | | | | | |
| | 29 | 2dpi 183A&T fish 3 gills | N/A | N/A | | | | | | |
| | 30 | 2dpi 183A&T fish 3 heart | N/A | N/A | | | | | | |
| | 31 | 2dpi 183A&T fish 4 gills | N/A | N/A | | | | | | |
| | 32 | 2dpi 183A&T fish 4 heart | N/A | N/A | | | | | | |
| | 36 | 2dpi 183A&T fish 6 heart | N/A | N/A | | | | | | |
| | 62 | 4dpi 183A&T fish 1 heart | N/A | N/A | | | | | | |
| | II-26 | 2dpc 183A&T fish 1 heart | N/A | N/A | | | | | | |
| | II-27 | 2dpc 183A&Tfish 2 gills | N/A | N/A | | | | | | |
| | II-29 | 2dpc 183A&T fish 3 gills | N/A | N/A | | | | | | |
| | II-30 | 2dpc 183A&T fish 3 heart | N/A | N/A | | | | | | |
| | II-32 | 2dpc 183A&T fish 4 heart | N/A | N/A | | | | | | |
| | II-34 | 2dpc 183A&T fish 5 heart | N/A | N/A | | | | | | |
| | II-68 | 6dpc 183A&T fish 4 heart | N/A | N/A | | | | | | |
| | II-142 | 14dpc 183A&T fish 5 heart | N/A | N/A | | | | | | |

Notes:

Samples highlighted in grey (17 samples) were qPCR negative (without any viral charge), they were not included in SNP genotyping.

Samples highlighted in red (20 samples) have a viral charge ranging from 1 to $1\times10^2$ initial copies per reaction and require enrichment by conventional PCR (50 cycles) before SNP genotyping using rhPCR.

Samples highlighted in orange ( 17 samples) had a viral charge ranging from $1\times10^2$ to $1.4\times10^3$ initial copies per reaction. These samples required enrichment by conventional PCR (40 cycles) before SNP genotyping using rhPCR.

Samples highlighted in blue (20 samples) had a viral charge ranging from $2\times10^3$ to $3\times10^4$ initial copies per reaction. These samples required enrichment by conventional PCR (30 cycles) before SNP genotyping using rhPCR.

Samples highlighted in green (10 samples) had a viral charge greater than $4\times10^4$ initial copies per reaction. They did not required enrichment by conventional PCR before SNP genotyping using rhPCR.

Cq values are mean of duplicates from rhPCR with 50 cycles instead of 40 cycles in the standard protocol.

# Discussion - Perspectives

Since the first description of CyHV-3 in the late 1990s, this virus has been causing important economic losses in the common and koi carp industries worldwide. Nowadays, CyHV-3 is the archetype of the family *Alloherpesviridae*. Complete genome sequences of 4 strains (KHV-U, KHV-J, KHV-I and KHV-GZ11) were available in the Genbank at the beginning of this PhD project, but no comparisons of the biologic properties of these strains were reported. Even if these strains represent the two existing lineages (Asian and European) described for CyHV-3, genome sequencing of additional strains was required to estimate the genetic diversity within this viral species and so to enhance the understanding of CyHV-3 evolution and pathogenesis.

Coupling genomic and biologic comparisons of viral strains

In the first chapter, we collected and sequenced seven strains of CyHV-3 from different geographical origins. Genomic comparisons of the full-length genome sequences of 11 CyHV-3 strains revealed a high level of similarity (> 99% identity). This high degree of sequence similarity was also confirmed by comparing full-length genome sequences of CyHV-3 obtained directly from infected fish tissues using targeted viral genomic enrichment (Hammoumi *et al.*, 2016). Despite the high degree of genome sequence similarity, phylogenetic analysis of CyHV-3 full-length genome sequences revealed the existence of two major genetic lineages, initially named European and Asian (Dong *et al.*, 2013). Interestingly, analysis of one isolated strain (CyHV-3 GZ11), suggested the existence of a third yet unidentified lineage. Furthermore, recombination analysis revealed that GZ11 could be a recombinant strain with a European genotype but an Asian geographical origin. This inconsistency between geographic origin and genotype was also found in the M3 strain. Such phenomenon had already been reported before in other isolates (Boutier *et al.*, 2015a). One possible reason regarding this phenomenon is due to international fish trading, so that fish infected by different strains and from different geographical origins could co-infect the same host (Rodgers *et al.*, 2011).

The first part of this study is the only example of comparative study of genomic and biological properties of different CyHV-3 strains. Importantly, the comparison of virulence among these strains leads to the identification of low, moderate and high virulence strains. Inter-lineage polymorphisms do not explain the differences of virulence observed, i.e. low and high virulence strains were found in both lineages. Of note, a negative correlation was observed between viral growth *in vitro* and virulence *in vivo* inferring that, in general, the more a viral strain is adapted to cell culture, the more attenuated it is *in vivo*. This was the case for the highly cell culture adapted CyHV-3 T strain (Mletzko *et al.*, 2017). The apparent low genomic diversity found among CyHV-3 strains can be considered as an advantage in terms of vaccine development. Indeed, cross protection following vaccination is expected but has not been studied, with the exception of a single report (Boutier *et al.*, 2017). More importantly, the difference in virulence found among CyHV-3 strains, likely the consequence of cell culture, highlights the importance of selecting the correct strains for the development of vaccine. This selection applies at

the beginning of the development when selecting the parental strain that will be used to produce the attenuated strain, and later when selecting a challenge strain to test the efficacy of the vaccine developed.

Passage of viruses in cell culture has been used as a simple approach for producing live attenuated vaccine candidates (cell culture adapted). It allows the amplification and selection of variants encoding randomly acquired mutations in genes essential for virulence *in vivo* but dispensable for replication *in vitro*. In the second chapter of this thesis, FL strain ORF131 SNPs exposed a more complex situation in which the 183A allele conferred adaptation to cell culture but reduced virulence *in vivo*. Recently, an attenuated recombinant CyHV-3 vaccine using the FL strain as parental strain was produced in the host lab (Boutier *et al.*, 2015b). The ORF131 183A allele encoded by this strain could explain, at least in part, the limited spread of this virus *in vivo* due to its partial attenuation. This observation also has implications for the future rational design of vaccines against CyHV-1 and CyHV-2, both of which encode ORF131 orthologs. SNPs within these orthologs may produce phenotypes similar to those described in the present study for CyHV-3.

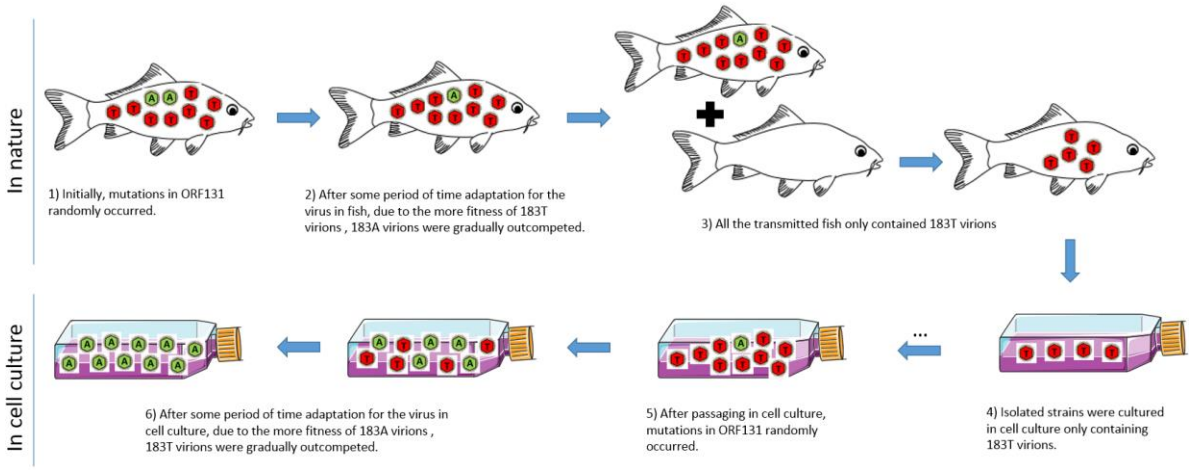
Syncytium formation associated with expression of viral glycoproteins

Syncytium formation induced by viral infection has been reported in other viral species associated with the expression of viral glycoproteins, such as retroviruses (Compton & Schwartz, 2017), ebolaviruses (Bär *et al.*, 2006), and Cytomegaloviruses (Vo *et al.*, 2020). In the second chapter of this thesis, we identified syncytial plaque formation *in vitro* as a common trait of CyHV-3 strains that exhibited higher fitness in cell culture, and that this likely represents separate and independent adaptions to cell culture. The genetic determinism of this trait was linked to a SNP in ORF131, which encodes an essential type 1 virion membrane glycoprotein. Syncytial plaque formation induced by the ORF131 183A allele was associated with higher fitness in cell culture.

Interestingly, the association between these two phenotypic traits has been demonstrated previously for members of the family *Herpesviridae*, such as HSV-1, for which point mutations in virion fusion glycoprotein B (gB) induce both traits (Kuny *et al.*, 2020; Parsons *et al.*, 2015). There is no detectable orthologue of gB in alloherpesviruses, and the CyHV-3 ORF131 protein shows no sequence similarity to gB. In the case of HSV-1, it has been proposed that the enhanced replication of syncytial viruses in cell culture is a consequence of syncytium formation promoting the spread of infection in the cell monolayer. In the case of CyHV-3, we observed that syncytium formation occurred at the late stages of infection and involved infected cells only. Consequently, we hypothesised that the higher titer of syncytial CyHV-3 strains likely reflected more efficient virion production or higher infectivity of virions in cell culture. The observed purifying selection of the ORF131 183A allele *in vivo* demonstrated that this allele confers less fitness than the ORF131 183T allele. The latter also exhibited higher resistance against the innate immune components of epidermal mucus and serum, which may also contribute to

greater fitness observed *in vivo*.

To illustrate the concepts of the mutation-selection of CyHV-3 ORF131, we used a graphical flow below to draw our hypothesis of the antagonistic selection of ORF131 SNP.




Sequences of all the strains and tissues from infected fish available in NCBI have 183T genotype. This observation is consistent with our observation that this genotype confers a higher fitness in natural conditions. However, it is rational to postulate that the 183A genotype appears *in vivo* because of random mutation. But 183T mutants have more fitness than 183A mutants, therefore 183A mutants would be gradually outcompeted. Supporting this hypothesis, we observed that the more recently infected fish from the transmission of fish with 183T dominant or pure 183T mutants will only contain 183T mutants. When researchers isolated strains from infected fish and cultured in cell culture, only 183T mutants presented in the very early passage. After continuous passaging in cell culture, mutations in ORF131 would randomly occur and generate the 183A genotype. Due to the higher fitness of 183A mutants in cell culture, once 183T to A mutation occurred, 183T mutants would be gradually outcompeted. Collectively, these observations provide scope for further investigations into the role of ORF131 and the impact of the SNP on its functions.

Putative roles of ORF131 protein

To the best of our knowledge, no studies have been reported on the role of CyHV-3 ORF131 or its orthologues. Extensive bioinformatic analyses did not suggest possible functions. CyHV-3 pORF131 is known to be an essential virion transmembrane protein (Vancsok *et al*., 2017), and it is conserved in all the strains available excepted for the point mutation at the position 183 reported in this study (Michel *et al*., 2010b; Yi *et al*., 2014; Vancsok *et al*., 2017). Here we listed some interesting results from predicted protein softwares below:

i. DNA of ORF131 contains 3 exons. With splicing of the mRNA, pORF131 encodes 429 aa. From the prediction of software SignalP V4.0, aa 1-22 form the signal peptide.
ii. From the prediction of software TMHMM V2.0, pORF131 is a Type I Membrane Protein. Aa 23-393 locate outside of the membrane, and aa 394-416 form a transmembrane helix with the left aa 417-429 locating inside of the membrane.
iii. From the prediction of softwares of NetNGglyc V and NetOglyc V3.1, pORF131 from 183A variant contains 2 N-glycosylation sites (aa 46 and 76) and 48 O-glycosylation sites (located at aa 187-361).
iv. Based on the chemical function of the aa, aa T (Threonine) 183 to A (Alanine) mutation could lead to increasing of binding affinity on cell membrane owing to that Threonine is hydrophilic and Alanine is hydrophobic, which could explain to some extent that 183A variant induces syncytium formation more efficiently in cell culture.
v. From the prediction of DEPP (Disorder Enhanced Phosphorylation Predictor), pORF131 from 183A variant has 3 out of 30 phosphorylated Serines, 31 out of 63 phosphorylated Threonines, and 3 out of 9 phosphorylated Tyrosines. 183 T to A mutation could lead to the decreasing of phosphorylated Threonines which could potentially affect the syncytium formation.

Syncytial plaque formation induced by the ORF131 183A allele was associated with higher fitness in cell culture but lower fitness in fish. Assuming that syncytium phenotypes observed *in vitro* reflect what happens *in vivo*, the absence of syncytium formation correlates with a higher virulence. A possible explanation to this correlation could be that CyHV-3 spreading *in vivo* occurs through mobility of infected cells. Supporting this hypothesis, the Incucyte experiments performed in this study demonstrated that infected cells exhibited an increased mobility in the early stage of infection. The formation of syncytia *in vivo* could impair the mobility of infected cells. Interestingly, recent study on Myxoma virus inducing syncytia formation supported a correlation between a reduced virus spreading *in vivo* and syncytium formation (Burton *et al.*, 2019).

Different hypotheses can be raised concerning the roles of pORF131:

i. pORF131 is expressed on the envelope of the viral particle

We used FL BAC recovered virus as a backbone, inserted a mCherry ORF just after the signal peptide of ORF131 and produced a FL BAC 131mCherry recombinant expressing mCherry and pORF131 as a fusion protein. After the genetic characterization of this recombinant by full-length genome sequencing, firstly, we performed multiple viral growth assay and plaque size assay *in vitro*, and confirmed the mCherry insertion did not affect the biological properties in cell culture compared to the parental strain. Then we performed viral attachment on CCB cells at 4 °C with this recombinant,

after fixation with 4% PAF, a polyclonal antibody (pAb) against mCherry was used to stain the virions bound on the cell surface, with the staining of secondary antibody Alexa Fluor™ 488, we could clearly see the signal on the cell surface, which proved directly that pORF131 is located on the envelope of the viral particles.

ii. pORF131 accumulates in the center of syncytium

In the process of FL BAC 131mCherry infection on CCB cells, mCherry protein was clearly detected in the cytoplasm and cell membrane. In the late stage of viral infection, we could clearly see syncytial plaque formation, and in the center of the syncytium, mCherry protein was accumulating, which may indicate that organelles get together to form a structure for more efficient protein processing due to cell fusion associated syncytium formation. In this way, the syncytial strain could produce higher titer of virions.

iii. Antibody against pORF131 is able to neutralize the virus

We tested the neutralizing ability of the serum from rabbit immunized by a plasmid encoding pORF131. The result showed clearly that the immunized serum can neutralize CyHV-3 FL BAC strain more efficiently than mock serum, which was double confirmed by the pAb against mCherry that can neutralize FL BAC 131mCherry virion. Altogether, we conclude that antibody against pORF131 is able to neutralize the virus.

iv. pORF131 is likely a fusion protein

We used pAb against mCherry to perform a pre-binding and post-binding assay on FL BAC 131mCherry virion. The result showed that the antibody can neutralize the virus to a similar extent during pre-binding and post-binding. Given that glycoproteins have been mainly found to play important roles in viral attachment and/or fusion with cell membrane, these observations indicate that CyHV-3 pORF131 is involved in virus-cell fusion. Thus, we concluded that pORF131 is likely to be a fusion protein.

There are at least three types of viral fusogenic proteins (Backovic & Jardetzky, 2009; Barrett & Dutch, 2020). Class I fusion proteins are predominantly composed of α-helices containing N-terminally located hydrophobic fusion peptides, and the post-fusion conformation of the fusogenic domain is characterized by a prominent trimeric α-helical coil. Hemagglutinin is one of the best studied viral Class I fusogenic proteins. Class II fusogenic proteins are mostly made of β-sheets and the fusion peptides are located in internal loops. Dengue virus E protein is an example of a well studied Class II viral fusogenic protein. Class III fusogenic proteins are composed of five domains with their molecular architecture being very distinct from class I and II fusion proteins. Examples of Class III viral fusiogenic

proteins include those of rhabdoviruses, baculovirus and herpesviruses (Backovic & Jardetzky, 2009). Due to the distant phylogenetic relationship between alloherpesviruses and avian and mammalian herpesviruses, there is no detectable orthologue of HSV-1 gB within the *Alloherpesviridae* family and without detailed structural information, it is difficult to establish to which class of viral fusogenic protein CyHV-3 ORF131 belongs to. Based on the domain comparison, there are broad similarities in general domain content and organization among CyHVs. There is less in common with HSV-1 gB, but the common features that are present do exhibit a similar organization. It should be noted that the detection of additional domains in HSV-1 gB may be linked to the fact that it is better studied, as the additional domains are all gB-specific. The CyHV-3 ORF131 SNP identified in this study occurs just before the first low complexity region in CyHV-3 ORF131 protein. Further scanning against other databases may provide further predictions for this region. Further work is needed to unravel the roles of ORF131 and identify the type of fusion protein.

Superinfection inhibition *in vitro*

In a competitive model in cell culture with two fluorescent CyHV-3 recombinants which allowed us to visualize the phenomenon of superinfection inhibition during the co-infection, we observed the selection and fixation of the syncytial genotype *in vitro*. Using an incucyte imaging system, we confirmed that superinfection inhibition is a general phenomenon not restricted to syncytial genotype. Given that viral progeny originating from a double positive cell formed a mosaic plaque with rare co-infection events, we hypothesized that the superinfection inhibition happened at the very early stage of viral infection. Inhibition of superinfection *in vitro* at a cellular level has also been reported for many viral families, including members of the family *Herpesviridae*, and can occur as early as 2-6 hpi (Meurens *et al.*, 2004, 1; Banfield *et al.*, 2003). To the best of our knowledge, such rapid and extreme inhibition of superinfection that we observed with CyHV-3 has not been described for other viruses, and thus opens up interesting avenues for further investigation into the underlying mechanisms.

Superinfection inhibition could be caused solely by the virus, solely by the cell, or both. Previously, it has been observed that for vaccinia virus, early expression of two viral proteins A33 and A36 played the crucial roles for virion repulsion inducing superinfection inhibition which was able to increase viral spreading in cell culture (Doceul *et al.*, 2010). It has also been observed that HSV-1 infection would induce innate immune response of the host cell, with virus-cell membrane fusion that could be sensed as a danger signal with potential implications for defending against enveloped viruses with specifically stimulate a type I interferon signaling pathway (Holm *et al.*, 2012). As viruses are dependent upon host transport systems to navigate within the cytoplasm, different viral species have evolved various strategies to manipulate cytoskeletal functions after entry. Actin functions in a wide range of attachment and entry processes that are critical to the very earliest stages of virus infection. Actin remodelling also inhibits superinfection by preventing viral entry into cells that are already

infected (Walsh *et al.*, 2019; Mueller *et al.*, 2014). The observations from this part of the study provide ample scope for further investigations into the mechanisms surrounding CyHV-3 superinfection inhibition.

At which stage of primary infection does superinfection inhibition occur: Attachment and/or fusion? We could use virus-like particles (VLPs, without genetic material) or inactivated virions (UV irradiation to break down the viral DNA) to mimic viral attachment/entry, so that we could reveal whether viral attachment and fusion is enough to initiate superinfection inhibition, through for example inhibition of translocation of viral capsid into nucleus. During primary infection or superinfection, from the cellular level, actins remodelling and receptors rearrangement could also be observed. Specific viral gene transcription or expression? We could use specific drugs to inhibit viral gene transcription or expression to establish if superinfection inhibition relies on the transcription or expression of specific viral genes.

Purifying selection and lower genetic diversity in CyHV-3

Our recent evidences supported a general low degree of diversity among cyprinivirus species clades compared to species clades within the family *Herpesviridae* (Donohoe *et al.*, 2021). This observation suggests that a high degree of purifying selection has occurred after species clade divergence among cyrpinivirus species. We speculated that this difference could be linked, at least in part, to fundamental differences in biology between members of the two distantly related virus families. The present study on CyHV-3 provides further insights into this previous observation. Co-infection experiments using two viruses encoding different ORF131 alleles showed both *in vitro* and *in vivo* that the more fit virus rapidly contributes to the purifying selection of the less fit virus during short-term lytic infections, and that inhibition of superinfection may act as a means of accelerating this process. Both ORF131 alleles induced rapid inhibition of superinfection *in vitro*. In contrast to the situation *in vitro*, inhibition of superinfection *in vivo* at the host level took much longer to develop and revealed differences between the ORF131 alleles, in that the 183T allele induced inhibition more rapidly and efficiently. Notably, inhibition of superinfection could contribute, at least in part, to the lower diversity observed within the CyHV-3 species clade (Donohoe *et al.*, 2021). This may occur through a reduction in the occurrence of co-infected cells, thus inevitably reducing genetic shift (inter-strain recombination). This reduction in the generation of diversity may be compounded by the inhibition of superinfection by variants with higher fitness. Thus, in addition to simply outcompeting less-fit variants, those with higher fitness may also rapidly deplete the repertoire of cells or highly permissive hosts available to less fit variants. The effects of this depletion may be more pronounced in highly competitive biological niches, for example in a situation where competition exists for potentially rare sub-populations of cells that support CyHV-3 latency. Furthermore, superinfection inhibition may also be important factor in tipping

the balance and dictating the outcome of directional selection where the difference in replicative fitness between competing variants is minimal.

Superinfection inhibition *in vivo* induced by mucosal innate immune response

Our results suggested that as early as 24h after primary infection by immersion, the entry through superinfection by immersion was significantly blocked in fish. In the meanwhile, we also tested after 24h of primary infection by immersion, superinfection by intraperitoneal injection was not affected regarding viral replication in fish (data not shown), which implied that innate immune response induced by primary infection is mainly on skin most probably related to mucosal immunity.

The perspective for this part could be another offspring project on superinfection inhibition *in vivo*. Our main hypothesis is that this phenomenon relies on the development of an innate mucosal immune response blocking the entry of the superinfecting virus. Firstly, we could test whether after one day after primary infection on fish, the mucus neutralization ability is significantly increased or not. As we know that the mucosal immune system of fish is a complex network of immune cells and molecules that are constantly sensing the environment and protecting the host from pathogen infection (Salinas & Magadán, 2017). There are different components of the immune system found in fish mucus, like mucins, enzymes, proteases, antimicrobial peptides, lectins and proteins (Ángeles 2012). CyHV-3 infection could lead to seroconversion and mucosal components changing for long time at either a non-permissive temperature or permissive temperatures (Cano *et al.*, 2020). The neutralizing capacity of the mucus could reveal a modification of its composition. Secondly, after one day of primary infection on fish, we could collect epithelial skin cells and use RNA seq to unravel gene expression modifications induced by the primary infection.

Conclusion

In conclusion, the present study demonstrates the power of coupling genomic and biologic comparisons of viral strains. Two SNP alleles of Cyprinid herpesvirus 3 ORF131 reveal opposite and antagonistic selection *in vitro* and *in vivo*. Superinfection inhibition plays a crucial role for the antagonistic selection which goes against the traditional evolutionary scenario to obtain more diversity of the viral genomes by recombination. CyHV-3 may have an intrinsic ability to actively contribute to the purifying selection of less fit variants by stimulation of superinfection inhibition at both the cellular and the host level. Many herpesviruses (or variants thereof) and other viruses also have the ability to inhibit superinfection. However, more widely, our observations demonstrate how the fundamental biology of some (perhaps many) viruses and their hosts may have a profound impact on the degree of diversity that arises within viral populations. This study also provides a good model to investigate the journey into virus evolution and a firm basis for the potential safety of a viral strain and its vaccine

strain.

The observation that CyHV-3 induces a strong inhibition of superinfection *in vitro* and *in vivo* together with the more general observation of a low genetic diversity in cypriniviruses suggest that these phenomenons could reflect a selective advantage of the host. Indeed, a reduction of genetic diversity of the virus seems incompatible with a selective advantage for the virus. It is therefore more likely that these observations are the consequences of the ability of host cells to reduce superinfection and so the diversity of alloherpesviruses. It will be very interesting in the future to identify the mechanisms of the inhibition of superinfection. This future project could reveal original mechanisms of cell autonomous immunity and a strategy developed by the host to slow down the evolution of its pathogen.

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