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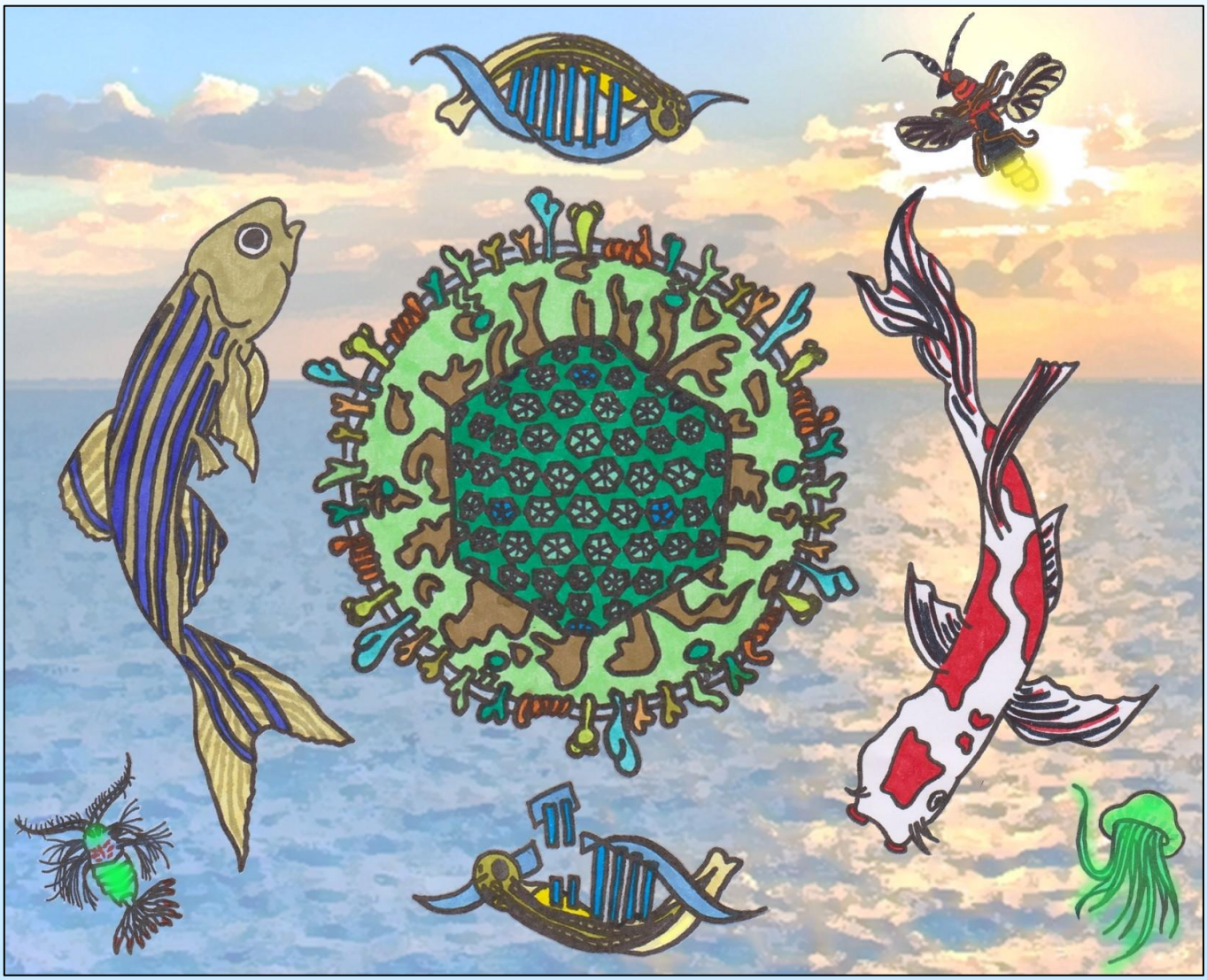
Sensibilité et permissivité des larves de poisson-zèbre (*Danio rerio*) aux cyprinivirus

Susceptibility and permissivity of zebrafish (*Danio rerio*) larvae to cypriniviruses



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SENSIBILITÉ ET PERMISSIVITÉ DES LARVES DE POISSON-ZÈBRE (*DANIO RERIO*) AUX CYPRINIVIRUS

SUSCEPTIBILITY AND PERMISSIVITY OF ZEBRAFISH (*DANIO RERIO*) LARVAE TO CYPRINIVIRUSES

Thèse dirigée par **M. Alain VANDERPLASSCHEN, UNIVERSITÉ DE LIÈGE**

Et par **M. Stéphane BETOULLE, UNIVERSITÉ DE REIMS CHAMPAGNE-ARDENNE**

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"Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less."
Maria Salomea Skłodowska-Curie (1867-1934)

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Abbreviations

AciHV-1	Acipenserid herpesvirus 1
AciHV-2	Acipenserid herpesvirus 2
AGM	Aorta-gonad-mesonephros
AngHV-1	Anguillid herpesvirus 1
ANOVA	Analysis of variance
BAC	Bacterial artificial chromosome
BF 2	Bluegill fin cell line
BGK	Black grouper kidney cell line
CAB	*Carassius auratus* blastula embryonic cells
CCB	*Cyprinus carpio* brain cell line
CCF	Colored carp fin cell line
CCG	Colored carp gill cell line
CCO	Channel catfish ovary cell line
CCT	Colored carp testis cell line
CF	Common carp fin cell line
CHIKV	Chikungunya virus
CHSE-214	Chinook salmon embryo cell line
CHT	Caudal hematopoietic tissue
CMC	Carboxymethylcellulose
CMV	Cytomegalovirus
CNEs	Conserved noncoding elements
CPE	Cytopathic effect
Crfb5	Cytokine receptor family member b 5
CRP	C-reactive protein
CSV	Chum salmon reovirus
CyHV-1, 2, 3, 4, 5	Cyprinid herpesvirus 1, 2, 3, 4, 5
CyHVs	Cyprinid herpesviruses
DAMPs	Damage-associated molecular patterns
DEGs	Differentially expressed genes
dGK	Deoxyribonucleoside kinase
DMEPF	Diseased Marbled eel pectoral fin cell line
Dpf	Day(s) post-fertilization
Dph	Day(s) post-hatching
Dpi	Day(s) post-infection
dsDNA	Double-stranded deoxyribonucleic acid
E gene	Early gene
EBV	Epstein-Barr virus
EHNV	Epizootic hematopoietic necrosis virus
EK-1	Eel kidney 1 cell line
EL	European eel liver cell line
ELISA	Enzyme-linked immunosorbent assay
EO-2	Japanese eel ovary cell line
EPC	Epithelioma papulosum cyprinid cell line
ERV	Endogenous retrovirus
EsHV-1	Esocid herpesvirus 1
ESV	European sheatfish virus
EVEs	Endogenous viral elements
EZRC	European Zebrafish Resource Centre
FHM	Fathead minnow cell line
G+C	Guanine plus cytosine
gB, H, L	Glycoproteins B, H, and L
GCHD	Grass carp hemorrhagic disease
GCRV	Grass carp reovirus

GFP	Green fluorescent proteins
GHNV	Goldfish hematopoietic necrosis virus
GiCB	Gibel carp brain cell line
GIMAPs	GTPases of immunity-associated proteins
GO	Gene ontology
GPCR	G-protein coupled receptor
HCV	Hepatitis C virus
HHV-1	Human herpesvirus 1
Hpi	Hour(s) post-infection
HVA	Herpesvirus Anguillae
HVEM	Herpes virus entry mediator
HVHND	Herpesviral hematopoietic necrosis disease
I.p.	Intraperitoneal
I.v.	Intravenous
IAV	Influenza A virus
IcHV-1	Ictalurid herpesvirus 1
IcHV-2	Ictalurid herpesvirus 2
ICTV	International Committee on Taxonomy of Viruses
IE gene	Immediate early gene
IFN	Interferon
Ig	Immunoglobulin
IHNV	Infectious hematopoietic necrosis virus
IL-10	Interleukin 10
IL-1B	Interleukin 1β
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IPNV	Infectious pancreatic necrosis virus
IRF	Interferon regulating factors
IRFs	Interferon response factors
ISGs	Interferon-stimulated genes
ISKNV	Infectious spleen and kidney necrosis virus
ISRE	IFN-stimulated response element
IVIS	*In vivo* bioluminescent imaging system
kbp	Kilobase (pair)
KEGG	Kyoto Encyclopedia of Genes and Genomes
KHV(D)	Koi herpesvirus (disease)
KO	Knock−out
KSHV	Kaposi's sarcoma-associated herpesvirus
L gene	Late gene
LAT	Latency associated transcript
LCDV	Lymphocystis disease virus
Ma	Million years
MAPK	Mitogen-activated protein kinase
MECF	Marbled eel fin cell line
MGNNV	Malabaricus grouper nervous necrosis virus
MHC	Major histocompatibility complex
miRNA	MicroRNA
MOI	Multiplicity of infection
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NCC	Non-specific cytotoxic cells
NK	Natural killer cells
NLRs	Nucleotide-blinding oligomerization domain-like receptors
NNV	Nervous necrosis virus
NOD	Nucleotide-blinding oligomerization domain

NV	Nonvirion
OIE	World Organization for Animal Health
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PFU	Plaque forming unit
PKR	dsRNA-dependent protein kinase
PKZ	Z-DNA-dependent protein kinase
Poly (I:C)	Polyinosinic-polycytidylic acid
PRRs	Pattern recognition receptors
qPCR	Quantitative PCR
RaHV-1	Ranid herpesvirus 1
RaHV-2	Ranid herpesvirus 2
RFLP	Restriction fragment length polymorphism
RGNNV	Redspotted grouper nervous necrosis virus
RIG-I	Retinoic acid-inducible gene I
RLRs	Retinoic acid-inducible gene I-like receptors
ROI	Region of interests
ROS	Reactive oxygen species
R_S	Inverted repeats
RTG-2	Rainbow trout gonad cell line
RT-PCR	Reverse transcription PCR
SalHV-1, 2, 3	Salmonid herpesvirus 1, 2, 3
SG	Stress granule
SGNNV	Sevenband grouper nervous necrosis virus
SHRV	Snakehead rhabdovirus
SHVV	Snakehead fish vesiculovirus
SINV	Sindbis virus
SJD.1	Zebrafish caudal fin cell line
SVCV	Spring viremia of carp virus
$TCID_{50}$	50% tissue culture infectious dose
TeHV-3	Testudinid herpesvirus 3
TiLV	Tilapia lake virus
TIRAP	Toll-interleukin 1 receptor domain–containing adaptor protein
TLRs	Toll-like receptors
TNF(R)	Tumor necrosis factor (receptor)
TO 2	Tilapia ovary cell line
$TR_{L, S, T}$	Long, short, third terminal repeat
TRMs	Tissue resident macrophages
$U_{L, S, T}$	Long, short, third unique region
VER	Viral encephalopathy and retinopathy
VHSV	Viral hemorrhagic septicemia virus
VNN	Viral nervous necrosis
WB	Western blotting
WGD	Whole genome duplication
Wpf	Week(s) post-fertilization
WT	Wild type
Z-DNA	Left-handed dsDNA
ZF4	1-day-old zebrafish embryo fibroblast like cell line
ZFERV	Zebrafish endogenous retrovirus
ZFIN	Zebrafish Information Network
ZfPV	Zebrafish picornavirus
ZIRC	Zebrafish International Resource Center
ZNIRE 1-5	Zebrafish non-coding infection response element 1-5
Z-RNA	Left-handed dsRNA

Résumé - Summary

Résumé

Le cyprinid herpèsvirus 3 (CyHV-3), également connu sous le nom de koï herpèsvirus (KHV), est un membre du genre *Cyprinivirus*, de la famille *Alloherpesviridae*, et de l'ordre des *Herpesvirales*. Ce virus responsable d'une maladie mortelle chez les carpes communes et koï (*Cyprinus carpio*) et entraînant des pertes économiques massives, est aujourd'hui le virus le plus étudié parmi les herpèsvirus des poissons. Outre le CyHV-3, deux autres herpèsvirus, l'anguillid herpèsvirus 1 (AngHV-1) affectant les anguilles et le cyprinid herpèsvirus 2 (CyHV-2) ciblant les carassins, ont également des impacts économiques significatifs. Une étude récente a démontré que le CyHV-3 induit une infection avortée après son inoculation chez le poisson-zèbre adulte (*Danio rerio*). Il est également capable d'infecter des lignées cellulaires de cette même espèce. Membre de la famille des *Cyprinidae*, le poisson-zèbre est un modèle expérimental de plus en plus utilisé en virologie, notamment pour l'étude des maladies infectieuses affectant les poissons d'élevage. Les objectifs généraux de la présente thèse étaient de comparer les capacités d'infection des virus AngHV-1, CyHV-2 et CyHV-3 chez le poisson-zèbre tant sur des modèles *in vitro* qu'*in vivo*.

Tout d'abord, la sensibilité et la permissivité de la lignée cellulaire ZF4 et des larves de poisson-zèbre à l'infection par l'AngHV-1, le CyHV-2 et le CyHV-3, ont été étudiées en utilisant des souches recombinantes exprimant des protéines fluorescentes vertes (GFP) et la luciférase en tant que gènes rapporteurs. La réplication virale a été suivie à l'aide de la microscopie à fluorescence à long terme (time-lapse) et de l'imagerie par bioluminescence (IVIS). Nos résultats ont montré qu'*in vitro*, les cellules étaient sensibles à l'infection par les trois virus, mais seulement permissives au CyHV-2 et au CyHV-3, tandis qu'*in vivo*, les larves n'étaient sensibles qu'au CyHV-2 et au CyHV-3 après inoculation par micro-injection intra-cardiaque. En outre, bien que sensibles, le degré de permissivité des larves aux CyHV-2 et CyHV-3 est resté incertain. Cependant, tant *in vitro* qu'*in vivo*, les infections établies par tous ces virus sont éliminées rapidement après l'inoculation, la microscopie en temps réel indiquant l'implication potentielle de la mort cellulaire programmée dans cette clairance du virus.

Afin de mieux caractériser la réponse à l'infection par le CyHV-3 dans ce modèle, une analyse transcriptomique a été menée sur des larves de poisson-zèbre infectées par le CyHV-3. Cette analyse a révélé la régulation positive de nombreux gènes stimulés par l'interféron, en particulier ceux codant pour des capteurs d'acides nucléiques, des médiateurs de la mort cellulaire programmée et des gènes apparentés. Des gènes d'ARN non codants non caractérisés et des rétrotransposons figuraient également parmi les gènes les plus régulés positivement. Il est intéressant de noter que cette réactivation/régulation des rétrotransposons en réponse à l'infection peut être bénéfique, car leurs intermédiaires génomiques d'ARN cytoplasmique et/ou d'ADN peuvent potentiellement servir de ligands pour les récepteurs de

reconnaissance de motifs moléculaires (PRR), renforçant ainsi la réponse immunitaire innée à l'infection virale.

L'analyse transcriptomique a mis en évidence les implications potentielles de la protéine kinase R (PKR) et d'une protéine kinase apparentée possédant des domaines de liaison à l'ADN Z (PKZ) dans la réponse de mort cellulaire programmée. L'impact des kinases PKR et PKZ sur la clairance du CyHV-3 a ainsi été étudié en utilisant des poissons-zèbres mutants PKR-KO et PKZ-KO générés par la technologie CRISPR/Cas9. Les résultats de ces expériences ont révélé que l'inactivation de PKR et PKZ n'entrave pas la clairance des infections par le CyHV-3 chez les larves de poisson-zèbre. Nous émettons l'hypothèse que le système immunitaire présente suffisamment de redondance pour monter une réponse appropriée en l'absence de ces gènes, malgré le stade de développement précoce des poissons-zèbres. Notre travail constitue un premier rapport sur la génération et l'utilisation de poissons-zèbres mutants PKR et/ou PKZ-KO. Ces modèles représenteront des éléments utiles pour une caractérisation plus poussée des modalités de l'infection des Cyprinidés par les herpèsvirus et pout l'étude d'autres virus en s'appuyant sur des modèles poisson-zèbre.

En conclusion, ces résultats montrent l'importance de la réponse immunitaire innée dans l'élimination de l'infection virale et soulignent le haut degré d'adaptation que les cyprinivirus ont subi pour faciliter leur persistance et leur transmission au sein de leurs hôtes naturels respectifs. Enfin, ce projet met en évidence la valeur potentielle complémentaire du modèle CyHV-3-poisson-zèbre par rapport aux modèles CyHV-3-carpe pour étudier les caractéristiques fondamentales des interactions entre le virus et l'hôte.

Summary

The cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), is a member of the genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales*. It is responsible of a lethal disease in common and koi carp (*Cyprinus carpio*), resulting in massive economic losses, and is now the most studied virus among fish herpesviruses. In addition to CyHV-3, the genus *Cyprinivirus* contains two other economically important viruses, namely the anguillid herpesvirus 1 (AngHV-1, affecting eel species) and cyprinid herpesvirus 2 (CyHV-2, affecting goldfish, Crucian carp, and Gibel carp). A recent study demonstrated that CyHV-3 induces an abortive infection after intraperitoneal inoculation of adult zebrafish (*Danio rerio*), in addition to being able to infect zebrafish cell lines. The zebrafish, a member of the family *Cyprinidae*, is an experimental model which has become increasingly used in virology, notably for studying infectious diseases affecting reared fish. The broad objectives of the present thesis were to compare AngHV-1, CyHV-2, and CyHV-3 in terms of their ability to infect zebrafish models both *in vitro* and *in vivo* and to investigate the potential of the zebrafish model to study the pathogenesis of these three cypriniviruses.

Firstly, the susceptibility and permissivity of the ZF4 cell line and zebrafish larvae to infection with AngHV-1, CyHV-2, and CyHV-3, was investigated, using recombinant strains expressing green fluorescent proteins (GFP) and luciferase as reporter genes. Viral replication was monitored using time-lapse fluorescence microscopy, and bioluminescence imaging (IVIS). Our results showed that *in vitro,* cells were susceptible to infection with all viruses, but only permissive to CyHV-2 and CyHV-3, while *in vivo,* larvae were only susceptible to CyHV-2 and CyHV-3 following inoculation by microinjection. Furthermore, while susceptible, it was unclear to what extent larvae were permissive to CyHV-2 and CyHV-3. However, both *in vitro* and *in vivo,* infections established by all these viruses were ultimately cleared rapidly after infection, with live microscopy indicating the potential implication of programmed cell death in this response.

Then, a transcriptomic analysis of CyHV-3-infected larvae was conducted in order to further characterize the response to CyHV-3 infection in this model. It revealed up-regulation of many known interferon-stimulated genes, in particular those encoding nucleic acid sensors, mediators of programmed cell death and related genes. Also, uncharacterized non-coding RNA genes and retrotransposons were among those most up-regulated. Interestingly, this retrotransposon re-activation/up-regulation in response to infection may be beneficial, as their cytoplasmic RNA and/or DNA genome intermediates may potentially act as ligands for PRRs, thus enhancing the innate immune response to viral infection.

The transcriptomic analysis supported a potentially important role of protein kinase R (PKR) and a related protein kinase containing Z-DNA binding domains (PKZ) in the programmed cell death response. The impact of these kinases on CyHV-3 clearance was investigated, using CRISPR/Cas9

generated PKR-KO and PKZ-KO zebrafish mutants. The results of these experiments revealed that the knockout of PKR and PKZ did not impair the clearance of CyHV-3 infections in zebrafish larvae. We hypothesize that even at this early developmental stage, the immune system exhibits enough redundancy to mount a sufficient response in the absence of these genes. This is the first report of the generation and use of PKR and/or PKZ-KO zebrafish mutants, and they will represent useful subjects for further characterization and the study of other viruses in zebrafish models.

In conclusion, these findings show the importance of the innate immune response alone in clearing viral infection and emphasize the high degree of adaptation that cypriniviruses have undergone to facilitate successful persistence and transmission within their respective natural hosts. Finally, this project highlights the potential value of the CyHV-3-zebrafish model *versus* CyHV-3-carp models to study the fundamental features of virus-host interactions.

Preamble

Preamble

The anguillid herpesvirus 1 (AngHV-1), cyprinid herpesvirus 2 (CyHV-2) and cyprinid herpesvirus 3 (CyHV-3) are three economically important cypriniviruses causing diseases solely in their natural host species. However, cells originating from non-natural host species can be infected, even inefficiently, by cypriniviruses. Both CyHV-2 and CyHV-3 are capable of infecting cell lines derived from species within the family *Cyprinidae* that are not their natural hosts (Boutier *et al.*, 2015a; Thangaraj *et al.*, 2021), with CyHV-3 already known to infect zebrafish cell lines (Rakus *et al.*, 2019). Similarly, AngHV-1 can infect at least one cell line derived from a member of the family *Cyprinidae* (Ueno *et al.*, 1996). Thus, it has been suggested that the ability of cypriniviruses to induce diseases only in their natural host species may be related to complex host-virus interactions downstream of host cell susceptibility. Recent work by Rakus *et al.* (2019) also demonstrated that CyHV-3 induces an abortive infection after intraperitoneal inoculation of adult zebrafish.

In the present project, we conducted an in-depth evaluation and comparison of AngHV-1, CyHV-2, and CyHV-3 in terms of their ability to infect zebrafish models both *in vitro* and *in vivo*. These experiments involved the exploitation of recombinant viruses expressing reporters, timelapse epifluorescence microscopy *in vitro*, live imaging and transcriptomics *in vivo*; and finally, the generation of CRISPR/Cas9 mutant zebrafish.

Accordingly, the structure of this manuscript is as follows. It starts with an introduction divided into three sections. The first section is a general description of viruses belonging to the order *Herpesvirales*, with a specific focus on the family *Alloherpesviridae*, which infects fish hosts. This section is adapted from the review published by the host lab in Fish and amphibian Alloherpesviruses in the *4th edition of the Encyclopedia of Virology*. The second section is devoted to the three economically important cypriniviruses AngHV-1, CyHV-2, and CyHV-3. Finally, the last part of the introduction focuses on the zebrafish, and more specifically its immune system during early larval stages and its use as a model to study fish viruses. The objectives of the thesis are then described, followed by the experimental section. It is composed of three chapters:

The first study aimed to evaluate the susceptibility and permissivity of the ZF4 cell line and zebrafish larvae to infection with AngHV-1, CyHV-2, and CyHV-3, using recombinant strains expressing green fluorescent protein (GFP) and luciferase as reporters, timelapse epifluorescence microscopy and live imaging. The second chapter describes a transcriptomic analysis of CyHV-3-infected larvae in order to characterize the antiviral response to CyHV-3 infection and to establish the extent of CyHV-3 gene transcription in this model. The third chapter describes the generation of CRISPR/Cas9 mutant hosts to investigate the potential modulation of zebrafish

permissivity to infection. These three chapters were part of a study published in *Viruses* (https://doi.org/10.3390/v15030768). In the last section of this manuscript, the main results are discussed together with perspectives.

Introduction

Introduction générale

La production aquacole et la croissance de ce secteur sont gravement entravées par les épidémies de maladies infectieuses. Au cours de la dernière décennie, les infections causées par les herpèsvirus ont été responsables de mortalités importantes chez les poissons. Des herpèsvirus ont été identifiés dans une grande variété d'espèces aquacoles importantes, notamment la carpe, le poisson-chat, l'anguille, le saumon et l'esturgeon.

À la fin des années 1990, les taux de mortalité élevés chez les carpes d'élevage causés par le Cyprinid Herpèsvirus 3 (CyHV-3, récemment rebaptisé Cyvirus cyprinidallo 3) (Bretzinger *et al.*, 1999; Hedrick *et al.*, 2000; Waltzek *et al.*, 2005) ont incité la communauté scientifique à étudier cette maladie émergente. Le CyHV-3 est un virus à ADN double brin du genre *Cyprinivirus* (récemment rebaptisé *Cyvirus*), appartenant à l'ordre des *Herpesvirales* et plus précisément à la famille des *Alloherpesviridae*, qui comprend les herpèsvirus qui infectent les poissons et les amphibiens (Davison *et al.*, 2013). Bien que la nomenclature des herpèsvirus ait été récemment modifiée par le Comité international pour la taxonomie des virus (ICTV), nous avons utilisé la nomenclature en vigueur juste avant cette dernière version dans ce manuscrit.

En raison de son impact économique sur l'élevage de la carpe et de sa propagation rapide dans le monde entier, le CyHV-3 a été inscrit sur la liste des maladies à déclaration obligatoire de l'Organisation mondiale de la santé animale (OIE) (Michel *et al.*, 2010a). Depuis lors, la nécessité de protéger les élevages de carpes communes (*Cyprinus carpio*) et de carpes koï (*Cyprinus rubrofuscus* var. "*koi*") de cette infection dévastatrice a suscité un intérêt accru pour l'étude du CyHV-3. En outre, la carpe commune est un modèle adéquat pour étudier les interactions hôte-virus, étant déjà une espèce largement étudiée dans le domaine de l'immunologie des poissons (Adamek *et al.*, 2014a; Rakus *et al.*, 2013). Par conséquent, les progrès réalisés dans notre compréhension du CyHV-3 dépassent aujourd'hui de loin ceux de tout autre membre de la famille des *Alloherpesviridae*. En général, l'impact d'un virus sur la faune, les pertes financières qu'il cause à l'industrie piscicole ou son importance en tant qu'objet de recherche fondamentale représentent les principales sources d'intérêt scientifique pour un virus spécifique. L'un des rares cas où ces trois raisons sont réunies est la problématique globale du CyHV-3, ce qui explique que le CyHV-3 soit aujourd'hui considéré comme l'archétype des alloherpèsvirus de poissons (Boutier *et al.*, 2015a).

Ce virus est l'agent étiologique d'une maladie hautement transmissible affectant la carpe, aussi bien en aquaculture que dans la nature, les épidémies se produisant principalement lorsque la température de l'eau est comprise entre 18 et 28°C (Gotesman *et al.*, 2013; Rakus *et al.*, 2013). Chez la carpe commune, la maladie peut entraîner une morbidité et une mortalité dans les 6 à 10 jours suivant l'infection (Adamek

et al., 2014b). Chez les poissons atteints, la progression de l'infection par le CyHV-3 est associée à une léthargie, une perte d'appétit, une hyperémie progressive à la base des nageoires, une augmentation de la production de mucus, et une érosion des extrémités des nageoires et des lésions cutanées (Hedrick *et al.*, 2000; McDermott and Palmeiro, 2013; Rakus *et al.*, 2013; Walster, 1999). Les changements histopathologiques comprennent l'érosion de l'épiderme (Adamek *et al.*, 2013; Miwa *et al.*, 2015), l'hyperplasie, l'hypertrophie et la nécrose de l'épithélium des branchies (Miyazaki *et al.*, 2008; Ouyang *et al.*, 2013; Pikarsky *et al.*, 2004) ainsi que la nécrose et l'inflammation des reins, de la *lamina propria* de l'intestin, du foie et de la rate (Hedrick *et al.*, 2000). L'altération de la fonction d'osmorégulation et du système immunitaire de l'hôte, augmentant la vulnérabilité à l'infection par d'autres microbes pathogènes (Negenborn *et al.*, 2015; Rakus *et al.*, 2013), est considérée comme étant à l'origine de la mortalité provoquée par l'infection avec le CyHV-3.

Outre le CyHV-3, le genre *Cyprinivirus* contient deux autres virus d'importance économique : l'anguillid herpèsvirus 1 (AngHV-1, récemment rebaptisé Cyvirus anguillidallo 1 par l'ICTV) et le cyprinid herpèsvirus 2 (CyHV-2, récemment rebaptisé Cyvirus cyprinidallo 2 par l'ICTV) (Donohoe *et al.*, 2021). L'AngHV-1 infecte l'anguille européenne (*Anguilla anguilla*), l'anguille japonaise (*Anguilla japonica*) et l'anguille américaine (*Anguilla rostrata*) (Delrez *et al.*, 2021). Ce virus est l'un des principaux agents pathogènes affectant les populations d'anguilles en aquaculture et dans la nature (Bandín *et al.*, 2014; Haenen *et al.*, 2010; Kullmann *et al.*, 2017; McConville *et al.*, 2018; Nguyen *et al.*, 2016; Van Beurden *et al.*, 2012a). L'AngHV-1 provoque une maladie hémorragique associée à des taux de mortalité pouvant atteindre 30 % chez les anguilles élevées dans des conditions d'aquaculture intensive, ce qui entraîne des pertes financières importantes (Chang *et al.*, 2002; Haenen *et al.*, 2002; Sano *et al.*, 1990; Van Beurden and Engelsma, 2012). Isolé pour la première fois au Japon en 1985 à partir d'anguilles européennes moribondes d'élevage et d'anguilles japonaises (Sano *et al.*, 1990), l'AngHV-1 est aujourd'hui fréquemment détecté chez les anguilles sauvages et d'élevage dans toute l'Europe (Haenen *et al.*, 2009; Nguyen *et al.*, 2017). Malgré les alertes scientifiques sur l'impact potentiel de cet herpèsvirus sur la population sauvage vulnérable (Bandín *et al.*, 2014; Haenen *et al.*, 2002; Nguyen *et al.*, 2017; Van Beurden *et al.*, 2012a), les interactions hôte-virus font encore l'objet de peu de recherches.

Le CyHV-2 infecte le poisson rouge (*Carassius auratus*), la carpe de Prusse (*Carassius gibelio*) et le carassin commun (*Carassius carassius*). Ce virus est l'agent étiologique d'une maladie hautement contagieuse, appelée nécrose hématopoïétique herpétique virale (HVHND). L'infection par le CyHV-2 est la plus grave chez les poissons rouges, où elle peut provoquer des taux de mortalité de 100 % à tous les stades de la vie, en particulier à des températures de l'eau comprises entre 15 et 20 °C (Jung and Miyazaki, 1995; Sahoo *et al.*, 2016). En outre, un nouveau type d'épizootie provoquant des taux de mortalité élevés chez le carassin commun allogynogénétique, appelée maladie hémorragique des

branchies, a été associé au CyHV-2 (Zhu *et al.*, 2018). Depuis les premiers rapports en 1992 et 1993 dans l'ouest du Japon (Jung and Miyazaki, 1995), des foyers de CyHV-2 ont été signalés dans le monde entier (Boitard *et al.*, 2016; Groff *et al.*, 1998; Jeffery *et al.*, 2007; Kalayci *et al.*, 2018; Stephens *et al.*, 2004). Bien que les épidémies de CyHV-2 causent des dommages massifs à la production de *Carassius* spp., les études sur la pathogenèse du CyHV-2 sont encore rares.

Les cyprinivirus ne provoquent des maladies que chez leurs espèces hôtes naturelles, ce qui suggère l'existence de restrictions liées à la sensibilité des cellules hôtes (c'est-à-dire la capacité à supporter l'entrée du virus) et à la permissivité des cellules hôtes (c'est-à-dire la capacité à supporter la réplication virale et la transmission d'une descendance virale viable à de nouvelles cellules, bien que la première puisse se produire sans la seconde). Notamment, des expériences reposant sur l'infection de lignées cellulaires ont démontré la capacité des cyprinivirus à infecter, même de manière inefficace, des cellules provenant d'espèces hôtes non naturelles. En effet, les CyHV-2 et CyHV-3 sont tous deux capables d'infecter des lignées cellulaires dérivées d'espèces de la famille des cyprinidés qui ne sont pas leurs hôtes naturels (Boutier *et al.*, 2015a; Thangaraj *et al.*, 2021), le CyHV-3 étant déjà connu pour infecter des lignées cellulaires de poisson-zèbre (*Danio rerio*) (Rakus *et al.*, 2019). De même, bien qu'il n'infecte pas naturellement des espèces n'appartenant pas à la famille des *Anguillidae*, il a été démontré que l'AngHV-1 peut infecter au moins une lignée cellulaire dérivée de la carpe commune (Ueno *et al.*, 1996).

Membre de la famille des cyprinidés, le poisson-zèbre (*Danio rerio*) est un modèle de plus en plus populaire en virologie. Contrairement à d'autres espèces de poissons telles que la carpe ou le carassin, le poisson-zèbre peut être facilement et rapidement manipulé génétiquement. Par ailleurs, la disponibilité du génome de référence du poisson-zèbre (Howe *et al.*, 2013) et d'une large gamme de lignées mutantes (Kettleborough *et al.*, 2013) facilite les recherches sur la fonction des gènes ou leur contribution à divers processus biologiques. Son stade larvaire transparent se prête facilement à l'imagerie *in vivo*, ce qui le rend particulièrement bien adapté à l'étude des interactions hôte-pathogène, y compris lors d'une infection virale (Levraud *et al.*, 2014).

Le poisson-zèbre possède un système immunitaire bien développé, composé des réponses immunitaires innées et adaptatives (Traver *et al.*, 2003; Trede *et al.*, 2004). Malgré quelques différences notables et bien que les sites de maturation diffèrent (Meeker and Trede, 2008), de nombreux types de cellules du système immunitaire des mammifères possèdent des homologues chez le poisson-zèbre (Balla *et al.*, 2010; Lugo-Villarino *et al.*, 2010). En outre, les orthologues de nombreux récepteurs de reconnaissance de motifs moléculaires (PRR) des mammifères, des cytokines, des protéines adaptatrices pour la transduction des signaux et d'autres composants importants ont été identifiés chez le poisson-zèbre (Li *et al.*, 2017; Meeker and Trede, 2008; Van Der Vaart *et al.*, 2012), ce qui indique que ce poisson représente un modèle utile pour l'étude des maladies infectieuses.

Bien que le poisson-zèbre juvénile et adulte utilise à la fois les branches innées et adaptatives du système immunitaire, l'immunité des stades embryonnaire et larvaire repose uniquement sur la composante innée, qui est détectable et active dès le premier jour de l'embryogenèse du poisson-zèbre, alors que le système adaptatif est complètement mature 4 à 6 semaines après la fécondation (Herbomel *et al.,* 1999; Lam *et al.,* 2004). Au cours de ces premiers stades de la vie, l'immunité cellulaire est médiée par les seules cellules myéloïdes, les macrophages et les neutrophiles étant les principales cellules effectrices (Le Guyader *et al.,* 2008; Lieschke *et al.,* 2001). Comme chez les mammifères, la réponse antivirale du poisson-zèbre est orchestrée par les interférons (IFN) de type I induits par les pathogènes. Ceux-ci sont appelés IFNΦ1, IFNΦ2, IFNΦ3 et IFNΦ4 (Stein *et al.,* 2007) (désignés ci-après par les symboles génétiques respectifs *ifnphi1*, *ifnphi2*, *ifnphi3* et *ifnphi4*) et sont structurellement similaires aux IFN de type I (α et β) des mammifères (Hamming *et al.,* 2011). Comme chez tous les vertébrés, les IFN de type I induisent chez le poisson-zèbre l'expression de gènes antiviraux, généralement appelés gènes stimulés par l'interféron (ISG). Cependant, la réponse IFN chez les larves de poisson-zèbre est uniquement médiée par *ifnphi1* et *ifnphi3*, *ifnphi2* n'étant exprimé que chez les adultes et *ifnphi4* n'étant que peu actif (Aggad *et al.,* 2009; Levraud *et al.,* 2007). La famille des IFN de type II du poisson-zèbre comprend deux membres, IFNγ1 et IFNγ2, qui sont également responsables de l'induction des ISG induits par les IFN de type I (Aggad *et al.,* 2010).

Ainsi, le poisson-zèbre représente un modèle pertinent et utile pour l'étude de la pathogénicité virale, la réponse immunitaire de l'hôte vertébré et les interactions entre l'hôte et le virus. Remarquablement, peu de virus sont connus pour infecter naturellement le poisson-zèbre (Balla *et al.,* 2020; Bermúdez *et al.,* 2018; Binesh, 2013; Shen and Steiner, 2004). Ce poisson poïkilotherme étant capable de survivre à une large gamme de température, plusieurs virus de mammifères peuvent infecter le poisson-zèbre dans des conditions expérimentales, ces hôtes présentant des degrés variables de sensibilité et de permissivité à l'infection. Cette propriété a également été exploitée pour étudier des virus humains tels que le virus de la grippe A, le virus du Chikungunya (CHIKV), le virus herpès simplex de type 1 (HHV-1) et le norovirus humain (Burgos *et al.,* 2008; Gabor *et al.,* 2014; Palha *et al.,* 2013; Van Dycke *et al.,* 2019). En outre, l'utilisation du poisson-zèbre a été explorée pour étudier le coronavirus du syndrome respiratoire aigu sévère 2 (SARS-CoV-2) (Laghi *et al.,* 2022; Tyrkalska *et al.,* 2023).

Le poisson-zèbre peut également être infecté par plusieurs virus de poissons importants sur le plan économique (Langevin *et al.,* 2013; LaPatra *et al.,* 2000; Martín *et al.,* 2015; Novoa *et al.,* 2006; Phelan *et al.,* 2005a; Rakus *et al.,* 2020; Sanders *et al.,* 2003). Parmi ceux-ci, le virus de la virémie printanière de la carpe (SVCV), l'un des virus les plus fréquemment utilisés dans les infections pour l'étude de la réponse immunitaire antivirale chez les larves et les adultes de poisson-zèbre (Aggad *et al.,* 2009; Bello-Perez *et al.,* 2020; Levraud *et al.,* 2007; López-Muñoz *et al.,* 2010; Sanders *et al.,* 2003). Par ailleurs,

les travaux récents de Rakus *et al.* (2019) ont démontré que le CyHV-3 pouvait induire une infection avortée après inoculation intrapéritonéale du virus chez le poisson-zèbre adulte.

Dans ce contexte, l'objectif général de la présente thèse est d'analyser le potentiel du modèle du poisson-zèbre pour étudier l'AngHV-1, le CyHV-2 et le CyHV-3, trois virus économiquement importants de la famille des *Alloherpesviridae*. L'objectif est de comparer la capacité de ces trois virus à infecter les cellules et les larves de poisson-zèbre et de caractériser la réponse à l'infection dans le modèle de larve de poisson-zèbre. En conséquence, cette thèse comporte deux objectifs principaux.

Le premier objectif de la thèse est de comparer les virus AngHV-1, CyHV-2 et CyHV-3 en termes de capacité à infecter des modèles de poisson-zèbre à la fois *in vitro* et *in vivo* en testant la sensibilité et la permissivité de la lignée cellulaire de poisson-zèbre ZF4 et des larves de poisson-zèbre à ces trois cyprinivirus. Actuellement, nous savons que l'inoculation intrapéritonéale de CyHV-3 induit une infection avortée chez le poisson-zèbre adulte et que CyHV-3 peut infecter les lignées cellulaires de poisson-zèbre. Cependant, jusqu'à présent, aucune étude n'a comparé la capacité d'AngHV-1, CyHV-2 et CyHV-3 à infecter des lignées cellulaires ou des larves de poisson-zèbre, et nous avons donc cherché à combler cette lacune. Pour atteindre cet objectif, nous avons utilisé plusieurs souches virales recombinantes exprimant des protéines fluorescentes vertes (GFP) et la luciférase comme rapporteurs.

Le deuxième objectif visait à mieux comprendre la réponse antivirale à l'infection par CyHV-3 chez les larves de poisson-zèbre. Cet objectif principal comprend tout d'abord la première étude de la réponse du poisson-zèbre au CyHV-3 en utilisant la technologie de séquençage de l'ARN du transcriptome entier (RNA-seq). Le but est de caractériser la réponse à l'infection par le CyHV-3 en termes d'expression différentielle des gènes stimulés par l'interféron (ISG) et d'implication potentielle de la mort cellulaire programmée, et d'établir l'étendue de la transcription des gènes du CyHV-3 dans le modèle larve de poisson-zèbre. Finalement, le but de la dernière partie de la thèse est de tester l'impact de deux protéines antivirales sur la clairance du CyHV-3 en utilisant des mutants de poisson-zèbre générés par l'outil biotechnologique CRISPR/Cas 9.

En réalisant ces deux objectifs principaux, nous avons souhaité (i) répondre à des questions importantes concernant la sensibilité et la permissivité de modèles *in vitro* et *in vivo* de poisson-zèbre aux alloherpesvirus, (ii) effectuer la première caractérisation approfondie des réponses immunitaires innées du poisson-zèbre à un cyprinivirus, et (iii) utiliser ces informations pour explorer la modulation potentielle de la permissivité du poisson-zèbre à l'infection. Le but ultime est d'exploiter ces connaissances dans de futurs projets de recherche axés sur les aspects fondamentaux de l'interaction virus-hôte en utilisant le modèle CyHV-3-poisson-zèbre confronté aux modèles CyHV-3-carpe commune. En outre, les mutants de poisson-zèbre générés pour cette étude constitueront des modèles utiles pour d'autres études.

En conséquence, la structure de ce manuscrit est la suivante :

Il commence par **une synthèse bibliographique** divisée en trois sections. La première section est une description générale des virus appartenant à l'ordre des *Herpesvirales*, en mettant l'accent sur la famille des *Alloherpesviridae*, qui infecte les poissons. Cette section est adaptée de la revue publiée par le laboratoire hôte dans *Fish and amphibian Alloherpesviruses* (*Encyclopedia of Virology* 4ème édition). La deuxième section est consacrée aux trois cyprinivirus d'importance économique AngHV-1, CyHV-2 et CyHV-3. Enfin, la dernière partie de l'introduction se concentre sur le poisson-zèbre, et plus particulièrement sur son système immunitaire au cours des premiers stades larvaires et sur son utilisation comme modèle pour étudier les virus des poissons.

Les **objectifs de la thèse** sont ensuite décrits, suivis de la **section expérimentale**. Elle est composée de trois chapitres :

Le premier chapitre présente une étude menée avec la plateforme *Zebrafish* du centre de recherche biomédicale interdisciplinaire GIGA (GIGA - *Zebrafish Facili*ty, Université de Liège, Belgique) et la plateforme d'imagerie du GIGA (GIGA - *Cell Imaging Core Facility*, Université de Liège, Belgique). Cette étude vise à évaluer la sensibilité et la permissivité de la lignée cellulaire ZF4 et des larves de poisson-zèbre à l'infection par AngHV-1, CyHV-2 et CyHV-3, en utilisant des souches recombinantes exprimant des protéines fluorescentes vertes (GFP) et la luciférase en tant que gènes rapporteurs. La réplication virale est suivie à l'aide de la microscopie à fluorescence, d'un système d'imagerie cellulaire automatisée en temps réel Incucyte, de l'imagerie par bioluminescence *in vivo* (IVIS) et de la microscopie à nappe de lumière, dédiée aux mesures dites de time-lapse.

Le deuxième chapitre présente une étude menée avec la plateforme *Zebrafish* et la plateforme de génomique du GIGA (GIGA - *Genomics*, Université de Liège, Belgique). Cette étude vise à effectuer une analyse transcriptomique des larves infectées par le CyHV-3, en utilisant l'approche RNA-Seq, afin de caractériser la réponse antivirale à l'infection par le CyHV-3 et d'établir l'étendue de la transcription des gènes du CyHV-3 dans ce modèle.

Le troisième chapitre présente une étude menée en collaboration avec le Dr. Isabelle Manfroid (*Zebrafish Development and Disease Models Laboratory*, *GIGA-Molecular Biology of Diseases*, Université de Liège, B-4000 Liège, Belgique) et la plateforme *Zebrafish* du GIGA. Cette étude décrit la génération de lignées mutantes de poisson-zèbre par le biais de la technologie CRISPR/Cas9 pour étudier la modulation potentielle de la permissivité du poisson-zèbre à l'infection par le CyHV-3. La réplication virale est suivie à l'aide de la microscopie à fluorescence, de l'IVIS et de la quantification relative du génome virale par qPCR.

Ces trois chapitres font partie d'une étude publiée dans la revue *Viruses* (https://doi.org/10.3390/v15030768).

Dans la dernière section de ce manuscrit, les principaux résultats ainsi que les futures perspectives sont discutés.

1. The order *Herpesvirales*

Widely distributed in nature, the order *Herpesvirales* consists of a large group of viruses, commonly referred to as herpesviruses, that share similar structural, genetic, and biological characteristics. Phylogenetically, the order *Herpesvirales* is split into three related families that infect a wide variety of host species (Pellett *et al.*, 2012a). The nomenclature of herpesviruses has been recently changed by the International Comity for Taxonomy of Viruses (ICTV). In this manuscript, we used the nomenclature in place just before this last version. The family *Herpesviridae* (renamed *Orthoherpesviridae* by the ICTV) consists of mammalian, avian and reptilian herpesviruses (Pellett *et al.*, 2012b). It is by far the most important family, considering both the number of known members and our fundamental understanding of their biology and viral-host interactions. The family *Malacoherpesviridae* contains bivalve herpesviruses (Davison *et al.*, 2009; Renault, 2016). Finally, the family *Alloherpesviridae* includes herpesviruses infecting fish and amphibians (Hanson *et al.*, 2011, 2016; Pellett *et al.*, 2012c). Species within the latter two families are much more divergent than species within the family *Herpesviridae* (Davison, 2002). The substantial genetic distance between these species and genera within the family *Malacoherpesviridae* and *Alloherpesviridae* indicates that these viruses have undergone extensive evolution either within their current or closely related hosts, enabling their replication within a specific or small subset of host species (Davison, 2002; Roizman and Pellett, 2001).

1.1. Virion morphology

Historically, the virion structure has been used as evidence to classify viruses as belonging to the order *Herpesvirales*. From the center outwards, the virion structure is as follows: (*i*) a densely packed genome composed of a single copy of linear double-stranded DNA genome (dsDNA) encapsulated in a distinct icosahedral nucleocapsid (T=16) of 100 to 130 nm in diameter; (*ii*) an amorphous proteinaceous matrix called the tegument; (*iii*) a lipid bilayer envelope acquired from the host and containing various viral glycoproteins (Fig. 1), which are both shorter and far more abundant than those found on other enveloped viruses (Wildy and Watson, 1962). While the families *Herpesviridae*, *Malacoherpesviridae*, and *Alloherpesviridae* are phylogenetically quite distant from one another (Davison *et al.*, 2009), viruses throughout the order *Herpesvirales* exhibit remarkable conservation of this virion architecture. For instance, it has been shown that the nucleocapsid of Human herpesvirus 1 (HHV-1, also known as herpesvirus simplex 1, family *Herpesviridae*) and that of Ictalurid herpesvirus 1 (IcHV-1, also known as channel catfish virus, family *Alloherpesviridae*) are strikingly similar (Booy *et al.*, 1996).

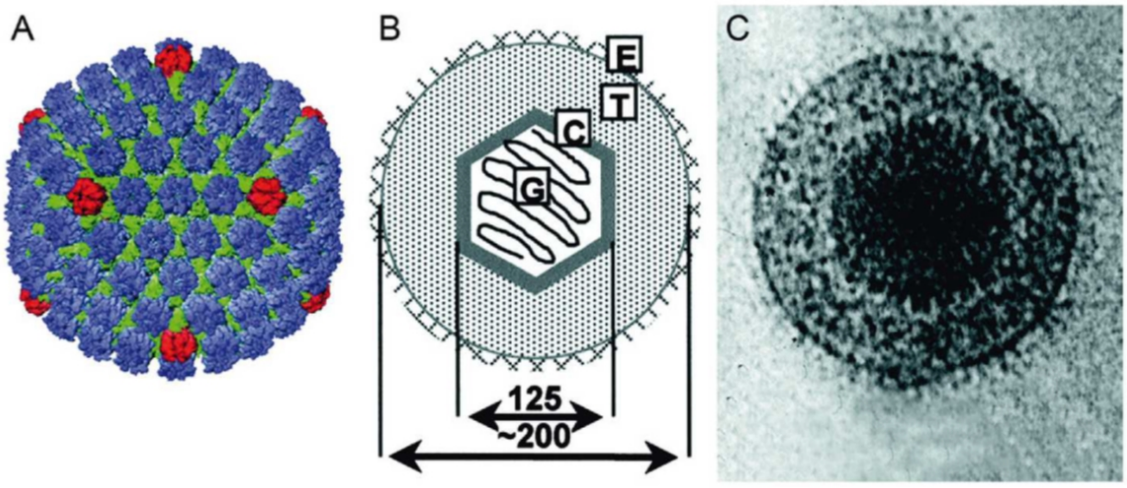

Figure 1 – Herpesvirus morphology.
(A) Reconstruction of HHV-1 capsid generated from cryo-electron microscope images, viewed along the 2-fold axis. The hexons are shown in blue, the pentons in red, and the triplexes in green (from Zhou *et al.* (2000) with permission from AAAS). (B) Schematic representation of a virion with diameters in nm. G=genome, C= capsid, T=tegument, E=envelope. (C) Cryo-electron microscope image of HHV-1 virion (from Rixon (1993) with permission of Elsevier). Adapted from Pellett *et al.* (2012a).

1.2. Main biological properties

In addition to shared morphological traits, common biological properties have been identified in all members of the order *Herpesvirales* (Ackermann, 2004; Pellett *et al.*, 2012a): (*i*) herpesviruses produce virions with the above-mentioned structure (Fig. 1); (*ii*) they encode their own DNA synthesis machinery, including a wide variety of genes involved in the metabolism and synthesis of nucleic acid, such as thymidine kinase, thymidylate synthase, dUTPase, ribonucleotide reductase, DNA polymerase, primase and helicase; (*iii*) viral DNA replication and nucleocapsid assembly takes place in the nucleus while additional processing to give rise to mature virions occurs in the cytoplasm; (*iv*) the production of new infective viral progeny usually results in lysis of the host cells; (*v*) they can establish lifelong latent infection, which has been described as an absence of regular viral transcription and replication, and a lack of production of infectious virus particles, but presence of intact viral genomic DNA and transcription of latency-associated genes; (*vi*) their ability to undergo long-term latency in immunocompetent hosts is the result of immune evasion mechanisms targeting major elements of the immune system; and (vii) the general herpesvirus replication cycle consists of seven main steps, attachment, penetration, DNA replication, capsid assembly, envelopment, and release (Pellett *et al.*, 2012a). This cycle is further described in the following section.

1.3. Herpesvirus biological cycle

The herpesvirus biological cycle can be split into three main phases: initiation of infection, lytic replication and latency (Pellett and Roizman, 2013). The description of these phases is mainly based on extensive investigations focusing on the members of the *Herpesviridae* family.

The lytic replication strategies of viruses belonging to the *Herpesviridae* family all share common traits (Johnson and Baines, 2011). Initiation of the infection process begins with viral entry, where surface glycoproteins in the viral envelope attach to cellular receptors, resulting in cell penetration. In *Herpesviridae*, at least 3 types of conserved glycoproteins appear to be crucial for this process, which are glycoproteins B, H, and L (gB, gH, and gL). Other glycoproteins taking part in cell surface interaction differ between herpesviruses, which may determine host cell specificity. In the family *Herpesviridae*, two non-exclusive mechanisms for viral penetration have been identified, i.e., direct fusion between the viral envelope and the plasma membrane, or endocytosis followed by fusion with the endosome membrane. Both mechanisms result in the introduction of the viral nucleocapsid, surrounded by tegument proteins, to the cytoplasm (Fig. 2).

Due to the macromolecular density and highly structured nature of cytoplasm, the migration of capsids from the cell surface to the nucleus cannot occur by diffusion alone. By latching onto a network of microtubules, the nucleocapsid coated by the tegument is transported towards the nuclear pores, where the viral linear genome is injected from the capsid into the nucleus (Pellett and Roizman, 2013). Circularization and partial chromatinization of the viral DNA starts almost immediately after entry into the nucleus (Garber *et al.*, 1993; Nevels *et al.*, 2011). From there, the infection initiates a lytic replication cycle if conditions are permissible, i.e., availability and state of particular viral and cellular transcription regulation and nature of chromatinization (Nevels *et al.*, 2011). Otherwise, herpesviruses can establish latent infections, which can eventually be interrupted by reactivation, leading to lytic replication and excretion of infectious particles.

During lytic replication, progeny infectious virions will be synthesized and able to infect other cells and hosts. The cellular transcription machinery is exploited for viral gene expression that occurs as a classic regulatory cascade conserved across all herpesviruses. This cascade involves the initial expression of immediate early genes (IE), followed by early (E) and late (L) genes. In the absence of *de novo* viral protein synthesis, IE gene expression begins. It is initiated by the interaction of tegument proteins with cellular transcriptional proteins, like RNA polymerase II, leading to the activation of transcription. The mains functions performed by IE gene products are regulatory, involving the inhibition of IE gene expression and the activation of E gene transcription (Pellett and Roizman, 2013). Occurring during the early stages of lytic replication, virus DNA replication is mediated by viral DNA polymerase and gives rise to the synthesis of branching concatemers, which are long continuous DNA molecules containing multiple copies of the same DNA sequence linked in tandem.

In the final phase, L gene expression results in the generation of structural proteins involved in assembly of new virion components, i.e., capsids, tegument, and envelope glycoproteins. Following capsid assembly in the nucleus, concatemerized genomes are cleaved before individual viral genomes are packaged into newly formed capsids coated with an initial tegument layer. To continue the

maturation process, immature virions must migrate from the nucleus to the cytoplasm. Primary envelopment, the first step of nuclear egress, involves the budding of preformed capsids at the inner nuclear membrane, followed by the release of enveloped immature virions in the perinuclear space. From there, fusion of the primary envelope with the outer nuclear membrane leads to the de-envelopment and release of nucleocapsids into the cytoplasm. Once in the cytoplasm, the next assembly steps consist of acquisition of additional tegument proteins to the nucleocapsid. Tegument addition and secondary envelopment of capsids, with acquisition of the glycoprotein-studded envelope, occur *via* budding into vesicles belonging to the trans-Golgi network. Finally, mature enveloped virions are released from the cell by exocytosis, involving transport to the cell membrane in cytoplasmic vesicles that fuse with the plasma membrane, or cell lysis (Fig. 2) (Mettenleiter *et al.*, 2009; Pellett and Roizman, 2013).

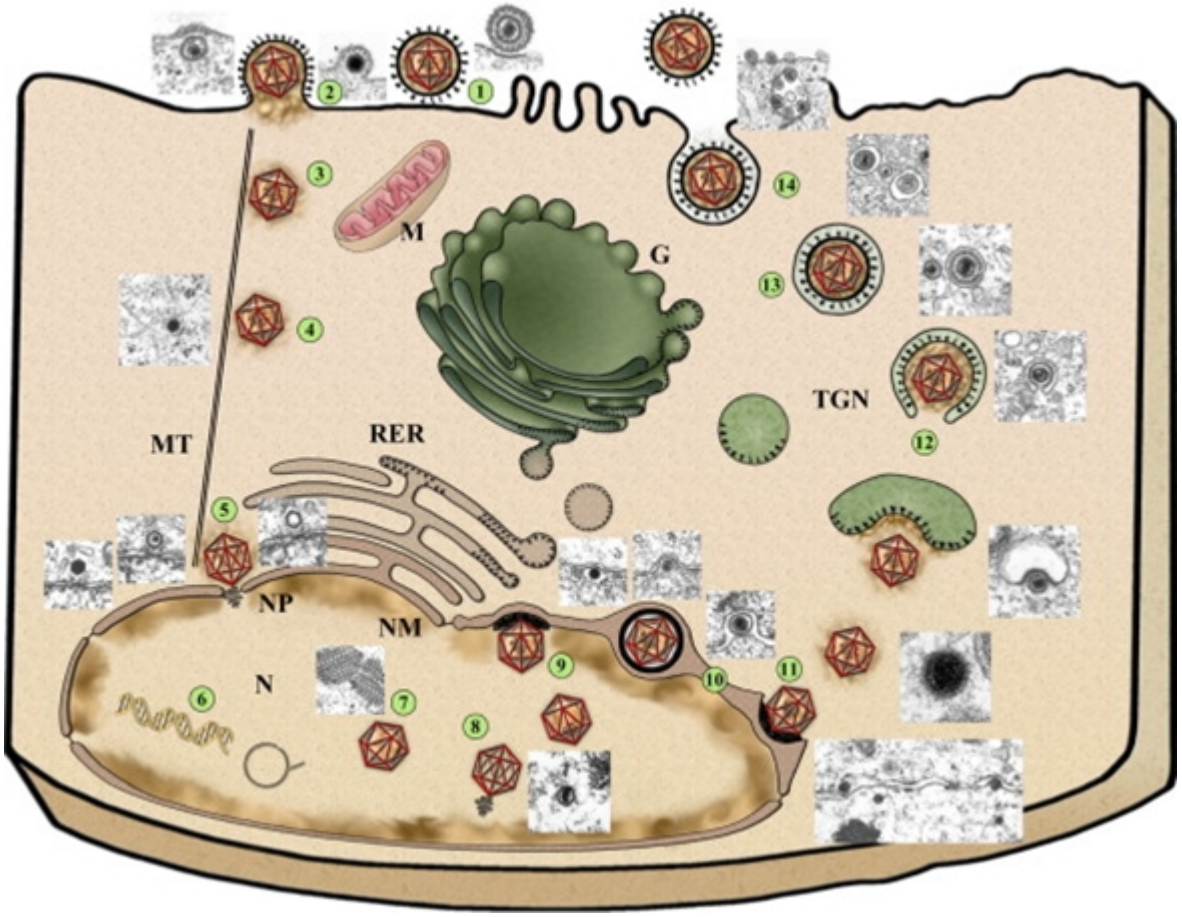

Figure 2 – Virion structure and replication cycle of herpesviruses.

Replication cycle of the alphaherpesvirus pseudorabies virus (PrV). A diagram of the replication cycle is shown together with electron micrographs of the respective stages. After attachment (1) and penetration (2), capsids are transported to the nucleus (N) (3) *via* interaction with microtubules (MT) (4), docking at the nuclear pore (NP) (5) where the viral genome is released into the nucleus. Here, transcription of viral genes and genome replication occur (6). Concatemeric replicated viral genomes are cleaved to unit-length during encapsidation (8) into preformed capsids (7), which then leave the nucleus by budding at the inner nuclear membrane (NM) (9) followed by fusion of the envelope of these primary virions located in the perinuclear space (10) with the outer nuclear membrane (11). Final maturation then occurs in the cytoplasm by secondary envelopment of intracytosolic capsids *via* budding into vesicles of the trans-Golgi network (TGN) (12) containing viral glycoproteins (black spikes), resulting in an enveloped virion within a cellular vesicle. After transport to the cell surface (13), vesicles fuse with the

plasma membrane, releasing a mature, enveloped virion from the cell (14). Rough endoplasmic reticulum (RER); mitochondrion (M); Golgi apparatus (G). Adapted from Mettenleiter *et al.* (2009).

Alternatively, a latent infection can be established by herpesviruses. Latency refers to a type of infection where the viral genome persists in the host cell without production of viral particles. During this stage, herpesvirus genomes are maintained as non-integrated closed circular episomes in the nucleus of latently infected cells. Only a small subset of viral genes – involved in the long-term maintenance of latency – are expressed, as genes involved in lytic replication are turned off. An important aspect of latent infections that makes them distinct from abortive infections is the ability to revert to productive lytic replication under permissive conditions. The specific mechanisms involved in the establishment of and reactivation from the latent state has been mainly investigated in the family *Herpesviridae* but are still evolving (Frappier, 2015; Pellett and Roizman, 2013). Interestingly, illness or detectable symptoms are not systematically observed following reactivation.

For the virus to ensure its sustainability, it must typically remain in the infected host and spread among the target species. Latent infections established by each herpesvirus can only take place in specific types of cells (Roizman and Pellett, 2001). This tissue tropism being potentially indicative of the general biology of herpesviruses, it has been best investigated among members of the subfamilies within the family *Herpesviridae*. For example, HHV-1 (family *Herpesviridae*, subfamily *Alphaherpesvirinae*) is latent in post-mitotic neuronal cells, thus reactivation from the latency is the only way to prevent extinction. For Kaposi's sarcoma-associated herpesvirus (KSHV, or Human gammaherpesvirus 8, subfamily *Gammaherpesvirinae*), a virus that establishes latency in dividing lymphoblastoid cells, the viral episome is replicated with the cellular genomic DNA and copies of the episome are subsequently distributed to daughter cells (Lomonte, 2017). This strategy has been linked to the expression of specific latent proteins tethering latent viral episomes to chromosomes of the host cell during cell division (Cardin *et al.*, 2009; Cohen, 2020). A connection between the establishment of a latent state and the expression of long noncoding RNAs has been demonstrated (Cohen, 2020). For example, the induction of latency by alphaherpesviruses is mediated by the non-coding viral-latency-associated transcript (LAT), suppressing lytic gene expression (Frappier, 2015). Additionally, virally encoded microRNAs (miRNAs), small non-coding RNAs participating in post-transcriptional gene regulation, are known to be involved in herpesvirus latent infection and are sometimes part of the few viral genes still expressed at this stage. As these small RNAs cannot be distinguished from host miRNAs, they may provide an ideal way for the virus to control gene expression during a long-term latent state without (or with little) use of viral proteins that are potentially immunogenic. Indeed, this may be linked to the fact that the prevalence of miRNA genes among members of the order *Herpesvirales* is higher than in other orders of viruses (Frappier, 2015; Grey, 2015).

Latency can be interrupted by cell differentiation or external stimuli, resulting in changes in the transcription factor environment of the cell harboring latent virus and leading to the expression of IE genes and entry into a productive cycle (Pellett *et al.*, 2012a). This property of the virus biology give rise to a certain spatial and temporal flexibility for viral sustainability in the host population. Furthermore, the ability of herpesviruses to undergo long-term latency may be a key factor that contributed to them becoming some of the most ubiquitous viral pathogens.

1.4. Genome features

The single dsDNA genome found in every member of the order *Herpesvirales* is characterized by a length that ranges from 124 to 295 kilobase pairs (kbp) and a percentage of guanine plus cytosine (G+C) that varies from 31 to 77% (Pellett and Roizman, 2013). Most members of the family *Herpesviridae* contain complex genomes, consisting of one long or sometimes two short unique regions flanked by internal and terminal repeats (Pellett *et al.*, 2012b). Internal and terminal reiterated sequences can differ in number of copies and can be lost or duplicated during cell subculturing (Pellett and Roizman, 2013). For years, the arrangement of these sequences resulted in the characterization of 6 classes of genome structure designated A to F (Fig. 3 A-F) (Pellett *et al.*, 2012a). Sequencing of Testudinid herpesvirus 3 (TeHV-3) (Gandar *et al.*, 2015) has led to the discovery of a new class of genome composed of a long unique region (U_L), extended at its right end by a short unique region (U_S) flanked by inverted repeats (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), resulting in the overall configuration TR_T-U_T-IR_T-U_L-IR_S-U_S-TR_S (Fig. 3 G). At their termini, the genomes of all herpesviruses contain conserved signals for packaging of the DNA into capsids and cleavage of concatemeric genomes to unit length (Pellett *et al.*, 2012a).

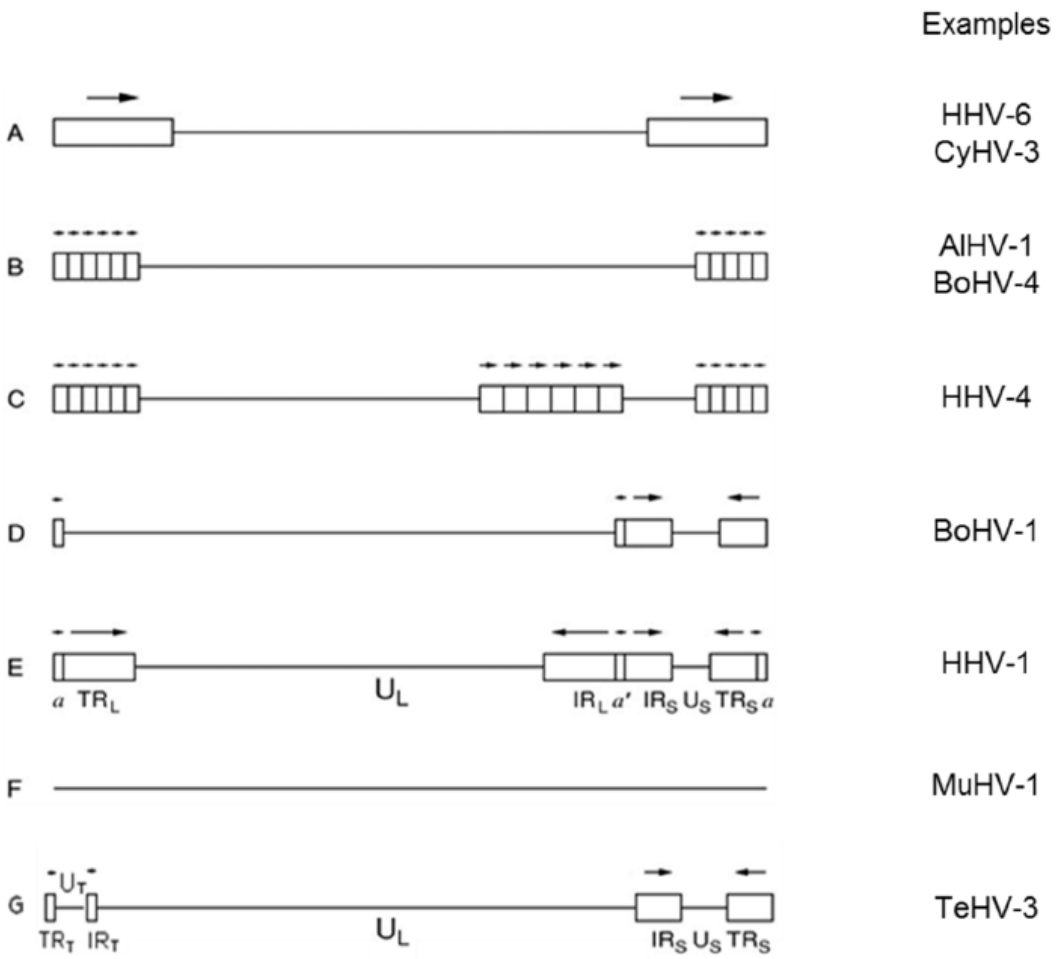

Figure 3 – Classes of herpesvirus genome structures.

Classes of herpesvirus genome structures. Unique and repeat regions are shown as horizontal lines and rectangles, respectively. The orientations of repeats are shown by arrows. The nomenclatures 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, themselves consisting of variable copy numbers 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) and Pellett *et al.* (2012b).

1.5. Phylogeny of the order *Herpesvirales*

Historically, the recognition of a virus has been based on virion morphology. Before the order *Herpesvirales* was created, the family *Herpesviridae* consisted of all known herpesviruses and the classification of herpesviruses into subfamilies relied on general biological criteria. Nowadays, the classification of an entity as herpesvirus is primarily based on its genetic content, as progress in

sequencing and bioinformatics were made. In retrospect, it was however discovered that the morphology-based phylogeny was generally consistent with the actual sequence-based phylogeny.

In 2008, the order *Herpesvirales* was created by the International Committee on Taxonomy of Viruses (ICTV, http://www.ictvonline.org) (Davison *et al.*, 2009; Pellett *et al.*, 2012a). It consists currently of three families: the already existing family *Herpesviridae* (recently renamed *Orthoherpesviridae* by the ICTV), which now encompasses herpesviruses of mammals, birds, and reptiles (Pellett *et al.*, 2012b), and the new families *Alloherpesviridae*, comprising herpesviruses of fish and amphibians (Pellett *et al.*, 2012c), and *Malacoherpesviridae*, including herpesviruses of invertebrates (Davison *et al.*, 2009; Renault, 2016).

While all members of the order *Herpesvirales* tend to conserve the same virion morphology (Hanson *et al.*, 2016), detectable genetic similarities within the three families are very low, indicating the very ancient origin of this order. In all herpesviruses, the most convincingly conserved gene is that encoding the putative ATPase subunit of the terminase, a complex involved in viral genome encapsidation. It is also conserved, to a lesser extent, in T4-like bacteriophages belonging to the family *Myoviridae* (Davison *et al.*, 2009; Pellett *et al.*, 2012a). The conservation of the predicted amino acid sequence of this protein in herpesviruses and tailed bacteriophages (Davison, 1992), along with the existence of conserved structural elements in other proteins, suggest that herpesviruses descend from ancient precursors having existed in bacteria (Rixon and Schmid, 2014).

The family *Herpesviridae* is divided into three sub-families *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*, containing five, five and seven genera, respectively. The ICTV currently lists 115 viral species in this family. Except for one (Iguanid herpesvirus 2), these species are all classified in one of the three subfamilies. The existence of 43 genes that are conserved among members of the family *Herpesviridae* supports the common ancestry of species within this family. These core genes were presumably present in the last common ancestor, which is thought to have existed 400 million years ago (McGeoch *et al.*, 2006).

The family *Malacoherpesviridae* comprises two genera, *Aurivirus*, and *Ostreavirus*, each containing one species, *Haliotid herpesvirus 1* (haliotid herpesvirus 1 or abalone herpesvirus), and *Ostreid herpesvirus 1* (ostreid herpesvirus 1 or oyster herpesvirus), respectively (Renault, 2016).

The next section contains a more in-depth description of the phylogeny of the family *Alloherpesviridae*, to which the three economically important viruses studied in this thesis, i.e., Anguillid herpesvirus 1 (AngHV-1, recently renamed Cyvirus anguillidallo 1), Cyprinid herpesvirus 2 (CyHV-2, recently renamed Cyvirus cyprinidallo 2), and Cyprinid herpesvirus 3 (CyHV-3, recently renamed Cyvirus cyprinidallo 3), belong.

1.6. The family *Alloherpesviridae*

1.6.1. Introduction

As previously pointed out, the taxonomy classification initially relied on virus morphology, and for many years, several herpesviruses infecting fish have been reported by the ICTV as members of the family *Herpesviridae*, based on morphology alone. The first alloherpesviruses to be studied in detail originated from the North American leopard frog (*Rana pipiens*). While the Ranid herpesvirus 1 (RaHV-1, initially named Lucké tumour herpesvirus), was identified as the etiological agent of renal adenocarcinoma or Lucké tumour (Fawcett, 1956), the frog virus 4, or Ranid herpesvirus 2 (RaHV-2), was isolated from the pooled urine of tumour-bearing frogs (Gravell *et al.*, 1968). During the same decade, IcHV-1 outbreaks began impacting the channel catfish (*Ictalurus punctatus*) industry in the United States in the late 1960's, and it subsequently became the first fish herpesvirus to be identified and classified in the order *Herpesvirales*. Furthermore, this virus was the first fish herpesvirus to be entirely sequenced. The subsequent phylogenetic analysis revealed that fish herpesviruses have evolved separately from mammalian, avian, and reptilian herpesviruses. Later, the genus *Ictalurivirus* (recently renamed *Ictavirus* by the ICTV) was created, as this virus was only very distantly related to other herpesviruses (Davison, 1992).

In the 1990s, an emerging virus, initially called carp interstitial gill nephritis necrosis virus, causing mass mortalities in common and koi carps (Ariav *et al.*, 1999; Bretzinger *et al.*, 1999) was characterized as a herpesvirus based on its virion structure (Hedrick *et al.*, 2000). This herpesvirus was later renamed Koi Herpesvirus (KHV) before its final nomenclature was established as CyHV-3 by the ICTV. Over the past two decades, this virus has become the most extensively studied fish herpesvirus (Boutier *et al.*, 2015a), providing substantial new insights into the biology of fish herpesviruses. The accumulation of further genomic data for a variety of herpesviruses infecting fish and amphibians allowed for a solid understanding of the phylogeny and evolution of the family *Alloherpesviridae* and its genera (Donohoe *et al.*, 2021; Waltzek *et al.*, 2009). It is described in the following section of this manuscript.

1.6.2. Phylogeny of the family *Alloherpesviridae*

Currently, the ICTV lists 13 virus species in the family *Alloherpesviridae*, distributed into four genera: *Cyprinivirus* (recently renamed *Cyvirus* by the ICTV), *Ictalurivirus* (recently renamed *Ictavirus* by the ICTV), *Salmonivirus* (recently renamed *Salmovirus* by the ICTV), and *Batrachovirus* (recently renamed *Batravirus* by the ICTV). The first three genera consist of fish herpesviruses while the last one (*Batrachovirus*) regroups amphibian herpesviruses. Table 1 includes the 13 classified alloherpesviruses of fish and amphibians according to the ICTV (Order *Herpesvirales*, Family *Alloherpesviridae*), plus 9 others not yet classified. A phylogenetic tree of the family *Alloherpesviridae*, based on a Bayesian analysis of the deduced amino acid sequences of DNA polymerase genes, is shown in Fig. 4.

The genus *Cyprinivirus* includes the alloherpesviruses of cyprinids (Cyprinid herpesvirus 1; 2 and 3 [CyHV]) and that of eel (AngHV-1). The genus *Ictalurivirus* contains viruses isolated from catfish (Ictalurid herpesvirus 1 and 2 [IcHV]) and sturgeon species (Acipenserid herpesvirus 2 [AciHV-2]), while the genus *Salmonivirus* consists of herpesviruses of salmonids (Salmonid herpesvirus 1; 2 and 3 [SalHV]) (Fig. 4A). Full genome sequences are available for nine of these viruses, and two unclassified members of the family *Alloherpesviridae*, representing all genera (Table 2).

The official taxonomical classification of at least nine more viruses is still awaiting, and for most of these only partial sequence data are available. However, these viruses undoubtedly belong to the family *Alloherpesviridae*, as shown by phylogenetic analyses. Some of them can be grouped within genera that are already existing. For example, SalHV-4 and SalHV-5 (Fig. 4B) will likely be included in the genus *Salmonivirus*). In addition, the novel cyprinid herpesvirus affecting sichel (*Pelecus cultratus*) and tentatively named CyHV-4 (Doszpoly *et al.*, 2015) clearly clusters within the genus *Cyprinivirus* (Fig. 4B). However, it currently remains unclassified by the ICTV (Table 1). In contrast, other viruses (e.g., Acipenserid herpesvirus 1 [AciHV-1]; Esocid herpesvirus 1 [EsHV-1]) do not cluster with viruses in any of the existing genera within the family *Alloherpesviridae* (Fig. 4B). Following detection by PCR assay and subsequent sequence analysis, another cyprinid herpesvirus named CyHV-5 was recently reported in roach (*Rutilus rutilus*) and asp (*Leuciscus aspius*). This virus was not included in the phylogeny analyses and is not yet classified (Sellyei *et al.*, 2020).

Interestingly, all currently known fish herpesviruses affect bony fish, with the exception of one herpesvirus reported in shark species (Hanson *et al.*, 2011). To determine their evolution and taxonomic classification, further genomic data from these virus species are required. Based on the description of additional members of the family *Alloherpesviridae*, the complexity and precision of the currently existing classification are likely to rapidly evolve in the future. Finally, the integration of alloherpesvirus sequences fused with transposons into the genome of several fish species has been demonstrated (Inoue *et al.*, 2017).

The presence of 12 convincingly conserved genes in full-length genome sequenced members of the family *Alloherpesviridae* supports the general genetic coherence of this family (Davison *et al.*, 2013). This limited number of core genes suggests that the last common ancestor of alloherpesviruses is considerably older than that of the family *Herpesviridae* (Van Beurden *et al.*, 2010). It is interesting to note that viruses belonging to the genera *Cyprinivirus* and *Ictalurivirus* can infect fish species from two different superorders (*Elopomorpha* and *Ostariophysi*) or subclasses (*Chondrostei* and *Neopterygii*), respectively. This indicates a more recent evolution of the family *Alloherpesviridae*, associated with a lower degree of coevolution of some alloherpesviruses with their hosts, compared with the family *Herpesviridae.*

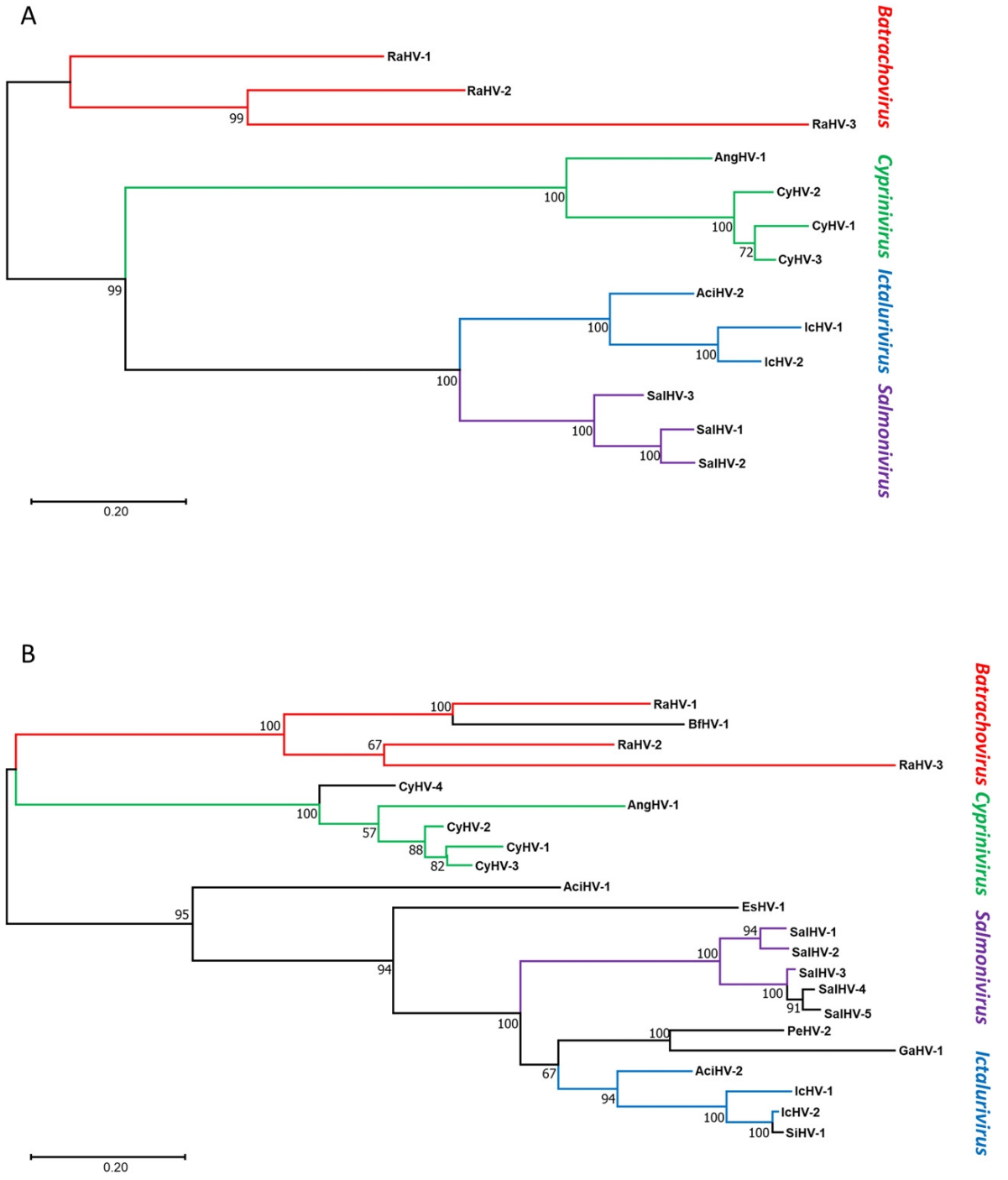

Figure 4 – Phylogenetic tree of the family *Alloherpesviridae*.

The analyses were based on the Bayesian analysis (WAG amino acid model) of the deduced amino acid sequences of DNA polymerase genes (134 and 113 amino acid residues, respectively for panel A and B). High statistical values confirm the topology of the trees. The four main genera within the family *Alloherpesviridae* are designated by different colored lines on the trees. Panel A: phylogenetic tree of the classified viral species in the family *Alloherpesviridae*. Panel B: phylogenetic tree of the classified and unclassified potential members of the family *Alloherpesviridae*. AciHV: acipenserid herpesvirus; AngHV: anguillid herpesvirus; BfHV: bufonid herpesvirus; CyHV: cyprinid herpesvirus; EsHV: esocid herpesvirus; GaHV: gadid herpesvirus; IcHV: ictalurid herpesvirus; PeHV: percid herpesvirus; RaHV: ranid herpesvirus; SalHV: salmonid herpesvirus; SiHV: silurid herpesvirus. GenBank and RefSeq accession numbers: AciHV-1: EF685903; AciHV-2: FJ815289; AngHV-1: NC_013668; BfHV-1: MF143550; CyHV-1: NC_019491; CyHV-2: NC_019495; CyHV-3: NC_009127; CyHV-4: KM357278; EsHV-1: KX198667; GaHV-1: HQ857783; IcHV-1: NC_001493; IcHV-2: NC_036579; PeHV-2: MG570129; RaHV-1: NC_008211; RaHV-2: NC_008210; RaHV-3: NC_034618; SalHV-1: EU349273; SalHV-2: FJ641908; SalHV-3: EU349277; SalHV-4: JX886029; SalHV-5: KP686090; SiHV-1: MH048901. From Boutier *et al.* (2021).

Table 1 – Classification of fish and amphibian alloherpesviruses according to the ICTV (Order *Herpesvirales*, Family *Alloherpesviridae*)

Genus	Viral species (ICTV list)	Viral species and acronym	Alternative viral name (s)	Susceptible host (s)
Batrachovirus	*Ranid herpesvirus 1*	Ranid herpesvirus 1 (RaHV-1)	Lucké tumor herpesvirus	Northern leopard frog (*Lithobates pipiens*)
	Ranid herpesvirus 2	Ranid herpesvirus 2 (RaHV-2)	Frog virus 4	Northern leopard frog (*Lithobates pipiens*)
	Ranid herpesvirus 3	Ranid herpesvirus 3 (RaHV-3)	-	Common frog (*Rana temporaria*)
Cyprinivirus	*Anguillid herpesvirus 1*	Anguillid herpesvirus 1 (AngHV-1)	European eel herpesvirus (HVA)	Japanese and European eel (*Anguilla japonica* and *A. anguilla*)
	Cyprinid herpesvirus 1	Cyprinid herpesvirus 1 (CyHV-1)	Carp pox herpesvirus	Common carp (*Cyprinus carpio*)
	Cyprinid herpesvirus 2	Cyprinid herpesvirus 2 (CyHV-2)	Goldfish haematopoietic necrosis virus	Goldfish (*Carassius auratus*) Gibel carp (*Carassius gibelio*)
	Cyprinid herpesvirus 3	Cyprinid herpesvirus 3 (CyHV-3)	Koi herpesvirus	Common carp (*Cyprinus carpio*)
Ictalurivirus	*Acipenserid herpesvirus 2*	Acipenserid herpesvirus 2 (AciHV-2)	White sturgeon herpesvirus 2	Sturgeon (*Acipenser spp.*)
	Ictalurid herpesvirus 1	Ictalurid herpesvirus 1 (IcHV-1)	Channel catfish virus	Channel catfish (*Ictalurus punctatus*)
	Ictalurid herpesvirus 2	Ictaluried herpesvirus 2 (IcHV-2)	Ictalurus melas herpesvirus	Black bullhead (*Ameiurus melas*)
Salmonivirus	*Salmonid herpesvirus 1*	Salmonid herpesvirus 1 (SaHV-1)	Herpesvirus salmonis	Rainbow trout (*Oncorhynchus mykiss*)
	Salmonid herpesvirus 2	Salmonid herpesvirus 2 (SaHV-2)	Oncorhynchus masou herpesvirus	Salmon and trout (*Oncorhynchus spp.*)
	Salmonid herpesvirus 3	Salmonid herpesvirus 3 (SaHV-3)	Epizootic epitheliotropic disease virus	Lake trout (*Salvelinus namaycush*)
Unclassified	N/A	Acipenserid herpesvirus 1 (AciHV-1)	White sturgeon herpesvirus 1	white sturgeon (*Acipenser transmontanus*)
	N/A	Cyprinid herpesvirus 4 (CyHV-4)	Sichel herpesvirus	Sichel (*Pelecus cultratus*)
	N/A	Esocid herpesvirus 1 (EsHV-1)	Blue spot disease virus	Northern pike (*Esox lucius*)
	N/A	Gadid herpesvirus 1 (GaHV-1)	Atlantic cod herpesvirus	Atlantic cod (*Gadus morhua*)
	N/A	Percid herpesvirus 2 (PeHV-2)	European perch herpesvirus	European perch (*Perca fluviatilis*)

	N/A	Salmonid herpesvirus 4 (SalHV-4)	Atlantic salmon papillomatosis virus	Atlantic salmon (*Salmo salar*)
	N/A	Salmonid herpesvirus 5 (SalHV-5)	Namaycush herpesvirus	Lake trout (*Salvelinus namaycush*)
	N/A	Silurid herpesvirus 1 (SiHV-1)	-	Glass catfish (*Kryptopterus bicirrhis*)
	N/A	Bufonid herpesvirus 1 (BfHV-1)	-	Common toad (*Bufo bufo*)

Table 2 contains the genomic features of the eleven alloherpesvirus genomes that have been fully sequenced so far. For the other two classified, and several unclassified fish herpesviruses as well, only partial sequence data is available. The size of alloherpesviruses genome ranges between 134-295 kbp containing 90-186 open reading frames (ORFs). The genome size of these viruses varies significantly depending on the genus to which they belong. While members of the *Ictalurivirus* genus have the smallest known alloherpesvirus genomes, ranging from 134-149 kbp, the genome of the CyHV-3 (family *Alloherpesviridae*) is the largest of all herpesviruses sequenced so far (Aoki *et al.*, 2007). Alloherpesvirus genomes have a simpler structure than that of most members of the *Herpesviridae* family. Most of them consist of only one unique region flanked by direct terminal repeats (TR). The TR of most fish herpesviruses are long compared to that of frog herpesviruses, which are either very short or undetectable (Table 2). The genome of SalHV-1, however, exhibits some peculiarities as its structure consists of a short unique region (U_S) flanked by an inverted repeats (R_S) and a longer unique region (U_L) that is not flanked by a detectable repeat. This arrangement is interestingly similar to mammalian alphaherpesviruses of the *Varicellovirus* genus, including bovine herpesvirus 1, equine herpesvirus 1, pseudorabies virus, and varicella-zoster virus (Davison, 1998).

Table 2 – Data on complete genome sequences of fish and amphibian alloherpesviruses (based on the ICTV classification)

Viral species	Genome size	Genome structure	Structures size	GC%	ORFs (No)	RefSeq
Anguillid herpesvirus 1	248.53 kbp	TR-U-TR	U: 226 kbp TR: 11 kbp	53.0	134	NC_013668.3
Cyprinid herpesvirus 1	291.14 kbp	TR-U-TR	U: 224 kbp TR: 33 kbp	51.3	143	NC_019491.1
Cyprinid herpesvirus 2	290.3 kbp	TR-U-TR	U: 260 kbp TR: 15 kbp	51.7	154	NC_019495.1
Cyprinid herpesvirus 3	295.15 kbp	TR-U-TR	U: 250 kbp TR: 22 kbp	59.2	163	NC_009127.1
Ictalurid herpesvirus 1	134.23 kbp	TR-U-TR	U: 97 kbp TR: 18 kbp	56.2	90	NC_001493.2
Ictalurid herpesvirus 2	142.92 kbp	TR-U-TR	U: 101 kbp TR: 20 kbp	53.8	91	NC_036579.1
Silurid herpesvirus 1	149.34 kbp	TR-U-TR	U :100 kbp TR :24 kbp	53.7	94	MH048901.1

Ranid herpesvirus 1	220.86 kbp	TR-U-TR	U: 219 kbp TR: 0.6 kbp	54.6	132	NC_008211.1
Ranid herpesvirus 2	231.8 kbp	TR-U-TR	U: 230 kbp TR: 1 kbp	52.8	147	NC_008210.1
Ranid herpesvirus 3	207.91 kbp	U	U: 207 kbp	41.8	186	NC_034618.1
Bufonid herpesvirus 1	158.25 kbp	U	U: 158	40.6	152	MF143550.1

1.6.3. Notable characteristics of fish alloherpesviruses

Fish alloherpesviruses appear to share several biological traits that distinguish them from herpesviruses infecting mammals, birds, and reptiles (*Herpesviridae*).

Firstly, while herpesviruses generally exhibit only mild pathogenicity in their natural immunocompetent hosts, fish alloherpesviruses can cause outbreaks with up to 100% mortality. This substantially higher virulence may be due to either a lower degree of fish herpesviruses adaptation to their hosts, or to elements linked to intensive fish farming, such as inbreeding and high-density rearing conditions. Furthermore, age-dependent sensitivity and permissivity have been reported for several alloherpesviruses of fish. For example, AciHV-1, AciHV-2, CyHV-1, CyHV-2, SalHV-2, SalHV-3 and IcHV-2 are especially pathogenic for recently hatched fish while CyHV-3 induces a lower infection in carp larvae (Hanson *et al.*, 2011; Van Beurden and Engelsma, 2012). Interestingly, it seems that as carp mature up to the juvenile stage, their susceptibility to CyHV-3 increases (Ronsmans *et al.*, 2014).

Secondly, the tropism of herpesviruses is known to be restricted to their natural host species or closely related species. Interestingly, several fish species have been thought to play a role in the epidemiology of alloherpesviruses, like AngHV-1 (Nguyen *et al.*, 2016) and CyHV-3 (Kempter *et al.*, 2012; Kielpinski *et al.*, 2010), as the presence of their genome was reported in a large number of sympatric fish species. However, the ability of these viruses to replicate in these species, and hence whether they may serve as vectors in viral transmission, remains to be confirmed.

Thirdly, as opposed to mammalian and avian hosts, the hosts of alloherpesviruses are poikilothermic, implying that their body temperature cannot be naturally maintained constant. Thus, the environmental temperature heavily influences the outcome of infection, both *in vitro* and *in vivo*. For example, the replication of AngHV-1 in eel kidney 1 (EK-1) cells occurs at temperatures between 15 and 30 °C, with an optimum around 20-25 °C (Sano *et al.*, 1990; Van Beurden *et al.*, 2012a). In contrast, low temperatures facilitates RaHV-1 replication *in vivo*, whereas induction of tumor metastasis is promoted by high temperatures (McKinnell and Tarin, 1984). Furthermore, CyHV-3 outbreaks typically occur in warm environmental water temperature (18-28 °C) (Ilouze *et al.*, 2012) while SalHV-3 outbreaks are reported when temperatures are low (6-15 °C) (Van Beurden and Engelsma, 2012). If the ambient water temperature is suboptimal for virus replication, infection caused by fish alloherpesviruses is generally less severe or even asymptomatic, providing an explanation for the seasonal occurrence of

some alloherpesviruses of fish (Gilad *et al.*, 2003). In practice, these biological traits have been exploited successfully for outbreaks management. As striking examples, successful natural immunization against CyHV-3 has been reported in carps (Ronen *et al.*, 2003), and a decrease in temperature induces the reduction of morbidity and mortality rates of AngHV-1 infections in eel culture systems (Haenen *et al.*, 2002).

And lastly, mounting data suggests that alloherpesviruses can establish latent infections. Evidence supporting this hypothesis has been provided for CyHV-1, CyHV-2, CyHV-3, SalHV-2, IcHV-1 and AngHV-1 (Chai *et al.*, 2020; Hanson *et al.*, 2011, 2016). Given that a latent state may be interrupted by transient virus reactivation, the balance between productive lytic infection and latency is intricately associated to host biology. This evidence indicates that for fish herpesviruses, water temperature plays a critical role in the transition from productive to latent infections (Hanson *et al.*, 2016).

1.7. Herpesviruses affecting fish

Over the last decade, herpesvirus infections have been responsible for high mortality outbreaks in fish. Herpesviruses have been presently identified in a wide variety of important aquaculture species, including carp, catfish, eel, salmon, and sturgeon. While our current knowledge of herpesviruses affecting fish is likely skewed towards economically important hosts, or viral species associated with significant disease, the existence of many additional alloherpesviruses is expected.

The first description of lesions caused by a fish herpesvirus can be traced to the 16th century, with the description of a pox disease of carp by the Swiss naturalist Conrad Gessner. Four hundred years later, these pox-like lesions were shown to be associated with herpesvirus-like particles (Schubert, 1966), later named CyHV-1 (Sano *et al.*, 1985). Prior to this, IcHV-1 was recognized as a fish herpesvirus, and for decades it was considered as the prototypic fish herpesvirus. Causing disease outbreaks with mass mortalities among catfish fry and fingerlings in the United States (Wolf, 1988), this virus was the first threatening herpesvirus for the fish farming industry. However, this virus affected the catfish industry only in restricted regions and the disease could be controlled through modification of management practices (Hanson *et al.*, 2011; Kucuktas and Brady, 1999).

In the late 1990s, high mortality rates in cultured carp caused by CyHV-3 (Bretzinger *et al.*, 1999; Hedrick *et al.*, 2000; Waltzek *et al.*, 2005) urged the scientific community to investigate this emerging disease. Due to its economic impact on carp farming and its rapid spread throughout the world, CyHV-3 was listed as a notifiable disease by the World Organization for Animal Health (OIE) (Michel *et al.*, 2010a). Since then, the desire to protect common and koi carp from this devastating infection prompted an increased interest to study CyHV-3. In addition, common carp (*Cyprinus carpio*) is a perfect model to study host-virus interactions, as it had already been a traditional species for fundamental research on

fish immunology (Adamek *et al.*, 2014a; Rakus *et al.*, 2013). Consequently, advances in our understanding of CyHV-3 now far surpass that of any other member of the family *Alloherpesviridae.* Generally, the impact a virus has on wildlife, the financial losses it causes to the fish farming industry, or its importance as a fundamental research object represent the main sources of scientific interest in a specific virus. One of the rare instances in which these three reasons are met is the CyHV-3 global issue, which explains why the CyHV-3 is now considered as the archetype of fish alloherpesviruses (Boutier *et al.*, 2015a). In addition to CyHV-3, the closely related CyHV-2 has been identified as the most problematic and frequently isolated virus of goldfish (*Carassius auratus*), a freshwater fish belonging to the *Cyprinidae* family, and AngHV-1 has recently been identified as an emerging threat for the critically endangered European eel (*Anguilla anguilla*). Although CyHV-2 outbreaks cause massive damage to *Carassius* spp. production and despite scientific alerts on the potential impact of AngHV-1 on the vulnerable wild eel population (Bandín *et al.*, 2014; Haenen *et al.*, 2002; Nguyen *et al.*, 2017; Van Beurden *et al.*, 2012a), host-virus interactions are still under-researched.

The cypriniviruses AngHV-1, CyHV-2 and CyHV-3 are the main protagonists of this thesis. They are described in the following section of this manuscript.

2. Three economically important cypriniviruses

AngHV-1, also known as Herpesvirus anguillae (HVA) and eel herpesvirus, is one of the main pathogens affecting eel populations in aquaculture settings as well as in the wild (Bandín *et al.*, 2014; Haenen *et al.*, 2010; Kullmann *et al.*, 2017; McConville *et al.*, 2018; Nguyen *et al.*, 2016; Van Beurden *et al.*, 2012a). This virus causes a hemorrhagic disease associated with mortality rates as high as 30% in eels reared under conditions of intensive aquaculture, resulting in significant financial losses (Chang *et al.*, 2002; Haenen *et al.*, 2002; Sano *et al.*, 1990; Van Beurden and Engelsma, 2012). Isolated for the first time in Japan in 1985 from farmed moribund European eels and Japanese eels (*Anguilla japonica*) (Sano *et al.*, 1990), AngHV-1 is now frequently detected in wild and farmed eels throughout Europe (Haenen *et al.*, 2009; Nguyen *et al.*, 2017).

CyHV-2 is the etiological agent of a highly contagious disease, named herpesviral hematopoietic necrosis disease (HVHND). Initially reported from goldfish, infections with CyHV-2 are also detected among closely related cyprinid fish species, including Crucian carp (*Carassius carassius*) (Fichi *et al.*, 2016; Zhao *et al.*, 2019) and Prussian carp (*Carassius gibelio*), also called Gibel carp (Doszpoly *et al.*, 2011; Xu *et al.*, 2013a). However, CyHV-2 infection is most severe in goldfish, where it can cause 100% mortality rates in all life stages, especially at water temperatures between 15 and 20°C (Jung and Miyazaki, 1995; Sahoo *et al.*, 2016). Additionally, a new kind of epizootic causing high mortality rates in allogynogenetic crucian carp, named hemorrhagic disease of gill, has been associated with CyHV-2 (Zhu *et al.*, 2018). Since the first reports in 1992 and 1993 in western Japan (Jung and Miyazaki, 1995),

CyHV-2 outbreaks have now been reported worldwide (Boitard *et al.*, 2016; Groff *et al.*, 1998; Jeffery *et al.*, 2007; Kalayci *et al.*, 2018; Stephens *et al.*, 2004).

CyHV-3 has become a major economic threat to both the common carp and koi farming sectors throughout the world. The highly contagious and extremely virulent disease caused by CyHV3, called KHV disease (KHVD), occurs in ponds during the spring and fall seasons, at water temperatures ranging from 18°C to 28°C (Gotesman *et al.*, 2013; Rakus *et al.*, 2013), resulting in mortality rates of 80% to 100% among the fish population (Ilouze *et al.*, 2006a). Since the first outbreaks in Germany in 1997 and in Israel and the USA in 1998 (Bretzinger *et al.*, 1999; Hedrick *et al.*, 2000; Perelberg *et al.*, 2003), the geographical range of the disease has become extensive as the disease is known to occur in, or has been reported among fish imported into, no fewer than 28 countries worldwide (OIE, 2012).

2.1. Genome

The complete viral genome sequences of AngHV-1, CyHV-2, and CyHV-3 strains derived from different geographical origins have been published (Aoki *et al.*, 2007; Davison *et al.*, 2013; Donohoe *et al.*, 2021; Li *et al.*, 2015a; Liu *et al.*, 2018a; Tang *et al.*, 2020; Van Beurden *et al.*, 2010; Wen *et al.*, 2017). The size of AngHV-1 genome (249 kbp) is close but smaller than that of its closest relatives in the genus *Cyprinivirus*. Sequencing of the CyHV-3 genome showed that it was 295 kbp in size and therefore the largest genome among the herpesviruses, followed by CyHV-1 (291 kbp) and CyHV-2 (290 kbp) (Aoki *et al.*, 2007; Davison *et al.*, 2013). Like all other fully sequenced genomes of alloherpesviruses, AngHV-1, CyHV-2, and CyHV-3 genomes all consist of one long unique region flanked by two copies of the TR, which are 11, 15, and 22 kbp in length respectively.

Van Beurden *et al.* (2010) initially described the taxonomic position and arrangement of ORFs in the AngHV-1 genome which are predicted to encode functional proteins, later refined on the basis of a transcriptomic study by the same author (Van Beurden *et al.*, 2012b). A feature map of the predicted AngHV-1 genes is shown in Fig. 5, in which the central part of the genome and the two duplicated TR regions encode for 124 and 5 ORFs, respectively.

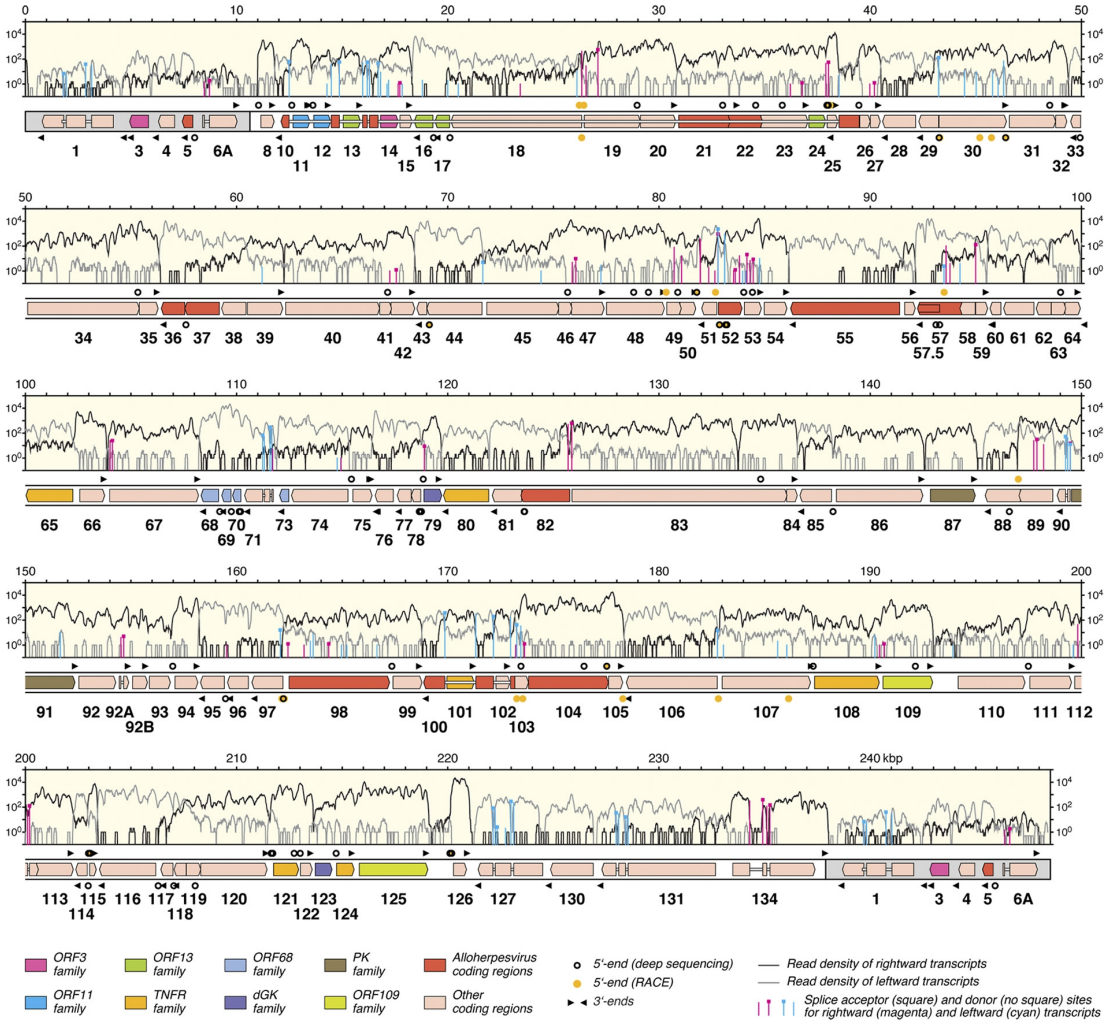

Figure 5 – Map of the AngHV-1 genome showing transcription and splicing of ORFs predicted to encode functional proteins.

The terminal direct repeats are shaded gray. ORFs are depicted as color-shaded arrows, with names (lacking the ORF prefix) below. The ORF colors indicate conservation among alloherpesviruses or families of related genes (see the key). Introns connecting spliced ORFs are shown as narrow white bars. The locations of transcript 5' and 3' ends are marked, rightward above the genome and leftward below. The light-yellow windows contain the transcriptome profile as two traces, separated into rightward (black) and leftward (gray) transcripts. The vertical lines indicate the locations of splice donor and acceptor sites supported by > 10 reads, divided into rightward (magenta) and leftward (cyan) splicing. The height of each line indicated the number of reads supporting transcription of the splice site, plotted on a log10 scale. Reproduced from Van Beurden *et al.* (2012b). Journal of Virology (2012) 86(18):10150-10161. DOI: 10.1128/jvi.01271-12.

The arrangement of ORFs in the CyHV-2 genome that are predicted to encode functional proteins was first described by Davison *et al.* (2013). A feature map of the predicted CyHV-2 genes is depicted in Fig. 6, showing the central part of the genome and the two duplicated TR regions, encoding 146 and 4 ORFs, respectively. Davison *et al.* (2013) revealed that the CyHV-2 genome contains an unusual 220-bp inverted repeat that is absent from its close relative CyHV-3, with copies to the right of ORF25C and ORF48. In CyHV-2, two ORFs (ORF25B and ORF151A) are fragmented, however it is not known whether the presumed mutations occurred *in vivo* or *in vitro*. Regarding the flanking genes, a translocation of the left end was shown, being positioned between ORF3 and ORF5, thus well within

TR in CyHV-3. As a result, the counterparts of some ORFs in TR in CyHV-3 (ORF1, ORF2, and ORF3) are found near the end of U in CyHV-2.

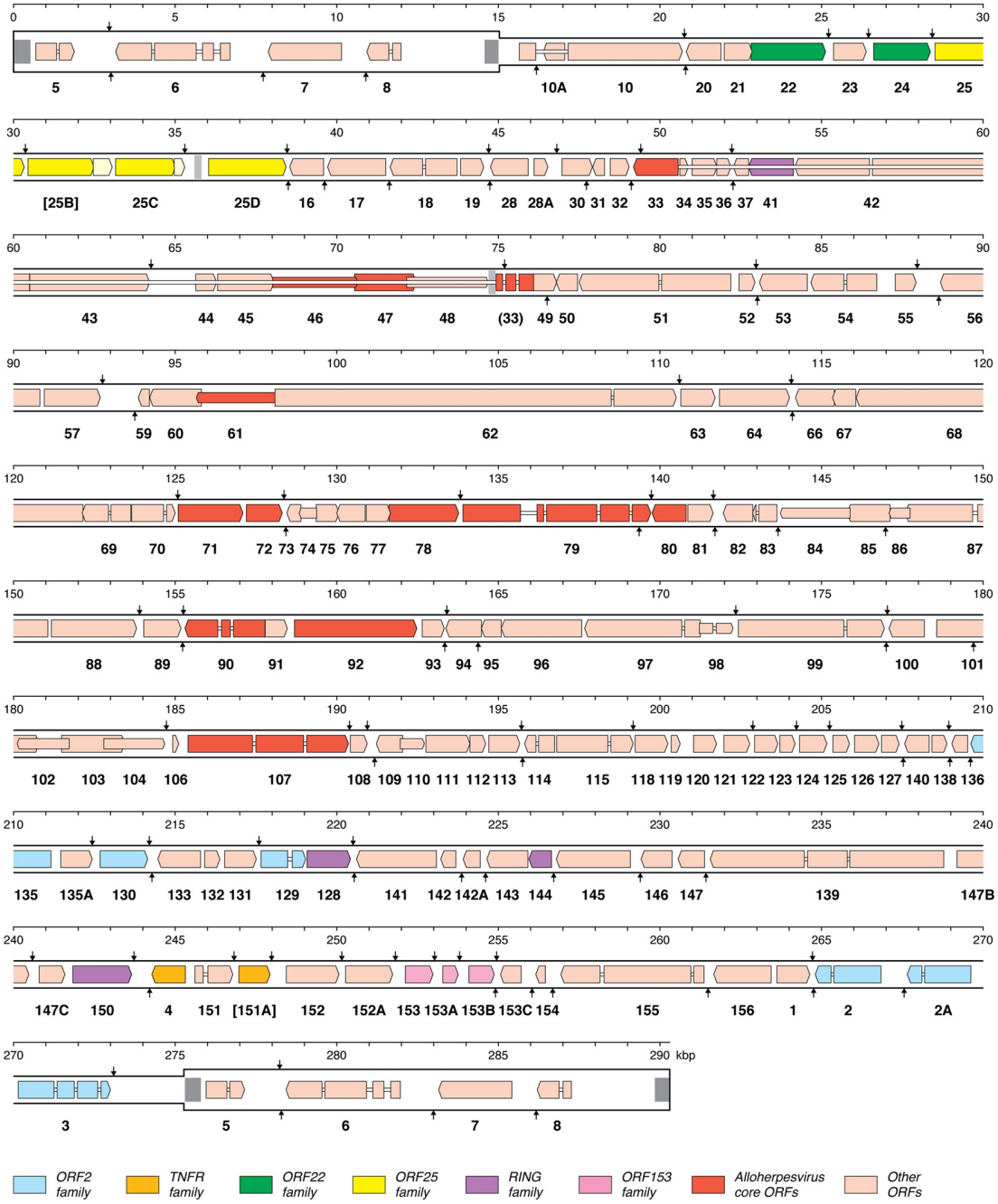

Figure 6 – Map of the CyHV-2 genome.

The terminal direct repeat (TR) is shown in a thicker format than the unique region (U). 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. Colors of protein-coding regions indicate core ORFs that are convincingly conserved among *Alloherpesviridae* species, families of related ORFs, and other ORFs. Predicted poly(A) sites are indicated by vertical arrows above and below the genome for rightward- and leftward-oriented ORFs, respectively. Inverted repeats at approximately 36 and 75 k bp are indicated by light-gray-shaded blocks. Names of fragmented ORFs are given in square brackets, with the ORFs depicted as intact. Pale yellow ORFs downstream from ORF25B and ORF25C represent remnants of additional ORFs in the ORF25 family. 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.

In the CyHV-3 genome, the annotation of ORFs predicted to encode functional proteins was first reported by Aoki *et al.* (2007), later refined based on a complete comparison with the genomes of other viruses in the genus *Cyprinivirus* and members of the other genera (Davison *et al.*, 2013). Fig. 7 contains a feature map of the predicted CyHV-3 genes, in which the central part of the genome and the two duplicated TR regions encode for 148 and 8 ORFs, respectively.

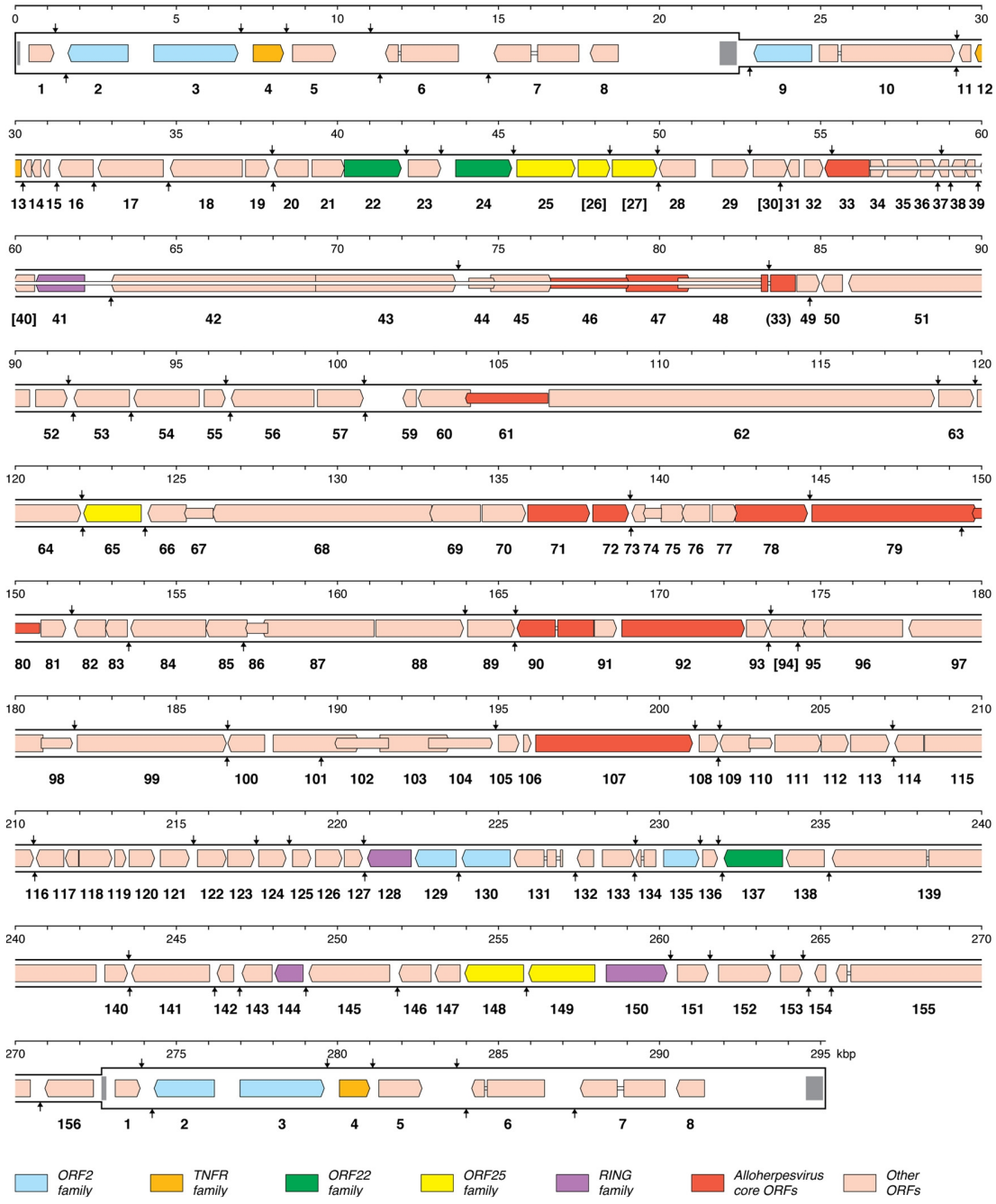

Figure 7 – Map of the CyHV-3 genome.
The terminal direct repeat (TR) is shown in a thicker format than the rest of the genome. ORFs predicted to encode functional proteins are indicated by colored arrows (see the key at the foot), with nomenclature lacking the ORF prefix given below. Introns are shown 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).

One of the peculiar features in the sequenced CyHV-3 genomes is the presence of fragmented ORFs (Aoki *et al.*, 2007), the precise set of which varies from strain to strain. Five ORFs (ORF26, ORF27, ORF30, ORF40, and ORF94) are frameshifted and likely non-functional in the reference strain KHV-U. Notably, CyHV-3 ORF26 and ORF27 are members of the ORF25 family, as is the fragmented CyHV-2 ORF25B. Evidence suggest that at least some fragmented ORFs originated *in vi*vo instead of in cell culture during viral propagation and isolation (Davison *et al.*, 2013). It has been proposed that the pathogenicity in fish farming settings has increased due to the loss of specific gene functions, consequently resulting in the emergence of the disease in common and koi carps.

Phylogenetically, the three cyprinid herpesviruses (CyHVs) are closely associated (Waltzek *et al.*, 2005), while AngHV-1 is the next most closely related to the CyHVs, and other alloherpesviruses are much more distantly related (Doszpoly *et al.*, 2008; Van Beurden *et al.*, 2010; Waltzek *et al.*, 2009). The degree of genome collinearity among the CyHVs is much higher than among the CyHVs and AngHV-1, confirming this observation (Donohoe *et al.*, 2021; Waltzek *et al.*, 2009). Consistent with their close relationships within the genus *Cyprinivirus*, the CyHVs share 120 conserved genes, of which up to 55 have counterparts in the more distantly related AngHV-1. However, as mentioned above, only 12 genes are conserved in all alloherpesviruses (Davison *et al.*, 2013) (see section 1.6.2.). The relevant ORFs in the AngHV-1 genome are highlighted in red in Fig. 5 (alloherpesvirus core ORFs 10, 21, 22, 36, 37, 52, 55, 57, 82, 98, 100, and 104). These genes encode proteins putatively involved in capsid morphogenesis (ORF36, ORF57, and ORF104), DNA replication (ORF21, ORF37, and ORF55), and DNA packaging (ORF10). The functions of the remaining 5 core genes encoding proteins (ORF22, ORF52, ORF82, ORF98, and ORF100) are unknown (Van Beurden *et al.*, 2010). Interestingly, comparisons based on three core genes conserved among all members of the *Alloherpesviridae* showed that AngHV-1 displays a slower rate of change and less positive selection than other cyprinivirus (Donohoe *et al.*, 2021).

The relevant ORFs in the CyHV-2 and CyHV-3 genomes are highlighted in red in Fig. 6 and Fig.7, respectively. Two additional genes (ORF42 and 67 in the AngHV-1 genome and ORF66 and 99 in the CyHV-2 and CyHV-3 genomes) might belong to this core set, however their conservation is less obvious. In addition to the 12 ORFs belonging to the core class and the two others mentioned above, ORF147B is possibly conserved among AngHV1, CyHV-1 and CyHV-2 (Davison *et al.*, 2013). The potential features and functions of CyHV-3 ORFs have been described in details (Boutier *et al.*, 2015a). Interestingly, there is evidence that the ancestors of CyHV-3 have captured several genes from the host cells or other viruses. Indeed, bioinformatics analysis of the CyHV-3 genome revealed the presence of genes that encode putative homologs of host or viral immune-related genes, including 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).

Cyprinivirus genomes contain gene families that have presumably arisen by gene duplication, a process generating genetic diversity that has been identified in herpesviruses of all three families. For instance, the AngHV-1 comprises eight gene families, which can be distinguished based on different colors in Fig. 5. These eight families include the ORF3 family (ORF3 which is duplicated, and ORF14), ORF11 family (ORF11 and 12), ORF13 family (ORF13, 16, 17, and 24), ORF68 family (ORF68, 69, 70 and 73), PK (Serine-threonine protein kinase) family (ORF87 and 91), TNFR (tumor necrosis factor receptor) family (ORF65, 80, 101, 108, 121, and 124), dGK (Deoxyribonucleoside kinase) family (ORF79 and 123), and ORF109 family (ORF109 and 125). These eight families are generally distant and the function of most of the proteins encoded by the ORFs remains unknown. The CyHV-2 and CyHV-3 genomes comprise six and five gene families that are shaded in distinguishingly different colors in Fig. 6 and Fig. 7, respectively. The CyHVs share the ORF2, TNFR, ORF22, ORF25 (encoding potential type I transmembrane proteins containing an immunoglobulin domain), and RING families. While being present in CyHV-2 and CyHV-3, ORF153 exists as a family of three only in CyHV-2 where it encodes potential type 3 membrane proteins. The AngHV-1 genome lacks all but the TNFR family, encoding potential immunomodulatory proteins, containing instead several other families that are different from the CyHVs (Davison *et al.*, 2013; Van Beurden *et al.*, 2012b). Recently, the host lab showed that CyHV-3 ORF12, belonging to this TNFR family, can inhibit the expression of behavioural fever by infected carp through neutralization of TNFalpha (Rakus *et al.*, 2017).

The genomes of herpesviruses are characterized as infectious because their transfection into permissive cells is enough to initiate replication of viral DNA and production of new infective viral progeny. This property has been used to generate recombinant viruses by recombination in eukaryotic and prokaryotic cells. The latter approach, used extensively for members of the family *Herpesviridae* (Tischer and Kaufer, 2012) and applied by the host lab to CyHV-3 (Costes *et al.*, 2008), is based on bacterial artificial chromosome (BAC) cloning of the whole viral genome and prokaryotic recombination technologies.

2.2. Pathogenesis

The infection cycle of all members of the family *Herpesvirales* contains two distinct phases, lytic replication, and latency (see section 1.3, Herpesvirus biological cycle). While lytic replication results in the production of progeny virions, latency consists in 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 state. Studies on several alloherpesviruses supported the existence of

these two types of infection. Most of these studies are related to CyHV-3 and provide the evidence that the switch between latency and lytic replication and *vice versa* can be regulated by the temperature of the water, enabling the virus to persist in the host population over the course of the seasons, even under non-permissive temperature conditions (Uchii *et al.*, 2014). The data related to CyHV-3 pathogenesis, as well as the limited data available for AngHV-1 and CyHV-2, are summarized below.

Productive infection - Portals of entry. Despite its importance both in cultured and wild eels, knowledge about the pathogenesis of AngHV-1, including the portal of entry, remains limited. Very recently, the host lab identified the gills and buccal mucosa as the major portals of entry and replication sites of AngHV-1 inro naïve eels inoculated by immersion in AngHV-1 contaminated water (Delrez, 2021). Indeed, *in vivo* bioluminescent imaging system (IVIS) analyses performed at 1 and 2 days post-infection (dpi) revealed expression of bioluminescent signal at the buccal mucosa, cephalic skin, gills, and tails while the rest of body and internal organs remained negative. Infection was shown to reach a systemic level at 3 dpi as bioluminescent signals were expressed in internal organs. Macro lens IVIS analysis and *in situ* immunodetection of virus replication further confirmed that the gills and the buccal mucosa covering the lips and the periodontal area are the portals of entry of AngHV-1 following inoculation by immersion in infectious water.

Studies documenting the key aspects of CyHV-2 pathogenesis (including the portal of entry) are limited. It has been previously suggested that either the gill or the skin might act as the portal of entry of CyHV-2 in goldfish (Giovannini *et al.*, 2016). The gut was also hypothesized to represent the portal of CyHV-2 entry in Gibel carp, supporting future strategies of oral vaccination (Dong *et al.*, 2022). Using a recombinant strain of CyHV-2 expressing bioluminescent and fluorescent reporter genes, the host lab recently demonstrated that the skin act as the major portal of entry of CyHV-2 in larvae (4 days post-fertilization [dpf]), juvenile (75 dpf) and adult (1.5 years old) Shubunkin goldfish following immersion in infectious water (He *et al.*, 2023). Indeed, IVIS analysis revealed that the skin was the most susceptible and permissive organ to infection at the earliest sampling points post-infection across all developmental stages. In juvenile and adult subjects, the main source of signal in the skin was on the caudal fin, suggesting a higher susceptibility to infection at this region and indicating that it may represent the main portal of entry at these life stages. Subsequent 3D renderings generated from confocal and light-sheet microscopy supported the exclusive viral localization in this tissue and histopathology examinations supported that the initial infection on the skin is followed by the spread of CyHV-2 to internal organs in larvae, resulting in a systemic infection and eventually to mortality (He *et al.*, 2023).

In early reports, CyHV-3 was suggested to enter the host through infection of the gills (Hedrick *et al.*, 2000; Ilouze *et al.*, 2006a; Miyazaki *et al.*, 2005, 2008; Pikarsky *et al.*, 2004; Pokorova *et al.*, 2005) and the gut (Dishon *et al.*, 2005; Ilouze *et al.*, 2006a). The two hypotheses were based on several observations: (*i*) the gills exhibit histopathological lesions earlier following virus inoculation by

immersion in water (Hedrick *et al.*, 2000; Pikarsky *et al.*, 2004), (*ii*) viral DNA is detected in gut and gills as early as 1 dpi (like 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 the IVIS showed that the skin is the major portal of entry for CyHV-3, following immersion in water containing virus (Fig. 8) (Costes *et al.*, 2009; Fournier *et al.*, 2012). A weak bioluminescent signal was reported 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 and the fins were reported to be the main site where the positive signal was observed (Costes *et al.*, 2009). Independent reports showing 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 further supported this finding (Miwa *et al.*, 2015). It has also been proved that fish epidermis can support early infection of a Novirhabdovirus (IHNV; infectious hematopoietic necrosis virus) in trout, which suggest that the skin represent an important portal of entry for viruses that affect fish (Harmache *et al.*, 2006). Although this mode infection mimics a natural route of infection, other epidemiological conditions could support virus entry through the digestive tract. In carp fed with material containing a CyHV-3 recombinant strain expressing luciferase as a reporter gene, bioluminescence imaging analyses performed at indicated time points (Fig. 8) showed that the major portal of entry through oral inoculation is the pharyngeal periodontal mucosa (Fournier *et al.*, 2012). This mode of infection resulted in the spread of the infection to the various organs tested, associated with clinical symptoms and mortality rates similar to the inoculation through immersion (Fournier *et al.*, 2012).

Following infection at the portal of entry, the spread of CyHV-3 in the infected fish has been demonstrated to be rapid, CyHV-3 DNA being detected in almost all tissues as early as 1-2 dpi (Gilad *et al.*, 2003; Ouyang *et al.*, 2013; Pikarsky *et al.*, 2004). Such a rapid spread could be explained by the tropism of this virus for white blood cells (Eide *et al.*, 2011). Following CyHV-3 infection, viral DNA can be isolated from blood as early as 1 dpi (Pikarsky *et al.*, 2004). During the early days post-infection, increasing viral charges are expressed by most of the organs (including those acting as portals of entry) according to time post-infection. While the cause of death is still under debate, it has been suggested that the severe CyHV-3 infection reported in kidneys and gills and the associated histopathological changes could be responsible for acute death (Gilad *et al.*, 2004; Hedrick *et al.*, 2000) and that the severe skin lesions may give rise to hypo-osmotic shock (Miwa *et al.*, 2015).

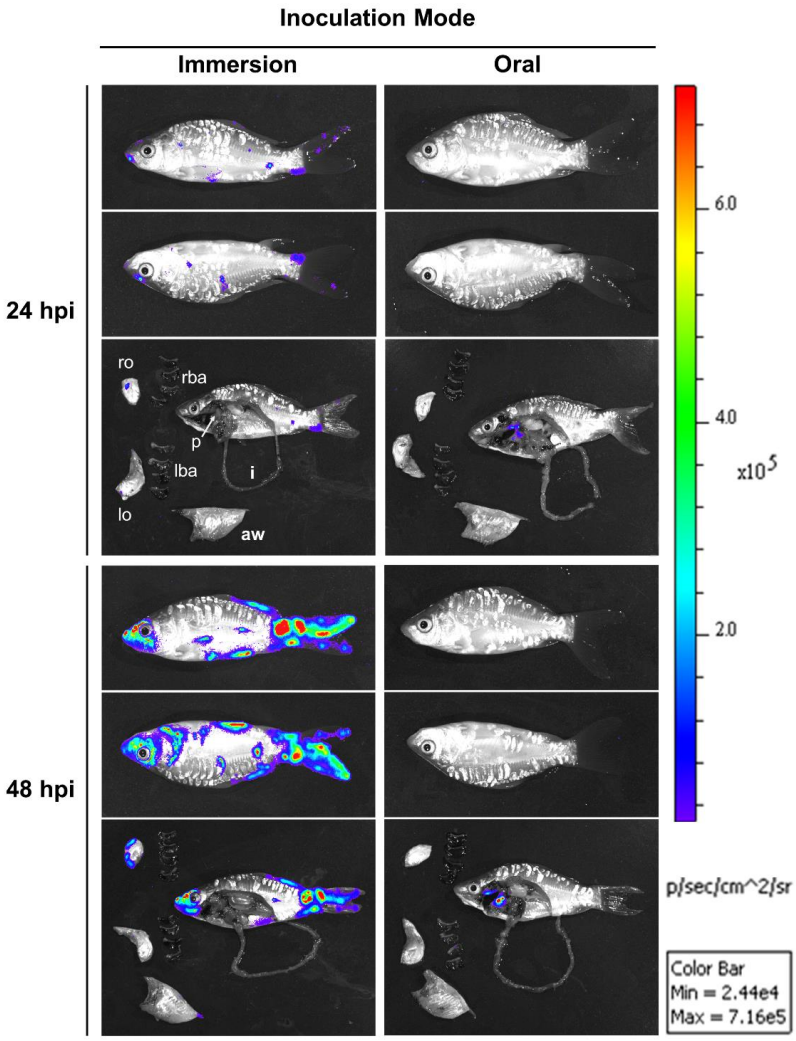

Figure 8 – The portals of entry of CyHV-3 in carp analyzed by *in vivo* bioluminescent imaging.
Two groups of fish (mean weight 10 g) were infected with a recombinant CyHV-3 strain expressing luciferase as a reporter gene either by bathing them in water containing the virus (Immersion, left column) or by feeding them with food pellets contaminated with the virus (Oral, right column). At the indicated times post-infection, six fish per group were analyzed by IVIS. Each fish was analyzed lying on its right and left side. The internal signal was analyzed after euthanasia and dissection. Dissected fish and isolated organs were analyzed for *ex vivo* bioluminescence using IVIS. One representative fish is shown for each time point and inoculation mode. Images collected over the course of the experiment were normalized using an identical 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.

Productive infection – Transmission. The nature of AngHV-1 transmission between hosts remains enigmatic. Studies focusing on the modes of transmission have been limited and the modes of inoculation used during experimental infections are sometimes unsuccessful in reproducing the infections reported during outbreaks in farming industries (Hangalapura *et al.*, 2007; Kobayashi and Miyazaki, 1997; Lee *et al.*, 1999; Ueno *et al.*, 1992). While water transmission may account for the prevalence of AngHV-1 in intensive aquaculture conditions, it may not be sufficient to enable efficient spread of this virus in the wild. Based on the *in vivo* bioluminescence imaging of luciferase expressed by a recombinant strain of AngHV-1, the host lab recently described two different modes of viral

transmission, indirectly, through water, and directly, through agonistic interactions, more specifically through biting between conspecifics (Delrez, 2021). Results showed that a high density of subjects releasing viral particles in a restricted environment is required for AngHV-1 infection through contaminated water. These conditions occur in intensive aquaculture and could have contributed partially to outbreaks and the rapid spreading of AngHV-1 within Asian and European eel farming industries (Chang *et al.*, 2002; Haenen *et al.*, 2002; Kim *et al.*, 2012; Park *et al.*, 2012; Van Beurden *et al.*, 2012a). On the contrary, viral transmission through biting is most likely acting as a mechanism to sustain transmission in natural habitats, where territorial eels engage in limited social interactions and where there is a low or declining density of hosts. Additionally, this mode of transmission is suggested to allow AngHV-1 to overcome the mucosal barrier. Whether vertical AngHV-1 transmission occurs in eels is still unclear.

Horizontal transmission of CyHV-2 within fish populations can occur by direct contact with fish that are either infected or asymptomatically carrying the virus (Goodwin *et al.*, 2009), or by indirect transmission, through an abiotic vector. However, the possible role of vectors in CyHV-2 transmission remain unexplored. At water temperatures of 13-15 °C, no resistance to disease was observed and no death occurred in CyHV-2-infected goldfish, however they were demonstrated to act as carriers to infect other fish (Ito and Maeno, 2014). In contrast to horizontal transmission, suggested to be the dominant mode of CyHV-2 transmission between fish, vertical transmission (i.e., from parent to offspring) remains understudied. Nevertheless, an epidemiological study documenting the occurrence of CyHV-2 in goldfish fingerlings, breeding fish, eggs, and fry indicates that this mode of transmission may also occur in this fish species (Goodwin *et al.*, 2009). Moreover, the presence of CyHV-2 in eggs of diseased fish has been revealed following RT-PCR, LAMP assay and electron microscopy examination, which further suggests that vertical transmission is possible (Zhu *et al.*, 2018).

Horizontal transmission of CyHV-3 can also occur by direct contact between fish or by indirect transmission. According to given epidemiological conditions, CyHV-3 has been demonstrated to enter carp through infection of the skin or infection of the pharyngeal periodontal mucosa (Fig. 8). Thus, skin to skin contact between acutely infected or carrier fish with naive ones, and cannibalistic and necrophagous behaviors of carp could represent two modes of direct transmission (Fournier *et al.*, 2012; Raj *et al.*, 2011). Interestingly, hot spots of carp breeding and mating seems to accentuate the horizontal transmission of CyHV-3 in natural ponds (Uchii *et al.*, 2011), which could involve this skin-to-skin contact (Raj *et al.*, 2011). Several potential vectors including plankton (Minamoto *et al.*, 2011), aquatic invertebrates (Kielpinski *et al.*, 2010), fish droppings (Dishon *et al.*, 2005), and mechanical transmission of sick fish by piscivorous birds (Ilouze *et al.*, 2011) have been reported for CyHV-3. Additionally, water is a major abiotic vector (Minamoto *et al.*, 2009), and virus replication in the gills, gut, and skin likely represent a source of viral excretion into the aquatic environment. The ability of CyHV-3 to

remain infective in water has been extensively investigated (Adamek *et al.*, 2013; Costes *et al.*, 2009; Dishon *et al.*, 2005; Pikarsky *et al.*, 2004). Using two experimental settings designed to enable transmission of CyHV-3 through infectious water or through infectious water and physical contact between infected and naive sentinel fish, showed that direct contact between fish promotes transmission of the virus as postulated (Boutier *et al.*, 2015b). However, this study also confirmed the high efficiency of transmission through infectious water. To date, CyHV-3 vertical transmission has not been reported.

Latent infection. Like all herpesviruses, evidence suggest that AngHV-1 can establish lifelong latent infections in eels, associated with reactivation during stressful events such as the migration back to the Sargasso Sea to spawn (Boutier *et al.*, 2021; Haenen *et al.*, 2009; Van Nieuwstadt *et al.*, 2001). The persistence of the AngHV-1 was demonstrated in cultured eels (150-200g) from a clinically healthy stock in which virus isolation appeared negative, however specific antibodies against AngHV-1 have been detected (Van Nieuwstadt *et al.*, 2001). Furthermore, reactivation of the virus could be enabled in several fish kept at 23°C, upon exogenous stress caused by dexamethasone treatment, proving that the healthy stock had been infected with AngHV-1 in the past (Van Nieuwstadt *et al.*, 2001).

Considering the length of the disease incubation period and the fact that disease is often precipitated by predisposing factors such as stress or shifts in water temperature, CyHV-2 has been generally regarded as a latent virus (Goodwin *et al.*, 2009). Chai *et al.* (2020) have recently confirmed the ability of CyHV-2 to cause latent infection in fish upon the primary infection, latency being reactivated by temperature stress *in vivo*. In addition, a novel cell line derived from the brain of Gibel carp, named GCBLat1, was identified as an *in vitro* model to study the CyHV-2 latent infection and reactivation. To date, the mechanism of latency and reactivation is however not fully established. Recent studies in cell lines have demonstrated the production of virus- and host-encoded miRNAs to promote CyHV-2 latency (Donohoe *et al.*, 2015; Lu *et al.*, 2017, 2018a, 2019). These findings indicate that CyHV-2 may establish a latent infection and/or persist in surviving fish. In *C. auratus* experimentally infected with CyHV-2, Wei *et al.* (2019) showed that the primary sites for persistent infection were the spleen and trunk kidney. Moreover, reactivation may be caused by several stress, including injuries and secondary infections, variations in water temperature, fish growth, or transport.

The vast majority of research investigating latency in alloherpesviruses has focused on CyHV-3. In addition to being detected from two months to as late as 1 year post-infection in fish surviving primary infection (Gilad *et al.*, 2004; Miwa *et al.*, 2015; Yuasa and Sano, 2009), CyHV-3 DNA can be routinely found in seemingly healthy fish (Cho *et al.*, 2014). Persistence of CyHV-3 was demonstrated in both farmed (Baumer *et al.*, 2013) and wild carp (Uchii *et al.*, 2009, 2014). At least 2 years after the initial outbreak, CyHV-3 DNA was found in the brain of both small-sized seronegative and large-sized seropositive carp from wild populations, suggesting that latently infected fish that had survived previous outbreak could transmit the virus to new naive fish (Uchii *et al.*, 2009). Netting stress and temperature

stress were reported to induce viral reactivation in fish several months after the initial exposure to CyHV-3, respectively without symptoms (Bergmann and Kempter, 2011) and with symptoms and mortality (St-Hilaire *et al.*, 2005). Notably, white blood cells have been suggested as a preferred latency site, as CyHV-3 ORF6 transcription was demonstrated to be associated with latent infection of IgM^+ B cells (Reed *et al.*, 2014). Interestingly, the similarity of one domain of ORF6 to the consensus sequences of proteins associated to alpha and gammaherpesvirus latency has been reported (Boutier *et al.*, 2015a). Additionally, CyHV-3 DNA was found in several tissues of fish harboring long-term infections, especially in the brain. However, whether or not the nervous system can be considered as an additional latency site (as observed in alphaherpesviruses) remains to be definitively proven (Boutier *et al.*, 2015a). Finally, miRNAs able to modulate viral gene expression were found to be encoded by CyHV-3 (Donohoe *et al.*, 2015). CyHV-3 miRNAs, like those encoded by members of the family *Herpesviridae* as a non-immunogenic mechanism of gene regulation, may also be involved in long-term maintenance of a latent infection. However, additional studies are necessary to determine the potential role of CyHV-3 miRNAs during latency.

2.3. Host-pathogen interactions

Susceptibility of hosts according to the developmental stage. Using *in vivo* bioluminescence imaging paired with an AngHV-1 strain expressing luciferase, the host lab has recently demonstrated that susceptibility to the virus increased according to the developmental stage of European eel, with glass eels being permissive but not susceptible to AngHV-1 (Delrez, 2021). This absence of susceptibility during the first stages of life can be explained by different hypotheses. Firstly, several cellular receptors mediating viral binding and entry are required for herpesvirus entry (Spear and Longnecker, 2003). These receptors could develop only during ontogenesis and thus be absent or not functional during early stages of fish (Ronsmans *et al.*, 2014). Secondly, early life stages of fish rely mainly on the innate immune system as the specific adaptive response mature later in life (Ronsmans *et al.*, 2014; Vadstein *et al.*, 2013). Thus, immune mechanisms in early developmental stages could be expressed at a higher or more efficient level than that in older stages.

While age-dependent susceptibility has been reported for some alloherpesviruses (Boutier *et al.*, 2021; Van Beurden and Engelsma, 2012), younger animals are more susceptible to diseases in most cases. For instance, CyHV-2 is particularly pathogenic for young fry (Groff *et al.*, 1998; Jeffery *et al.*, 2007; Jung and Miyazaki, 1995), at water temperatures between 15 and 25°C (Jeffery *et al.*, 2007). Indeed, while CyHV-2 can affect all life stages of goldfish, including egg, fry, fingerling and adult fish, the susceptibility of juveniles to infection was shown to be higher than that of adults, leading to severe mortality in less than 1 year old fish (Groff *et al.*, 1998). Interestingly, a recent study performed by the host lab showed that Shubunkin goldfish larvae are less susceptible but more permissive to CyHV-2

compared to juvenile and adult goldfish, ultimately resulting in rapid systemic infection associated to much higher mortality rates in the former (He *et al.*, 2023).

The susceptibility of common carp to CyHV-3 has been demonstrated to increase during ontogenesis. Indeed, the host lab reported that carp larvae are susceptible and permissive to CyHV-3 infection directly after hatching and that an increase in susceptibility is observed according to the developmental stage (Ronsmans *et al.*, 2014). Compared to juvenile and fingerling stages (>21 days post-hatching [dph]), the susceptibility of embryo and larval stages (>21 dph) to CyHV-3 infection was shown to be limited (Ronsmans *et al.*, 2014). This lower susceptibility reported for the early developmental stage was primarily due to the epidermal mucus being substantially more inhibitory to CyHV-3 entry in the host at this life stage than in older stages. The lower permissivity of earlier carp developmental stages to CyHV-3 replication relative to later stages, resulting in reduced mortality in the former, is in stark contrast to what was recently observed in earlier goldfish developmental stages upon CyHV-2 infection (He *et al.*, 2023). This difference between closely related CyHV-2 and CyHV-3 has been hypothesized to be the result of differing adaptation to their hosts, or host-habitat (He *et al.*, 2023).

Host immune response. There are still major gaps in our understanding of the host-pathogen interaction between AngHV-1 and its natural host. Very few studies examine the immune response of European eel, which hinders our knowledge on host-pathogen interactions (Nielsen and Esteve-Gassent, 2006). The molecular ontogeny of larval immunity in European eel at increasing temperatures was investigated by Miest *et al.*, with the aim of characterizing the response of specific immune genes potentially involved in both the innate and adaptive immune responses to AngHV-1 infection *in vitro* (Miest *et al.*, 2019). Pro-inflammatory responses were reported upon AngHV-1 infection of juvenile eel tail explants and were characterized by the up-regulation of cytokine interleukin 1β (IL1β), and major histocompatibility complex II (MHC-II). Interestingly, the up-regulation of antiviral responses markers, i.e., interferon regulating factors (IRF) 3 and 7, was not observed. This might be related to viral inhibition of interferon response, which has been reported elsewhere with other members of the family *Alloherpesviridae* (Adamek *et al.*, 2012; Adamek *et al.*, 2014b; Schulz *et al.*, 2019). Their findings are in line with the initial phase of mucosa response observed in CyHV-3 infection (Adamek *et al.*, 2014b; Miest *et al.*, 2019). In addition, the influence of AngHV-1 infection on the innate immunity of European eels was recently investigated through measurements of spleen phagocyte respiratory burst activity and potential killing activity, as well as pronephros lymphocyte proliferation stimulated by concanavalin A (lectin family) or lipopolysaccharide (Schulz *et al.*, 2019). In AngHV-1 infected fish, all measured parameters were shown to be significantly lower than in the control group, which suggests an immunosuppressive effect of the virus on cellular defense mechanisms in European eel. These observations are consistent with the similar research done on CyHV-3 infected carp (Siwicki *et al.*, 2012).

Studies investigating the host immune response in CyHV-2 infections in fish are very limited. Water temperature is a factor that plays a significant role in host-CyHV-2 interactions as high water temperature treatments are shown to promote immunity against CyHV-2 in survivor fish (Nanjo *et al.*, 2017). This could be related to an increased efficiency of the innate immune system at higher water temperature, leading to reduced mortality. Immunological analysis remains limited for CyHV-2 infections, as immunological tools available for goldfish, such as antibodies specific to goldfish T-cell subsets, are still lacking. Used in many fish immunology studies, the clonal ginbuna crucian carp *C. auratus langsdorfii* might be a promising model species for the study of CyHV-2 infection and immunity (Nanjo *et al.*, 2017). To provide insight on the development of antiviral therapies and crucial knowledge regarding viral pathogenicity, the differential gene expressions were studied in moribund and surviving CyHV-2-infected crucian carp using suppression subtractive hybridization (SSH) followed by the sequencing and analyses of expressed sequence tags (ESTs) (Xu *et al.*, 2014a). Large differences were noted in the differential gene expression profiles between the moribund and survivor fish group. The overexpression of keratin8, MPO, dusp1, NF-kB inhibitor, Rab GTpase (Rab21), and small GTP binding protein (Rac2) genes in response to CyHV-2 infection was confirmed following further characterization of these genes, demonstrating their potential as marker genes in disease investigations (Podok *et al.*, 2014; Xia *et al.*, 2016).

Using digital gene expression tag profiling (DGE) from both control and moribund fish, another study on the expression profiling of kidney tissue of silver crucian carp revealed that around 2912 genes were differentially expressed, among which 1422 were up-regulated and 1490 were down-regulated (Lu *et al.*, 2017). Moreover, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed that genes implicated in proteasome, neuro-active ligand-receptor interaction, calcium signaling pathway and peroxisome proliferator-activated receptors (PPAR) signaling pathways were enriched in infected fish. In addition, quantitative RT-PCR revealed an up-regulation of MHC-I, interferon regulatory factor 3 (IRF3) and mitogen-Activated Protein Kinase 7 (MAPK7) genes in CyHV-2 infected silver crucian carp (Lu *et al.*, 2017). In Gibel carps, host miRNAs were recently reported to be involved in CyHV-2 infection and to be implicated in the regulation of apoptosis and immune-related genes (Lu *et al.*, 2018a). Crucian carp IFNc (ccIFNc) were identified and characterized as members of the type I interferon family, with a potential role in countering CyHV-2 infection (Xia *et al.*, 2018). CyHV-2 miRC12 has been reported to suppress virus-induced apoptosis and to promote virus replication by targeting caspase 8 (Lu *et al.*, 2019). The authors also demonstrated that the expression of caspase 8 was reduced and that the apoptosis induced by CyHV-2 was inhibited following the over-expression of miRC12. Moreover, the complement C3 gene from Gibel carp, named CagC3, was recently cloned and sequenced and was proved to participate in the innate immune response of Gibel carp to CyHV-2 infection (Fan *et al.*, 2020).

The skin and/or pharyngeal periodontal mucosa of carp act as portals of entry of CyHV-3 (Fig. 8) (Costes *et al.*, 2009; Fournier *et al.*, 2012). These mucosal epithelia are covered by mucus, which is an immune component that plays a crucial role as a physical and chemical barrier against pathogens (Dash *et al.*, 2018; Ellis, 2001; Tiralongo *et al.*, 2020; Vadstein *et al.*, 2013). The mucus layer comprises a wide variety of proteins, including enzymes, mucins, immunoglobulins, antimicrobial peptides, and lytic agents, which have the capacity to neutralize microbes (Ellis, 2001; Shephard, 1994; Van Der Marel *et al.*, 2012). Interestingly, the skin mucus was shown to act as an innate barrier that inhibits, at least partially, CyHV-3 binding to epidermal cells through neutralization of viral infectivity as demonstrated by *in vitro* assay (Raj *et al.*, 2011). Ronsmans *et al.* (2014) showed that the low sensitivity of carp larvae to CyHV-3 infection was circumvented by a mucus removal treatment, which suggests a crucial role of skin mucus in protecting larvae against CyHV-3, especially at this developmental stage which does not yet benefit from a mature adaptive immune system. The immune response against CyHV-3 has been studied in the skin and gut of common carp (Adamek *et al.*, 2013; Syakuri *et al.*, 2013). In the skin, CyHV-3 infection results in the down-regulation of genes that encode 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), in addition to changes in the cutaneous bacterial flora and subsequent development of secondary bacterial infections (Adamek *et al.*, 2013). The activation of interferon class I pathways and the up-regulation of pro-inflammatory cytokine IL-1B and inducible nitric oxide synthase (iNOS) were also reported in the studies above (Adamek *et al.*, 2013; Syakuri *et al.*, 2013).

Interferons (IFNs) are secreted mediators that play crucial roles in the innate immune response against viruses. Secretion of IFN type I by common carp brain (CCB) cells was observed in response to spring viremia of carp virus (SVCV) but not CyHV-3 infection, suggesting that this crucial antiviral pathway can be inhibited by CyHV-3 *in vitro* (Adamek *et al.*, 2012). Moreover, activation of the IFN type I response and reduction of CyHV-3 spreading in CCB cells were reported when these cultured cells were stimulated with Poly (I:C) prior to CyHV-3 infection (Adamek *et al.*, 2012). Upon CyHV-3 infection *in vivo*, a systemic IFN type I response is induced in carp skin, intestine, and head kidney, and a correlation between the magnitude of IFN type I expression and the virus load is observed (Adamek *et al.*, 2013, 2014a; 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). Other *in vitro* studies suggested that, unlike SVCV, CyHV-3 does not induce apoptosis (Miest *et al.*, 2015), and that CyHV-3 inhibits the activity of stimulated macrophages and proliferative response of lymphocytes in a temperature-dependent manner (Siwicki *et al.*, 2012). Following CyHV-3 infection, the stimulation of the apoptosis intrinsic pathway *in vivo* was delayed to

14 dpi, as detected by the expression of pro-apoptotic proteins (Apaf-1, p53, and Caspase 9) (Miest *et al.*, 2015).

A further transcriptomic study revealed the wide array of immune-related genes implicated in the systemic immune response against CyHV-3 (Rakus *et al.*, 2012). The response of two carp lines (R3 and K) with different resistance to CyHV-3 was investigated by using DNA microarray and real time PCR. The more resistant carp line exhibited a significantly higher expression of several immune related genes, including those involved in development of adaptive mucosal immunity, complement activation, MHC-I restricted antigen presentation, and pathogen recognition. In addition, higher activation of $CD8^+$ T cells was demonstrated following real time quantitative PCR (qPCR)-based analysis. Thereby, a correlation between the differences in resistance to CyHV-3 and the differential expression of immune-related genes can be established (Rakus *et al.*, 2012). Regarding the acute phase response induced by CyHV-3 infection, an up-regulation of complement-associated proteins and C-reactive proteins was also reported by 3 dpi, indicating a strong and rapid innate immune response (Pionnier *et al.*, 2014).

The systemic adaptive immune response to CyHV-3 infection has been investigated through measurements of 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). A slight cross-reaction by enzyme-linked immunosorbent assay (ELISA) and western blotting (WB) of anti-CyHV-3 antibodies to CyHV-1 was reported, most likely because some epitopes are shared by these two closely related viruses (Adkison *et al.*, 2005; Davison *et al.*, 2013; St-Hilaire *et al.*, 2009). Detection of anti-CyHV-3 antibodies begins at 7-14 dpi, increases till 20-40 dpi, and finally gradually decreases with significant titers still measured at 150 dpi (Perelberg *et al.*, 2008; Ronen *et al.*, 2003). During these periods, protection against CyHV-3 challenge correlates with the anti-CyHV-3 antibody response. Moreover, while the titer of anti-CyHV-3 antibodies in previously infected fish is only slightly higher or comparable to that of naïve fish at 280 dpi, immunized fish remain resistant to a lethal challenge, this being possibly due to the subsequent rapid response of B and T memory cells to antigen re-stimulation (Perelberg *et al.*, 2008).

The discovery of a new immunoglobulin isotype, IgT (or IgZ) specialized in mucosal immunity, 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).

Cyprinivirus genes involved in immune evasion. Herpesviruses have evolved sophisticated immune evasion strategies to counter the host immune response (Alcami, 2003; Griffin *et al.*, 2010; Horst *et al.*, 2011). Within alloherpesviruses, two viral homologs of cellular interleukin (vIL-10) have been reported

in AngHV-1 and CyHV-3, encoded by ORF25 and ORF134, respectively (Aoki *et al.*, 2007; Van Beurden *et al.*, 2011). Highly conserved evolutionarily, cellular IL-10 is an essential multifunctional anti-inflammatory element of the regulatory response. In addition of being detected in nearly all infections in vertebrates, IL-10 has also been suggested to have an immunosuppressive role in fish species (Van Beurden *et al.*, 2011). Orthologues of cellular IL-10 are encoded by herpesviruses and poxviruses and appear to have been acquired from their host on various independent occasions throughout evolution (Ouyang *et al.*, 2014). Recently, common carp IL-10 was reported to exhibit prototypical activities identified in mammalian IL-10, including anti-inflammatory activities on neutrophils and macrophages, stimulation of $CD8^+$ memory T cells, and stimulation of the differentiation and antibody secretion by IgM^+ B cells (Piazzon *et al.*, 2015). It remains to be confirmed if the viral homologue of cellular IL-10 encoded by CyHV-3 ORF134 exhibits comparable properties to carp IL-10. CyHV-3 ORF134 was suggested to encode a functional IL-10 homologue in an *in vivo* study using a zebrafish (*Danio rerio*) embryo model (Sunarto *et al.*, 2012). Indeed, the number of lysozyme-positive cells was shown to increase to a similar level as observed with zebrafish IL-10 mRNA following injection of CyHV-3 IL-10 mRNA into zebrafish embryos. Moreover, the down-regulation of the IL-10 receptor long chain (IL-10R1), performed using a specific morpholino, resulted in the vanishment of this effect (Sunarto *et al.*, 2012). As revealed by the proteomic analysis of the viral supernatant from CyHV-3 infected cells, the vIL-10 encoded by ORF134 is the second most abundant protein of the CyHV-3 secretome (Ouyang *et al.*, 2013). This IL-10 homolog was however shown to be essential neither for viral replication *in vitro* nor for virulence *in vivo*, as a recombinant CyHV-3 strain with the deletion of ORF134 displayed a replication *in vitro* and a mortality rate *in vivo* that were comparable to that displayed by its parental and revertant strains (Ouyang *et al.*, 2013). As for AngHV-1, further structural and functional characterization of the vIL-10 encoded by ORF25 is necessary to investigate its potential role in immune evasion during infection.

In addition to IL-10, members of the TNFR superfamily of cytokines were also found to be targeted by viruses. Soluble viral TNFRs might inhibit the induction of apoptosis by blocking the activity of tumor necrosis factor (Alcami, 2003). Secreted forms of TNFR have been predicted in members of the genus *Cyprinivirus*. For instance, the potential immunomodulatory proteins encoded by AngHV-1 ORF101 and ORF124 both contain a conserved domain of TNFR (Van Beurden *et al.*, 2010). The secretome of CyHV-3 was characterized by Ouyang *et al.* (2013), revealing that ORF12 was the most abundant secreted viral protein in the supernatant of infected CCB cells. Rakus *et al.* (2017) demonstrated that common carp express behavioral fever in response to CyHV-3. This process, where ectotherms increase their body temperature by moving to warmer places to limit pathogen infection, appears to confer an advantage to infected hosts. Interestingly, a soluble decoy TNFR encoded by CyHV-3 ORF12 can bind to host TNF-alpha, which is a crucial component in the induction of behavioral fever. The manifestation of behavioral fever by the fish can be delayed upon the expression of this decoy

receptor, consequently promoting CyHV-3 replication in its host (Rakus *et al.*, 2017). Whether anguillid hosts of AngHV-1 also exhibit a similar type of temperature preference during infection remains unclear. If so, putative AngHV-1 encoded TNFRs (ORF101 and 124) may potentially act in comparable ways, however this area remains currently unexplored.

Furthermore, embryonic lethality, morphological defects, and increasing apoptosis were induced by the overexpression of CyHV-3 ORF4, which encodes a viral homologue of the herpes virus entry mediator (HVEM), a member of the TNFR superfamily, and CyHV-3 ORF12 in zebrafish embryos (Yi *et al.*, 2015). The ORF4, belonging to the TNFR family, was also found among the predicted functional proteins encoded by the CyHV-2 SH-01 strain (Yang *et al.*, 2022). This same study showed that the CyHV-2 ORF30 codes for a protein homologous to the late lytic protein BDLF3 of the Epstein-Barr virus (EBV). Interestingly, BDLF3 was recently identified as a protein implicated in EBV immune evasion, impairing $CD8^+$ T cell recognition by targeting cell surface MHC class I molecules for ubiquitination and proteasome-dependent down-regulation and contributing to evasion of CD4+ cell responses by targeting MHC class II molecules (Quinn *et al.*, 2016).

Analysis of the CyHV-3 transcriptome revealed that CyHV-3 ORF112 is expressed as an IE gene (Ilouze *et al.*, 2012) and that its 278 amino acid expression product is incorporated into the virion (Michel *et al.*, 2010b). Although no homology has been detected for the N-terminal end of the protein, the C-terminal part encodes a functional Zalpha domain which consists of 66 amino acids binding to left-handed dsDNA (Z-DNA) or left-handed dsRNA (Z-RNA) (Athanasiadis, 2012). Zalpha domains have been found in three vertebrate cellular proteins (ADAR1, DAI and PKZ) that belong to the host innate immune response and in three viral proteins (E3L encoded by most *Chordopoxviridae*, ORF112 encoded by CyHV-3 and I73R encoded by African swine fever virus), which act as immune evasion factors. One of the three cellular proteins is the Z-DNA-dependent protein kinase (PKZ), which is encoded only by Cypriniformes and Salmoniformes (Rothenburg *et al.*, 2005) and is a paralog of the dsRNA-dependent protein kinase (PKR) expressed by all vertebrates. PKR and PKZ are interferon-induced proteins which play crucial roles in antiviral innate immunity. As CyHV-3 ORF112 contains a Zalpha binding domain, it was suggested to be able to over-compete the binding of PKZ to Z-DNA and that the latter protein plays important roles in the innate immune response of carp against CyHV-3 (Tomé *et al.*, 2013). Interestingly, genome editing of CyHV-3 by Diallo *et al.* (2023) revealed that the expression of only the Zalpha domain of ORF112 was sufficient for normal viral replication *in vitro* and virulence *in vivo* in adult fish and that its deletion was lethal for the virus. Moreover, viral replication could be rescued only by Zalpha domains expressing Z-binding activity, the capacity to induce liquid-liquid phase separation (LLPS), and A-to-Z conversion.

Finally, the CyHV-3 genome encodes the ORF16, which codes for a potential G-protein coupled receptor (GPCR) and could also be involved in immune evasion (Aoki *et al.*, 2007; Michel *et al.*, 2010a).

However, no significant reduction of virulence was observed *in vivo* following deletion of CyHV-3 ORF16 using BAC mutagenesis (Costes *et al.*, 2008).

2.4. Host range

2.4.1. Cell lines susceptible to AngHV-1, CyHV-2, and CyHV-3

AngHV-1 can be grown in cultured cells, including cell lines derived from Japanese eel kidney (EK 1) (Chen *et al.*, 1982), Japanese eel ovary (EO-2) (Chen and Kou, 1981; Ueno *et al.*, 1992), marbled eel fin (MECF and DMEPF) (Pao *et al.*, 2018, 2019), and European eel liver (EL) (Zheng *et al.*, 2020). Cell lines permissive to AngHV-1 have also been derived from non-eel species, such as black grouper kidney (BGK) (*Epinephelus awoara*), colored carp fin (CCF), colored carp testis (CCT), common carp fin (CF), or tilapia ovary (TO 2) (*Serotherodon mossambica* X) (Ueno *et al.*, 1992, 1996). Other generally used cell lines like BF 2 (bluegill fin, *Lepomis macrochirus*), CCB, CCG (colored carp gill), CCO (channel catfish ovary), CHSE-214 (Chinook salmon embryo; *Oncorhynchus tshawytscha*), EPC (epithelioma papulosum cyprinid, *C. carpio*), and RTG 2 (rainbow trout gonad, *Salmo gairdneri*) are not susceptible to infection with AngHV-1 (Ueno *et al.*, 1992; Zheng *et al.*, 2020).

A cytopathic effect (CPE) is normally induced following viral replication in permissive cell lines, leading to the detachment of dead cells from the substrate. CPE gives rise to the formation of characteristic plaques, named viral plaques, which morphology evolve depending on time post infection. The morphology of viral plaques can vary from small foci of infection to large areas of CPE, and includes spindle-shaped plaques of varying sizes, called syncytial plaques. They are formed by the fusion of infected cells, resulting in the formation of multinucleated expanding cells. This event is enabled by the surface expression of viral proteins with fusion properties. Interestingly, syncytia is only observed during infections with viruses able to fuse directly on the cell surface without endocytosis (Salsman *et al.*, 2005). The fitness of viruses in cell culture is related to their reproduction rates *in vitro*. The adaptability of the virus to the cell can be determined by measuring the plaque size on the infected cell monolayer (Goh *et al.*, 2016).

Typical CPE is observed upon AngHV-1 infection, involving rounding of the infected cell, vacuolization, and increased cell volume. Characteristic viral plaques begin with many cells rounded, semi-suspended, fused, and result in syncytia formation and cell detachment (Ueno *et al.*, 1992; Zheng *et al.*, 2020). Isolation and adaptability of AngHV-1 in cultured cells can vary depending on the cell line and field strain used (Ueno *et al.*, 1996). Nevertheless, well-adapted laboratory strains usually can reach titers up to around 10^7 plaque forming units (PFU)/ml (Davidse *et al.*, 1999; Pao *et al.*, 2018; Ueno *et al.*, 1996; Zheng *et al.*, 2020).

CyHV-2 can be isolated from cultured cells. Early studies on viruses obtained from ill fish revealed that CyHV-2 could not be passaged more than five times, which posed a serious obstacle to *in vitro* experiments (Thangaraj *et al.*, 2021). In 1995, the virus was successfully isolated from goldfish samples using fathead minnow (FHM) cell line and CPE was observed (Jung and Miyazaki, 1995). Using a Koi-Fin cell line, Xu *et al.* (2013) isolated and identified CyHV-2 from Gibel carp. At present, a wide range of cell lines have been successfully developed from various tissues derived from CyHV-2 host species. For example, Ma *et al.* (2015) established and characterized a new cell line derived from the brain of Gibel carp, called GiCB. During the same period, a cell line derived from the fin of the Ryukin goldfish, named RyuF-2, was developed and enabled reproducible virus culture reaching a titer of $10^{5\text{-}6}$ $TCID_{50}$ ml^{-1} (Shibata *et al.*, 2015). More recently, Lu *et al.* (2018b) established the GiCF cell line, derived from the caudal fin of Gibel carp. In this cell line, apoptosis was observed upon CyHV-2 infection. Very recently, a highly sensitive cell line, called FtGF, was established from the Fantail goldfish Fin by Dharmaratnam *et al.* (2020). It was revealed that these cells could support more than 20 serial passages of CyHV-2 with a titer of $10^{7.8 \pm 0.26}$ $TCID_{50}$ mL^{-1} during early passage levels (Thangaraj *et al.*, 2021). In infected cells, CyHV-2 induces CPE which involve pyknosis, granulation, cytoplasmic vacuolization, syncytium formation in focal areas, cell rounding, formation of holes and complete detachment of the cell monolayer (Daněk *et al.*, 2012; Ito *et al.*, 2013; Jeffery *et al.*, 2007; Jung and Miyazaki, 1995; Ma *et al.*, 2015; Sahoo *et al.*, 2016; Xu *et al.*, 2013a).

CyHV-3 can be cultivated in a variety of cell lines derived from tissues of koi and common carp brain, fin, and gills. A comprehensive list of all cell lines that are known to be permissive or not to CyHV-3 replication is provided in Boutier *et al.* (2015a). Very recently, Rakus *et al.* (2019) demonstrated that CyHV-1 and CyHV-3 can replicate in the adult zebrafish cell line SJD.1 and in the 1-day-old zebrafish embryo cell line ZF4, however there was no CPE detected. Cytoplasmic vacuolization and increased cell volume are observed during typical CPE elicited by CyHV-3 (Pikarsky *et al.*, 2004). CyHV-3-infected cells form syncytial virus plaques, and different plaque sizes have been reported in recombinant strains with deletions of non-essential glycoproteins and essential virulence genes (Boutier *et al.*, 2017; Ilouze *et al.*, 2006b; Vancsok *et al.*, 2017). In later stages, infected cells become rounded and detach from the substrate. Most infectious viral particles can be recovered from the supernatant, or cell-free fraction, of infected cells (Gilad *et al.*, 2003). Fitness of CyHV-3 *in vitro* seems to vary between field and laboratory strains and according to the cell line used. However, well adapted laboratory strains can generally reach titers up to 10^6 to 10^7 PFU/ml (Ilouze *et al.*, 2006a), or even higher as shown in a recent study investigating the effect of parameters like the multiplicity of infection, time of infection and time of harvest on the production of virions in cultured cells (Mletzko *et al.*, 2017).

2.4.2. Fish species susceptible to AngHV-1, CyHV-2, and CyHV-3

To date, AngHV-1 has been detected in farmed American eel (*A. rostrata*), farmed and wild European eel, farmed Giant mottled eel (*A. marmorata*), farmed Japanese eel, and farmed Short finned eel (*A. australis*). Investigation of the presence of AngHV-1 in the native ichthyofauna in Poland revealed that 5 species out of 20, i.e., European perch (*Perca fluviatilis*), pikeperch (*Stizostedion lucioperca*), Prussian carp, round goby (*Neogobius melanostomus*), and sterlet (*Acipenser ruthenus*), appeared positive (Nguyen *et al.*, 2016). Even if herpesviruses are known to be host specific, the capacity of AngHV-1 to replicate or remain viable in these non-anguillid species has not been shown yet. Therefore, whether they can be considered healthy carriers or vector species is still unclear. Finally, the susceptibility of experimentally infected common carp to AngHV-1 has been demonstrated by Ueno *et al.* (1992).

In addition to goldfish, CyHV-2 can also cause outbreaks in other related cyprinid species. Following experimental challenge, all three varieties of goldfish, Ryukin, Edonishiki and Ranchu, were susceptible to CyHV-2 while no disease was observed in *C. auratus buergeri*, *C. auratus grandoculis*, *C. auratus langsdorfii*, and common carp (Ito and Maeno, 2014). The first reports of CyHV-2 infection in Gibel carp occurred in 2011 in Hungary (Doszpoly *et al.*, 2011). It was later demonstrated that CyHV-2 affect fish both in aquaculture settings and in the wild (Daněk *et al.*, 2012; Wang *et al.*, 2012a). There is evidence that this virus also infects other fish species, such as Crucian carp and hybrids resulting from the crossing of *C. gibelio* (female) and *C. carpio* (male) (Fichi *et al.*, 2013; Wu *et al.*, 2013). In China, diseased *Aristichthys nobilis* (Bighead carp), *Erythroculter ilishaeformis*, *Culter alburnus*, *Hypophthalmichthys molitrix* (Silver carp) and *Mylopharyngodon piceus* (Black carp) exhibiting clinical features akin to that of *C. auratus* suffering from gill hemorrhagic disease were shown to be positive for CyHV-2 following diagnosis by LAMP assay and electron microscopy examination (Zhu *et al.*, 2018). Taken together, these findings suggest that the host range of CyHV-2 may be broader than that of related viruses belonging to the *Cyprinivirus* genus.

CyHV-3 can infect a large range of species; however, it only causes diseases in common and koi carp. This virus can infect hybrids of koi × goldfish and koi × crucian carp, associated with mortality rates of 35% and 91%, respectively (Bergmann *et al.*, 2010a), and to a lesser degree, hybrids of common carp × goldfish, with a rather low mortality rate (5%) (Hedrick *et al.*, 2006). It has been suggested that cyprinid and non-cyprinid fish species, as well as freshwater mussels and crustaceans, can act as vector of CyHV-3, this virus having been detected by PCR in these species (Table 3) (El-Matbouli *et al.*, 2007; El-Matbouli and Soliman, 2011; Fabian *et al.*, 2013; Kempter *et al.*, 2009, 2012; Kempter and Bergmann, 2007; Kielpinski *et al.*, 2010; Radosavljevic *et al.*, 2012). Cohabitation experiments suggest that CyHV-3 can be carried asymptomatically and disseminated to permissive carp by 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) (Bergmann *et al.*, 2010b; El-Matbouli

and Soliman, 2011; Fabian *et al.*, 2013; Kempter *et al.*, 2012; Radosavljevic *et al.*, 2012). According to a growing body of research, CyHV-3 might infect goldfish asymptomatically (Bergmann *et al.*, 2010b; El-Matbouli and Soliman, 2011; Sadler *et al.*, 2007), despite notable discrepancies in the literature (Yuasa *et al.*, 2013). *In vitro* research supported this observation by demonstrating that CyHV-3 can proliferate and induce CPE in cell cultures derived from common and koi carp but also from goldfish and silver carp (Davidovich *et al.*, 2007). The OIE lists one KHVD susceptible species (*C. carpio* and its hybrids) and several fish species suspected to be carriers (Atlantic sturgeon, catfish, goldfish, grass carp, ide, and Russian sturgeon) (OIE, 2016). Interestingly, this virus was recently detected in migratory wild ducks in North America, pointing to a potential involvement of aquatic birds in the spread of CyHV-3 (Torres-Meza *et al.*, 2020). Finally, a recent study reported the detection of CyHV-3 viral mRNA at 1 dpi in kidney and at 1 and 3 dpi in spleen of adult zebrafish following infection by i.p. injection, followed by a quick elimination of the virus from fish (Rakus *et al.*, 2019). Up-regulation of the expression of genes encoding antiviral proteins was also demonstrated, suggesting that the antiviral response of zebrafish infection might contribute to this rapid clearance.

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

	Detection of CyHV-3			Detection of CyHV-3 genome in carp after cohabitation
Common name (species)	**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.* (2010b); [i] Radosavljevic *et al.* (2012); [j] Yuasa *et al.* (2013); [k] Kielpinski *et al.* (2010).

3. The zebrafish as a model to study fish diseases and immune response

Gene function or contribution to various biological processes, including response to viral infection, can be difficult to investigate in fish species such as carp or goldfish, in the absence of processes that facilitate easy manipulation of these cyprinid fish genomes. In contrast to carp and goldfish, the zebrafish can be easily and rapidly genetically manipulated, thus facilitating investigations into genes related to the host antiviral and immune response. Native to the floodplains of the Indian subcontinent and found in slow-flowing and shallow waters such as the Ganges and Brahmaputra River basins, the zebrafish is a small and shoaling teleost characterized by blue-black longitudinal stripes alternating with silver-stripes extending across its body (Lawrence, 2007; Spence et al., 2007). In addition to being a popular aquarium fish, it became a common and useful laboratory animal to study gene function and vertebrate development following pioneering works by George Streisinger. While papers studying zebrafish embryos exist since as early as the 1950s, a groundbreaking study describing the cloning of zebrafish and the successful large-scale forward genetic screens for zebrafish mutations, commonly known as the Tübingen/Boston screens, led to the establishment of this small fish as an important model organism (Eisen, 1996; Grunwald and Eisen, 2002; Streisinger et al., 1981). These events were later followed by the creation of The Zebrafish Information Network (ZFIN; http://zfin.org/), hosting a dedicated online database of genetic, genomic, and developmental information known as the Zebrafish Model Organism Database, and the Zebrafish International Resource Center (ZIRC), which is the first zebrafish genetic resource repository.

The zebrafish is an attractive and useful model for immunity research and infectious disease, this model being used to study the immune response against bacterial, viral, and eukaryotic pathogens

(Lieschke and Trede, 2009; Masud et al., 2017; Traver et al., 2003; Trede et al., 2004; Yoder et al., 2002). Zebrafish embryos and larvae are transparent, enabling the real time in vivo observation of immune cells interacting with pathogens (Herbomel and Levraud, 2004; Torraca et al., 2014). The fully sequenced and well-annotated zebrafish reference genome allows rigorous and accurate analyses of transcriptional changes in response to infection (Howe et al., 2013; Stockhammer et al., 2009). Mutants and transgenic zebrafish lines in which immune cell lineages are labeled by expression of fluorescent proteins have been generated to study immune cell function and in-vivo behavior in response to infection and injury (Masud et al., 2017). For example, transgenic lines expressing a fluorescent protein in macrophages or neutrophils have contributed to better understand how these immune cells migrate to and interact with sites of injury or infection (Ellett et al., 2011; Mathias et al., 2006; Renshaw et al., 2006; Sanderson et al., 2015; Walton et al., 2015). Moreover, the knockdown or knockout of immune genes in zebrafish has been used to study the role of genes involved in hematopoiesis, immune cell function, or host cell defense.

In this section, we will briefly describe the zebrafish phylogeny and taxonomy, then we will highlight the major features of the antiviral immune system of the zebrafish larvae, and finally we will review the naturally occurring infections in zebrafish as well as experimental infection studies with fish viruses.

3.1. The zebrafish phylogeny and taxonomy

The zebrafish was scientifically described as *Danio rerio* by the Scottish physician Francis Hamilton in 1822, along with other species of the genus from Eastern India (Hamilton, 1822). While recurring changes have been made since the first description of this fish, its correct scientific name remains *Danio rerio.* In addition to being one of more than 20 species within the genus *Danio*, the zebrafish belongs to the diverse order of *Cypriniformes*, which is comprised of more than 4,000 species (Parichy, 2015; Stout *et al.*, 2016). This sub-chapter includes a brief description of the phylogenetic position of zebrafish and a short overview of its relation to other fish model systems.

3.1.1. Phylogenetic position

More than 500 million years ago, the subphylum of vertebrates emerged within the chordate phylum (Pough and Janis, 2019) (Fig. 9). Among the vertebrates, the zebrafish belongs to the jawed vertebrates or gnathostomes lineage (Table 4). The emergence of the jaw apparatus was a major innovation of gnathostomes, enabling the exploration of new food sources and contributing to the evolutionary success of this lineage (Liem *et al.*, 2001). The acquisition of the pectoral and pelvic fins represents another crucial morphological innovation leading to the gnathostomes, as they can be found in a more derived form in zebrafish (Liem *et al.*, 2001). Gnathostomes are further separated into two

principal subgroups: the cartilaginous fishes (Chondrichthyes) and the bony vertebrates (Euteleostome or Osteichthyes), which include zebrafish and humans (Pough and Janis, 2019) (Fig. 9).

The lineages leading to zebrafish and human parted around 400-450 million years ago, with the ray-finned fishes (Actinopterygii), to which zebrafish belongs, diverging from the lobe-finned fishes (Sarcopterygii) which include the tetrapods. Within the ray-finned fishes, the zebrafish is part of the teleosts (Teleostei), which is the most species-rich clade (Fig. 10). It is comprised of more than 25,000 species, accounting for almost 50% of all living vertebrates (Helfman *et al.*, 2009; Nelson, 2006). Teleosts are grouped into the Neopterygii, together with the holostean fishes that include bowfin and gars. They are further separated into three main lineages: the osteoglossomorphs, the elopomorphs and the clupeocephalans that include zebrafish and most other fish models such as salmonids, medaka, carps and goldfish (Fig. 10). The interrelationships of these three major lineages are still a matter of ongoing investigation (Betancur *et al.*, 2017). Finally, two main radiations are recognized within the clupeocephalans, the percomorphs with more than 14,000 species and the ostariophysans, with more than 10000 species and including diverse fishes such as electric knifefishes, catfishes and the cypriniforms to which the zebrafish belongs (Alfaro *et al.*, 2009; Chakrabarty *et al.*, 2017; Near *et al.*, 2013).

Table 4 – Phylogenetic classification of zebrafish (*Danio rerio*). Classification below the gnathostome level is following Betancur *et al.* (2017).

Kingdom	Metazoa (Animalia)
Superphylum	Deuterostomia
Phylum	Chordata
Subphylum	Vertebrata
Infraphylum	Gnathostomata
Megaclass	Osteichthyes (Euteleostome)
Superclass	Actinopterygii
Class	Actinopteri
Subclass	Neopterygii
Infraclass	Teleostei
Supercohort	Clupeocephala
Cohort	Otomorpha
Subcohort	Ostariophysi
Superorder	Cypriniphysae
Order	Cypriniformes
Suborder	Cyprinoidae
Family	Danionidae
Genus	*Danio*
Species	*Danio rerio*

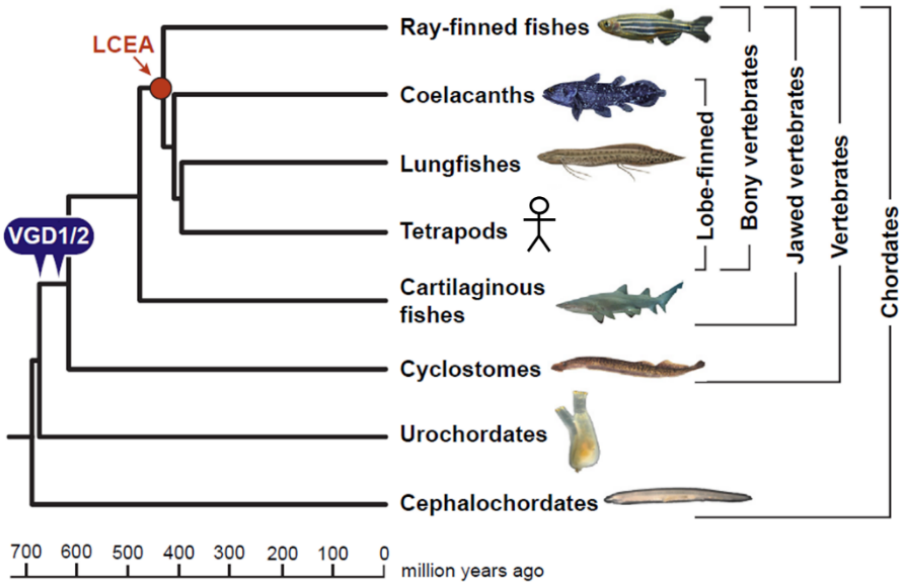

Figure 9 – Phylogeny of the vertebrate lineage.

Zebrafish belongs to the ray-finned fishes, with their phylogeny further detailed in Fig. 10. Reconstructing the last common euteleostome (i.e., bony vertebrate) ancestor (LCEA = *red dot*) is essential for the comparison of ray-finned and lobe-finned vertebrates, especially for the biomedical link of zebrafish to human. VGD1 and VGD2 indicate the likely occurrences of two rounds of vertebrate genome duplication at the base of vertebrates. From McCluskey and Braasch (2020).

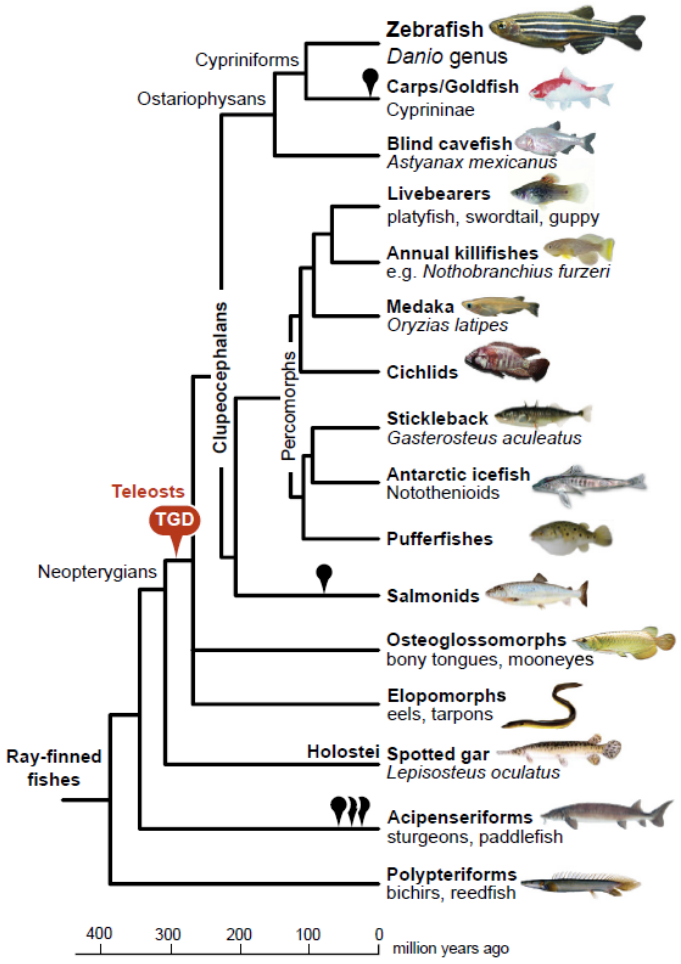

Figure 10 – Phylogeny of the ray-finned fish lineage.

TGD indicates the occurrence of the Teleost Genome Duplication at the base of teleosts. Black pin symbols show the occurrence of additional, lineage-specific genome duplication events. From McCluskey and Braasch (2020).

3.1.2. The zebrafish and its relation to goldfish and carp

The zebrafish belongs to the order of *Cypriniformes* which contains the largest group of freshwater fishes and includes ornamental species, such as rasboras and goldfish, and species important for aquaculture, such as grass carp and common carp (Stout *et al.*, 2016). Comparing zebrafish, which are extensively used in biomedical research, to other phylogenetically diverse fish species is essential to advance our understanding of zebrafish evolution in relation to their teleost, ray-finned, and bony vertebrate ancestors.

Goldfish and the closely related common carp are important aquaculture species which both share an additional, carp lineage-specific whole genome duplication (WGD) event that took place in their common ancestor ~8 to 12 million years (Ma) ago, thus being a quite recent event compared to the genome duplication event that occurred in teleosts (TGD) (320 to 350 Ma ago) (Glasauer and Neuhauss, 2014) (Fig. 10). Believed to have been an allotetraploidy event (i.e., a hybrid of two closely related species created with both chromosome sets of each parent being present in gametes) (Ma *et al.*, 2014), this fusion took place after the divergence from grass carp (*Ctenopharyngodon idella*) but before goldfish diverged from the common carp. To understand the genomic and morphologic effects of genome duplications in vertebrates, the evolutionary consequences of the carp genome duplication can be analysed using zebrafish as "unduplicated" outgroup in relation to the carp genome duplication (Chen *et al.*, 2019; Xu *et al.*, 2014b). Moreover, the relative evolutionary proximity of zebrafish to carp and goldfish provides supplementary reference sequences for the identification of conserved noncoding elements (CNEs) playing a role in gene regulation (Margulies *et al.*, 2003; Woolfe *et al.*, 2004), at sensitivities not obtainable from comparing other fish species which are much more distantly related. Overall, the zebrafish appears to be relatively close evolutionarily to the goldfish and common carp. Therefore, this fish has the potential to be a model organism even for viruses exhibiting a narrow host range. Having coevolved with their canonical hosts, these viruses often induce disease in single fish species (for instance alloherpesviruses in goldfish or carp).

3.2. The zebrafish antiviral immune response during early stages of development

3.2.1. The zebrafish immune system - Generalities

Similar to humans, zebrafish exhibit two main branches of immunity with fully fledged innate and adaptive components. The kidney marrow – equivalent to the bone marrow in mammals – and the thymus represent the two primary lymphoid organs, one secondary peripheral organ in adult fish being the spleen (Wattrus and Zon, 2018). Like other teleosts, the zebrafish lacks the secondary immune

organs called lymph nodes. Thus, the spleen is the main organ where antigen-presenting cells (APCs) and lymphocytes interact (Renshaw and Trede, 2012).

In addition, zebrafish mucosal barriers contain the mucosa-associated lymphoid tissues (MALTs) (Salinas, 2015; Yu *et al*., 2020), including the gut-associated lymphoid tissue (GALT), the gill-associated lymphoid tissue (GIALT), the nasal-associated lymphoid tissue (NALT) and the skin-associated lymphoid tissue (SALT). While these tissues show a well-defined spatial organization including organized-MALT structures in mammals, teleost fish MALTs are believed to be composed of dispersed leukocytes distributed across the mucosal tissues, resulting in what is known as diffuse MALT (Parra *et al*., 2015; Salinas *et al*., 2011; Salinas, 2014). Recent discoveries have challenged the belief that teleost fish lack organized mucosal lymphoid structures. Notably, in 2008, interbranchial lymphoid tissue (ILT) was identified within the gills of Atlantic salmon (*Salmo salar*) (Haugarvoll *et al*., 2008). In 2021, Dalum *et al.* used high-resolution three-dimensional (3D) imaging to examine the gills of zebrafish, revealing the organization of the GIALT. Its compartmentalization into segments was identified, with dispersed immune cells and two lymphoid aggregates, which resemble secondary lymphoid organs: the ILT and a newly discovered lymphoid structure termed the amphibranchial lymphoid tissue (ALT). Very recently, the lymphoid architecture of the zebrafish branchial cavity was investigated using a marker for T lymphocytes/natural killer (NK) cells (ZAP70) and advanced imaging techniques, leading to the identification of a sub-pharyngeal lymphoid organ, named "Nemausean lymphoid organ" (NELO) (Resseguier *et al*., 2023). Reminiscent of mammalian tonsils, this organ is enriched in T/NK cells, plasma/B cells, and antigen-presenting cells embedded in a network of reticulated epithelial cells and is thought to be represent a secondary lymphoid organ.

The zebrafish harbors a fundamental system of innate immunity with leukocytes like the macrophage lineage together with granulocytes such as eosinophils and neutrophils (Novoa and Figueras, 2012; Traver *et al.*, 2003). The innate immune system is also mediated by dendritic cells, mast cells, and there is growing support for natural killer (NK) cell lineages, as non-specific cytotoxic cells (NCCs), considered as an evolutionary precursor of mammalian NK cells, have been identified in zebrafish (Moss *et al.*, 2009). The innate immune response to local infections that is mounted by these cells is similar to the one seen in mammals, with the expression of TNF-alpha and IL-1B, which are characteristic cytokines that trigger the pathogens engulfment by macrophages (Secombes *et al*., 2001; Traver *et al.*, 2003). In zebrafish, 22 putative toll-like receptors (TLRs), including orthologs of all 10 human TLRs, have been discovered (Kanwal *et al.*, 2014; Li *et al.*, 2017). Innate immunity in zebrafish also includes homologues for adaptor proteins for signal transduction, cytokines, and many elements of the complement system (Crim and Riley, 2012). While the zebrafish genome encodes cytokines and TLRs sharing orthology with mammalian genes, it also encodes various "fish-specific" genes within

these families, with “fish-specific” receptor families predicted to function in immunity (Wcisel and Yoder, 2016).

The adaptive immune system is an evolutionary novelty of jawed fish (Kasahara *et al.*, 2004). In zebrafish, it is mediated by T and B lymphocytes which become functional after 3 to 6 weeks post-fertilization (wpf), T and B cell development occurring in the thymus of larval and adult fish and in the pronephros of larval fish and kidney marrow of adult fish, respectively (Langenau *et al.*, 2004; Trede *et al.*, 2004). Unlike humans, zebrafish B lymphocytes produce three immunoglobulin isotypes IgM, IgD, and IgZ (aka IgT) (Fillatreau *et al.*, 2013). Zebrafish cells also express both classes of MHC molecules, known as MHC class I and II, which indicates the conserved interactions between the innate and adaptive immune systems (Fischer *et al.*, 2013). As the fully functional adaptive immune response takes three to six weeks to develop, the zebrafish relies on the innate immune system, augmented by maternally derived immune molecules, for defense against pathogens during its early life stages (embryonic and larval stages) (Wang *et al.*, 2009, 2012b). This late maturation of the adaptive response allows for the study of specific immune system components at various stages of immunologic development. Moreover, infection of zebrafish at different lifecycle stages, from dependance on the innate immune system to reliance on combined innate/adaptive immune systems, can be easily achieved in large numbers of fish. No further details will be given regarding adaptive immunity as most or all of the experimental data collection usually occurs during the zebrafish first days postfertilization and the innate immune system is solely responsible for the immune functions observed in the zebrafish larvae during these early days.

In this subchapter, we will briefly describe how the immune system develop and mature in zebrafish, then we will highlight the major features of the components of the zebrafish larvae antiviral innate immune response.

3.2.2. Development and maturation of the immune system

During embryogenesis, developmental hematopoiesis takes place in four sequential waves and within shifting anatomical sites of blood cell production (Cumano and Godin, 2007; Martinez-Agosto *et al.*, 2007). The first two waves are called “primitive”, each one generating transient precursors giving rise respectively to embryonic myeloid cells and erythrocytes (Cumano and Godin, 2007; Davidson and Zon, 2004; Palis and Yoder, 2001). Then, the next two waves of development hematopoiesis consist of multipotent progenitors called “definitive” hematopoietic precursors, which generate adult cell types. Erythromyeloid progenitors (EMPs) are the first to appear, giving rise to erythroid and myeloid lineages (Bertrand *et al.*, 2007; Frame *et al.*, 2013; Palis, 2016). Multipotent hematopoietic stem cells (HSCs), which can both self-renew and produce all adult hematopoietic cell types, are generated during the fourth

and final wave of developmental hematopoiesis (Dzierzak and Speck, 2008; Medvinsky and Dzierzak, 1998; Morrison *et al.*, 1995).

The emergence of erythroid and myeloid lineages marks the start of the development of the immune system (Fig. 11). The primitive myeloid cells are comprised of both macrophages and neutrophils, the former appearing at around 20 hours post-fertilization (hpf) and distributing throughout the animal, thus providing a fist wave of phagocytes that play a role in host defense (Herbomel *et al.*, 1999; Herbomel and Levraud, 2004; Wattrus and Zon, 2018). Indeed, primitive macrophages from the yolk sac possess the ability to engulf pathogens and patrol the whole organism, following an expedited differentiation pathway (Herbomel *et al.*, 2001; Novoa and Figueras, 2012). Embryonic microglia, which are the tissue resident macrophages (TRMs) of the central nervous system, can also originate from primitive macrophages that have been observed to colonize the brain (Herbomel *et al.*, 2001). This primitive population of microglia is suggested to be later replaced by permanent, definitive precursors, which might indicate that each microglial subset varies in its ability to provide immune protection (Ferrero *et al.*, 2018; Xu *et al.*, 2015). By 33 hpf, some of these primitive macrophages will become neutrophils (Harvie and Huttenlocher, 2015). Multiple homologous genes as mammals, such as *lmo2*, *gata1a*, *scl*, and *cul4a* in the erythroid lineage and *pu.1* in the myeloid lineage, are expressed during these primitive waves of development in the zebrafish (Dooley *et al.*, 2005; Galloway *et al.*, 2005; Yamada *et al.*, 2001; Yang *et al.*, 2019; Zhu *et al.*, 2005). Following the appearance of erythroid and myeloid cells, lymphoid cell development occurs (Carroll and North, 2014; Herbomel *et al.*, 1999; Ivanovs *et al.*, 2017; Tavian *et al.*, 2010). Markers of lymphoid progenitors, such as *rag1* (encoding a protein involved in genomic rearrangement, named V(D)J recombination, of the TCR and Ig loci, used as a marker for maturing lymphocytes), *rag2*, *lck* and *ikaros* (encoding a transcription factor, used as an early lymphoid marker), are all detectable at the end of the primitive wave of hematopoiesis (Jing and Zon, 2011; Langenau *et al.*, 2004; Willett *et al.*, 1997, 2001). While lymphoblast/lymphocyte markers arise at 3 dpf, the zebrafish still depend on its innate immune system for defense against external threats. Starting at 4 dpf, the initiation of definitive hematopoiesis in the kidney is marked by the expression of four early hematopoietic markers, *c-myb*, *ikaros*, *runx2*, and *scl*, in this location (Murayama *et al.*, 2006). At a similar time, T cells develop in the thymus and enter circulation at around 8 dpf (Page *et al.*, 2013). The development of progenitor cells in the dorsal aorta and posterior cardinal vein occurs at around 20 dpf (Page *et al.*, 2013). As mentioned earlier, the full maturation of the adaptive immune system, as represented by circulating lymphocytes, does not occur until 3 wpf (Novoa and Figueras, 2012; Trede *et al.*, 2004; Willett *et al.*, 1999).

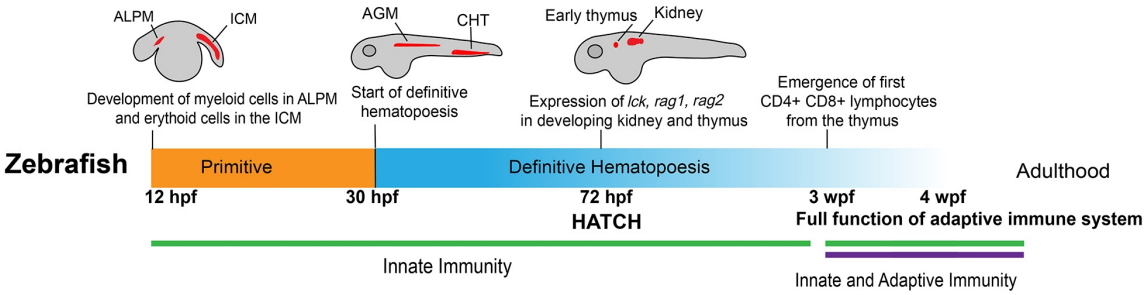

Figure 11 – Development of zebrafish immune system.
The development of the immune system starts with hematopoiesis at ~20 hpf in the zebrafish, with myeloid and erythroid cells arising in the ALPM and ICM, respectively (Jagannathan-Bogdan and Zon, 2013). In zebrafish, definitive hematopoiesis in the AGM and transition into the CHT start at 30 hpf (Jagannathan-Bogdan and Zon, 2013). At 72 hpf, vital markers for early lymphoid progenitors are present in developing immune organs, such as the early thymus and kidney in zebrafish (Langenau *et al.*, 2004; Trede *et al.*, 2004; Willett *et al.*, 1999). At 72 hpf, the zebrafish emerges from the chorion and into contact with the outside environment without fully developed CD4 + /CD8 + lymphocytes, which appear later at 3 wpf (Lam *et al.*, 2004). ALPM: anterior lateral plate mesoderm; ICM: intermediate cell mass; HSC: Hematopoietic stem cells; AGM: aorta-gonad-mesonephros; CHT: caudal hematopoietic tissue. From Miao *et al.* (2021).

Regarding the locations in which the hematopoietic lineages develop, early hematopoiesis begins in the intermediate cell mass (ICM) and the anterior lateral plate mesoderm (ALPM) of zebrafish embryos for erythroid cells and myeloid cells, respectively (Fig. 11) (Berman *et al.*, 2005; Hogan *et al.*, 2006; Jing and Zon, 2011; Willett *et al.*, 1999). Like in humans, the zebrafish hematopoiesis takes place in the aorta-gonad-mesonephros (AGM) during a transitional period. Then, the hematopoietic stem cells move to the venous plexus in the ventral tail, termed caudal hematopoietic tissue (CHT), which functions similarly to the fetal liver of mammals (Murayama *et al.*, 2006). The first HSC-derived leukocytes originate from the CHT and then distribute rapidly throughout the body. The cells finally move into the thymus and kidney, which are the definitive hematopoietic organs from where the development of lymphoid cells and their later emergence take place (Burns *et al.*, 2005; Langenau *et al.*, 2004; Murayama *et al.*, 2006). By 72 hpf, the formation of T lymphocytes is initiated following the colonization of the thymic rudiment by HSC daughters and the first circulating T lymphocytes are observed at 7-8 dpf (Trede *et al.*, 2004). While the CHT remains active for several weeks, the pronephros is colonized, with hematopoietic stem and progenitor cells (HSPCs) being observed within the developing kidney by 4-5 dpf. This organ harbors the definitive wave of hematopoiesis producing the adult immune system and becomes the sole site of hematopoietic production in the adult zebrafish as no immature precursors have been visualized in the spleen, or any other adult tissue (Jagannathan-Bogdan and Zon, 2013; Wattrus and Zon, 2018).

To conclude, the specific immune system, both cell-mediated and humoral immunity, is non-functional during early larval stages, and becomes fully competent following the maturation of both the lymphoid organs and cells, several weeks after hatching (Lam *et al.*, 2004). Thus, both adaptive and innate immunity, as well as leukocytes such as tissue macrophages, circulating monocytes, B and T

lymphocytes, neutrophils, eosinophils (Grzelak *et al.*, 2017) and dendritic antigen-presenting cells (Lugo-Villarino *et al.*, 2010) are observed in juvenile and adult zebrafish.

3.2.3. Antiviral innate immunity

The zebrafish innate immune system, including molecular, chemical, and physical components, provides a first line of defense against pathogens and infections. The antiviral innate immune response is crucial for the survival of an organism and its induction relies on the recognition of viral components by the host. Firstly, it includes physical barriers such as the mucus barrier and phagocytic cells, including neutrophils and macrophages that can kill virus particles and recruit phagocytes to infection sites.

As mentioned in 3.2.2., the development of the innate immune system of zebrafish larvae is rapid, with primitive macrophages at 20 hpf, primitive neutrophils at 33 hpf, and active neutrophils able to migrate and phagocytize pathogens at 52 hpf (Kanther and Rawls, 2010). Macrophages phagocytosing *Mycobacterium marinum* in infected zebrafish adults and embryos highlights the importance of phagocytes in zebrafish innate immunity (Davis *et al.*, 2002). In a similar study, the activity of macrophages in zebrafish embryos challenged with *E. coli* has been visualized using video microscopy (Herbomel *et al.*, 1999). Macrophages could be seen covered in adhered bacteria, actively phagocytosing cell corpse, within 15 min of *E. coli* infection, and the circulatory system was completely clear of bacteria within a few hours. Releasing reactive oxygen species (ROS) to degrade virus particles and recruit additional phagocytes to infection sites, the respiratory burst response is an important response of macrophages and neutrophils. Following this response, the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (PHOX) complex produces ROS, hydrogen peroxide (H_2O_2), and superoxide anion $O^{-}2$, the PHOX complex being conserved between humans and zebrafish (Kawahara *et al.*, 2007).

The antiviral innate immune response also involves pattern recognition receptors (PRRs) such as TLRs, retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide-blinding oligomerization domain (NOD)-like receptors (NLRs) and cytosolic DNA sensors. In addition to fish specific TLRs, homologues for mammalian TLRs (Kanwal *et al.*, 2014; Li *et al.*, 2017), gene homologues encoding adaptor proteins necessary for signal transduction such as toll-interleukin 1 receptor domain–containing adaptor protein (TIRAP) and myeloid differentiation primary response gene 88 (MyD88) (Jault *et al.*, 2004; Meijer *et al.*, 2004), and homologues for IRAK-4 and TRAF6, key proteins in the TLR signaling pathway (Phelan *et al.*, 2005b; Sullivan and Kim, 2008), have been identified in zebrafish. Antimicrobial defense mechanisms are initiated by PRRs through several conserved signaling pathways (Broz and Monack, 2013), resulting ultimately in the activation of cytokines, chemokines, cell adhesion molecules, and immunoreceptors (Akira *et al.*, 2006). In addition to orchestrating the early host response to infection, these components represent an important link to the adaptive response (Mogensen, 2009). The

existence of numerous viral sensing PRRs improves the cellular response, enabling an effective and rapid defense. PRRs that bind pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) and trigger antiviral IFN and cytokines expression through NF-kB and interferon response factors (IRFs) are critical to the immune response activation (Wu and Chen, 2014).

Several antiviral response-related genes are produced following the activation of IRFs. The IFN system plays a key role in the defense against viruses as it can control most, if not all, viral infections in the absence of adaptive immunity (Randall and Goodbourn, 2008). In acute viral infections, a potent response is elicited by IFN, activating a battery of interferon-stimulated genes (ISGs) which contribute to the overall effects against a given virus (Sadler and Williams, 2008; Schoggins and Rice, 2011). Gene transcription, including the transcription of cytokine genes, has been shown to be significantly altered in the developing zebrafish embryos or larvae following experimental infections with various pathogens such as betanodaviruses (Kim *et al.*, 2015; Morick and Saragovi, 2017; Ordas *et al.*, 2011; Saraceni *et al.*, 2016). Moreover, homologues to mammalian cytokines, such as IL-1B (Pressley *et al.*, 2005), type I (Altmann *et al.*, 2003) and type II (Igawa *et al.*, 2006) IFN, TNF-alpha (Praveen *et al.*, 2006; Pressley *et al.*, 2005), and several interleukins (Sullivan and Kim, 2008) have been identified in zebrafish. In addition to their roles in immunity, cytokines also play important developmental roles, altered cytokines levels adversely impacting numerous developmental processes in various systems, such as the central nervous system (Cui *et al.*, 2013; Deverman and Patterson, 2009).

Following virus recognition, the initiation of an inflammatory response is also important to clear viral pathogens. The transcription of pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1B, and TNF-alpha is triggered by the activation of NF-kB (Pang and Iwasaki, 2012). Inflammatory cytokines and chemokines can recruit phagocytes at the site of infection. Finally, the complement system plays an important role in innate immunity and neutralization of viruses. Mechanisms for the activation of the complement include recognition of PAMPs and DAMPs, and C-reactive protein (CRP). A study of CRP genes and proteins revealed that zebrafish *crp2*/Crp2 and *crp5*/Crp5 had the largest increases in expression in response to SVCV and viral hemorrhagic septicemia virus (VHSV) (Bello-Perez *et al.*, 2017). The recognition of motifs through three activation pathways by the complement system can lead to complement-mediated lysis (Zhang and Cui, 2014) in order to respond to microbial pathogens. The complement system is conserved in zebrafish, many of its components having been found in this fish, including B (Gongora *et al.*, 1998), C1q (Hu *et al.*, 2010), C1s (Nakao *et al.*, 2011), C3 (Samonte *et al.*, 2002; Vo *et al.*, 2009a, b), C4 (Samonte *et al.*, 2002), C6, C7, C8a, C8b, C8y, and C9 (Nakao *et al.*, 2011), H (G. Sun *et al.*, 2010), I, mannose-binding lectin–associated serine protease (MASP)-1, MASP-2, MASP-3, and mannose-binding lectin (Nakao *et al.*, 2011). Complements are activated through the classical, alternative and lectin pathways in teleost fishes (Holland and Lambris, 2002; Nakao *et al.*, 2011; Sunyer and Lambris, 1998).

3.2.4. Interferon response

Cytokines are small, soluble pleiotropic proteins secreted by virtually all cells in the body. Their primary role is to modulate the amplitude of immune responses and their expression levels are closely regulated because of the profound biological consequences of inappropriate up-regulation. Important antiviral cytokines include the type I IFNs, of which homologues have been observed in zebrafish (Altmann *et al.*, 2003). Upon recognition of viral infection, type I IFNs are secreted and function as autocrine and paracrine factors to protect other cells from further viral infections. Thus, the innate immune response to viral infection is governed by IFN and genes induced by IFN. Belonging to the class II helical cytokine family, IFNs can be divided into different classes in mammals: type I (α, β, ω, ε, and κ), type II (γ), and type III (λ) (Pestka *et al.*, 2004). While type I and type III IFNs have well established antiviral activities in mammals, the function of type II IFNs is associated with the response against bacterial infection.

In one of the first studies in zebrafish, IFN expression was shown to be induced in zebrafish liver cells upon infection with Snakehead Rhabdovirus (SHRV) (Altmann *et al.*, 2003). In addition to the IFN gene, now known as *ifnφ1*, that was first characterized in this study, three other IFN genes (*ifnφ2*, *ifnφ3*, *ifnφ4*) activated in response to viral infection are present in zebrafish (Table 5) (Aggad *et al.*, 2009; Zou *et al.*, 2007). Currently, three types of IFN have been characterized in zebrafish: type I (separated in group I, composed of *ifnφ1* and *ifnφ4*, and group II, composed of *ifnφ2* and *ifnφ3*), type II (including *ifnγ1* and *ifnγ2*) (Stein *et al.*, 2007; Zou *et al.*, 2007) and type IV (designated as IFN-υ [*ifnu*]) (Chen *et al.*, 2022). Within zebrafish type I IFN, group I is characterized by a pair of conserved cysteine residues forming a disulfide bridge and group II is characterized by two pairs of conserved cysteine residues forming two disulfide bridges (Hamming *et al.*, 2011). While the two groups signal through two different receptor complexes in zebrafish, each receptor complex is thought to include cytokine receptor family member b 5 (Crfb5) (Aggad *et al.*, 2009). Group I IFNs and Group II IFNS are thought to interact with Crfb1/Crfb5 complexes and Crfb2/Crfb5 complexes, respectively. Interestingly, Crfb1 IFN receptor clusters disruption caused by the knockdown of caveolin (Cav1) decreased antiviral immune responses in zebrafish (Gabor *et al.*, 2013).

Table 5 – IFN genes in zebrafish (adapted from Sullivan *et al.*, 2021).

Zebrafish Gene					Predicted Human Ortholog	Ensembl Human Gene ID	Orthology Resource(s)
Gene Symbol	**Ensembl Gene ID**	**Chr.**	**IFN Type**	**Induction by virus infection**			
Ifnφ1 [a]	ENSDARG00000025607	3	Type I (Group 1)	SHRV [a, b, c] SVCV [d, e, f, g, h] IHNV [e] IAV [i] TiLV [j] CHIKV [k] NNV [q, r, s, t]			-
ifnφ2 [l, m]	ENSDARG00000069012	3	Type I (Group 2)	SVCV [g] NNV [r]			-

ifnφ3 [l, m]	ENSDARG00000070676	3	Type I (Group 2)	NNV [r]			-
ifnφ4 [l, m]	ENSDARG00000100678	12	Type I (Group 1)	SVCV [l] IHNV [l]			-
ifnγ1 [n]	ENSDARG00000024211	4	Type II	SVCV [o, p] TiLV [j]	*IFNG*	ENSG00000111537	ZFIN, Ensembl
ifnγ2 [n]	ENSDARG00000045671	4	Type II		*IFNG*	ENSG00000111537	ZFIN, Ensembl

[a] Altmann *et al.* (2003); [b] Gabor *et al.* (2015); [c] (Phelan *et al.*, 2005a); [d] Espín-Palazón *et al.* (2016); [e] Langevin *et al.* (2013); [f] Levraud *et al.* (2007); [g] (Varela *et al.*, 2014a); [h] (Zhu *et al.*, 2020a); [i] Gabor *et al.* (2014); [j] Rakus *et al.* (2020); [k] Palha *et al.* (2013); [l] Aggad *et al.* (2009); [m] Zou *et al.* (2007); [n] Igawa *et al.* (2006); [o] Álvarez-Rodríguez *et al.* (2018); [p] García-Valtanen *et al.* (2014); [q] Lu *et al*. (2008); [r] Chen *et al*. (2015); [s] Jin *et al*. (2019); [t] Liu *et al*. (2020)

Two transcript isoforms can be expressed by the *ifnφ1* gene in zebrafish: a longer, constitutively-expressed transcript, lacking sequence encoding a secretion signal peptide, thus likely retained within the cells, and a shorter, virally-induced transcript, containing a signal peptide that causes the protein to be secreted (Levraud *et al.*, 2007). Discrete spatiotemporal patterns are also exhibited by the transcripts encoded by the *ifnphi1* gene (Aggad *et al.*, 2009). Compared to whole larvae, basal levels of *ifnphi1* are elevated in adult zebrafish. However, viral infection could induce increased *ifnphi1* expression levels in both adult and larval zebrafish. In the uninfected zebrafish larvae, a study showed that secreted isoform of *ifnphi1* was expression at very low level whereas *ifnphi3* and the intracellular isoform of *ifnphi1* were expressed at relatively high constitutive level (Briolat *et al.*, 2014). In the transgenic zebrafish line Tg(*ifnphi1:mCherry*), the expression of mCherry fluorescent protein driven by the *ifnphi1* promoter was observed in hepatocytes and neutrophils after infection with Chikungunya virus (CHIKV) (Palha *et al.*, 2013). While transcripts encoded by the *ifnphi2* gene were below the level of detection in zebrafish larvae, they were expressed at levels comparable to *ifnphi1* in adult spleens and their expression in the spleen was induced upon SVCV infection (Aggad *et al.*, 2009). In both whole larvae and adult spleens, transcripts encoded by the *ifnphi3* gene were expressed at elevated basal levels and were not induced following IHNV or SVCV infection (Aggad *et al.*, 2009). Finally, transcripts encoded by the *ifnφ4* were expressed at modest basal levels and were mildly induced in zebrafish larvae upon SVCV infection. Thus, the IFN response is mediated by *ifnphi1* and *ifnphi3* in zebrafish larvae, as *ifnphi2* is expressed only in adults and *ifnphi4* has little activity (Aggad *et al.*, 2009; Levraud *et al.*, 2007). The survival of zebrafish larvae injected intravenously with SVCV (Levraud *et al.*, 2007) and IHNV (Aggad *et al.*, 2009) was increased following treatment with recombinant zfIFNΦ1 and zfIFNΦ2, with zfIFNΦ1 indicated as the more protective protein (Aggad *et al.*, 2009). López-Muñoz *et al.* (2009) demonstrated that both group I and II IFNs could protect adult zebrafish against SVCV, group II IFNs eliciting a rapid and transient expression of antiviral genes and group I IFNs inducing antiviral and proinflammatory genes at higher levels, but much more slowly. Group I and II IFNs have thus been suggested to have complementary roles in viral infection, where group II IFNs would play a key role during the early

stages of these infections, while group I IFNs would be important for the viral clearance and resolution of the infection at later stages.

While many similarities are observed in the type I IFN signaling of zebrafish and humans, significant differences can also be found. For example, type I IFNs of fish (including zebrafish) have retained introns, while type I IFNs of mammals do not. Although mammalian type I IFNs are typically secreted upon viral induction, fish type I IFNs can be either transcribed in the presence or absence of signal peptides for extracellular expression (Levraud *et al.*, 2007).

The bioactivities of fish type I IFNs (mainly group I IFNs) have been well documented in fish species and the induction of a large set of genes encoding antiviral proteins like Mx, viperin, ISG15, and PKZ was described. The activation of the IFN receptor clusters signal through the Jak/STAT pathway to induce ISGs sharing an IFN-stimulated response element (ISRE) (Schoggins *et al.*, 2011). Type I IFNs induce the expression of a large set of ISGs in response to viral infection in zebrafish, several of them having mammalian orthologs that are ISGs in mammals, such as *mxa* (Altmann *et al.*, 2004), *rsad2* (Levraud *et al.*, 2007), *isg15* (Langevin *et al.*, 2013) and the two homologues of mammalian PKR, *pkr* and *pkz* (Rothenburg *et al.*, 2005, 2008). Like PKR, PKZ is thought to be able to inhibit viral replication through the phosphorylation of eIF2α. In zebrafish, PKR and PKZ coexist in a head-to-tail (parallel) orientation in the genome, which is suggested to be crucial for similar transcriptional activation after immunostimulation (Rothenburg *et al.*, 2008). Recently, Levraud *et al.* (2019) used deep RNA sequencing to produce a comprehensive list of ISGs of zebrafish larvae following inoculation of recombinant zebrafish type I IFN or infection with CHIKV. More than 400 zebrafish ISGs, either directly induced by IFN or induced by CHIKV in an IFNR-dependent manner, and 72 orthology groups including ISGs in both humans and zebrafish were identified. A significant portion of the interferon-stimulated gene (ISG) repertoire is lineage specific, approximately 40% of protein-coding zebrafish ISGs lacking human orthologs. Notably, multiple ISGs such as finTRIMs, *gig2*, and eight members of the very large NLR family were found among the 14 gene families unique to fish (Levraud *et al*., 2019).

In mammals, type II IFNs are typically secreted by $CD4^+$ TH_1 cells and NK cells. Apoptosis induction during viral infection and cell proliferation inhibition can be enabled by these IFNs (Zou and Secombes, 2011). The expression of many ISGs responding to type I IFNs was induced by fish *ifnγ* (Grayfer and Belosevic, 2009; Martin *et al.*, 2007), which suggest a cross-activation of the innate immune responses initiated by type I and type II IFNs. In adult zebrafish, zIFNγ1–2 failed to modulate the resistance of zebrafish to viral infection, being unable to increase the expression of proinflammatory and antiviral genes in response to SVCV infection (López-Muñoz *et al.*, 2009). Aggad *et al.* (2010) showed that *ifng1* and *ifng2* are induced by SVCV in adult zebrafish but not in larvae.

The presence of teleost fish type III IFNs has not been reported yet. However, a formerly unreported class II cytokine, designated as IFN-υ (*ifnu*), and its receptors IFN-υR1 (*ifnur1*, also known as *crfb12* in fish) and CRFB4/IL-10R2 were recently identified in the zebrafish genome (Chen *et al*., 2022). Interestingly, IFN-υ and IFN-υR1 are found at unique and highly conserved locations, distinct from the loci of the three other types of interferons. Phylogenetically, IFN-υ and IFN-υR1 group with class II cytokines and class II cytokine receptors, respectively. This discovery suggests that this IFN ligand-receptor system could be classified as a type IV interferon, expanding the current understanding of the three types of interferons in vertebrates. In addition, IFN-υ was found to respond to grass carp reovirus (GCRV) infections by regulating the expression of ISGs and inhibiting viral replication (Chen *et al*., 2022). IFN-υ and IFN-υR1 were also identified through bioinformatics across various vertebrate lineages, including fish, amphibians, reptiles, birds, and mammals. Furthermore, in the African clawed frog (*Xenopus laevis*), IFN-υ was found to use IFN-υR1 and IL-10R2 (a homolog of CRFB4) to induce an antiviral state (Chen *et al*., 2022).

3.3. The zebrafish as a model organism for research in virology

The zebrafish is a powerful model system for the study of viral infection and host immunity as it possesses numerous advantages, including near transparency during the embryonic and larval periods of development, experimental susceptibility to a wide range of viruses, a deeply sequenced genome, a large variety of mutant and transgenic lines, easy maintenance in the laboratory, low relative cost of experimentation, functional similarity to the immune system of other teleosts as well as mammals, and fluorescence and live imaging techniques (Crim and Riley, 2012; Sullivan *et al.*, 2021). Real time visualization enables the monitoring of a single infected zebrafish and can be performed during the first eight days postfertilization, meaning that no mature B or T cells are present yet. As mentioned in 3.2, the zebrafish displays innate immunity very early in development but lacks a fully functional adaptive immune response for the first 3-6 weeks of development (Lam *et al.*, 2004), allowing the study of the interaction of a pathogen with different components of the immune system at various developmental stages (i.e., study of innate components independently from the adaptive immune system) (Kanther and Rawls, 2010). The similarities between the zebrafish and mammalian immune system, including homologues for cytokines, adaptor proteins for signal transduction, mammalian toll-like receptors, and many elements of the complement system, offer great utility for zebrafish models of viral immunity and viral infection that can be applied to both human health and aquaculture. Indeed, zebrafish have been used as a model organism to study several human viruses, including RNA viruses such as the influenza A virus (IAV) (Gabor *et al.*, 2014), CHIKV (Palha *et al.*, 2013), Sindbis virus (SINV) (Boucontet *et al.*, 2018; Passoni *et al.*, 2017), and hepatitis C virus (HCV) (Ding *et al.*, 2011), as well as DNA viruses such as HHV-1 (Antoine *et al.*, 2014; Burgos *et al.*, 2008). In addition, the zebrafish model has been used to study several viral infections associated with mass mortalities of cultured fish species and

causing severe economic losses. Interestingly, there are only few reports of naturally occurring viral infections in zebrafish. Below is a summary of the naturally occurring viral diseases of zebrafish as well as a description of zebrafish susceptibility to experimental infection with fish viruses. Information about fish viruses studied *in vivo* using zebrafish can be found in Table 6.

3.3.1. Naturally occurring viral infections

Under natural conditions, the zebrafish is thought to be infected by pathogens through the gills, the damaged fish surface, or the gastrointestinal tract (O'Toole *et al.*, 2004). However, detailed knowledge about routes of infections in naturally occurring viral diseases remains scarce and experimental infections, such as intravenous (i.v.) or intracelomic injection using mutant or recombinant viruses, are often produced under unnatural conditions thus altering many aspects of infection. Moreover, experimental infection studies with virus that are not naturally occurring in zebrafish do not necessarily show the type of host-pathogen interactions that would be obvious when these pathogens naturally infect coevolved host species. The fact that few naturally occurring infections have been well characterized in zebrafish can be partially explained by the extraordinary fecundity of zebrafish. Indeed, most or all of the experimental data collection usually occurs during the first days postfertilization and the number of viable embryos produced is often sufficient to conduct studies (Crim and Riley, 2012). Thus, a level of morbidity and mortality is accepted by most researchers, unless there is a significant drop in the quality of embryos produced or in fecundity. Most pathogens recognized in zebrafish represent bacterial, fungal, or parasitic agents previously characterized in commercially important fish species. This lack of information about naturally occurring pathogens in zebrafish doesn't reflect an inability of viral pathogens to infect zebrafish but rather a lack of investigation in this area, as shown by studies investigating the experimental infection of zebrafish with viruses isolated from other fish species (LaPatra *et al.*, 2000; López-Muñoz *et al.*, 2010; Lu *et al.*, 2008; Ludwig *et al.*, 2011; Novoa *et al.*, 2006; Phelan *et al.*, 2005a; Sanders *et al.*, 2003; Seeley *et al.*, 1977; Xu *et al.*, 2008) and by the detection of endogenous retroviruses, retrotransposons, and retroid agents in the genome of zebrafish (Basta *et al.*, 2007; Shen and Steiner, 2004). Moreover, spontaneous occurring neoplastic lesions are fairly common in laboratory zebrafish and it is possible that some neoplasms are caused by unrecognized oncogenic viruses as viruses can be associated with tumorigenesis in other fish species (Bowser *et al.*, 2005; Francis-Floyd *et al.*, 1993; Sano *et al.*, 1991). Notably, other research groups can be exposed to increasing risks because of undiagnosed viral infections in zebrafish, as aquatic facilities become more centralized with shared systems (Crim and Riley, 2012; Kent *et al.*, 2009).

Redspotted grouper nervous necrosis virus (RGNNV). Nervous necrosis virus (NNV) is an important fish pathogen belonging to the family *Nodaviridae* which targets nervous tissues, preferentially brain and retina, and which produce important economic losses in aquaculture worldwide. NNV belongs to the *Betanodavirus* genus that is comprised of nonenveloped, spherical viruses with a

bipartite positive-sense RNA genome. Betanodaviruses are the causative agents of viral nervous necrosis (VNN) (Yoshikoshi and Inoue, 1990), also known as viral encephalopathy and retinopathy (VER) (OIE, 2019a), which is a highly destructive disease of hatchery-reared larvae and juveniles of marine fish. The first naturally occurring virus reported in zebrafish was the RGNNV (Binesh, 2013). RGNNV is a betanodavirus that is known to infect a wide variety of species, including at least 32 species of fish (Doan *et al.*, 2017) such as the zebrafish and other species used in biomedical research like the goldfish (Binesh, 2013), guppies (Hegde *et al.*, 2003) and Japanese medaka (Furusawa *et al.*, 2006). It is part of the *Nodaviridae* family which consists of nonenveloped, single-stranded RNA viruses with an icosahedral capsid composed of 32 capsomers and enclosing a linear, positive-sense, bi-segmented RNA genome comprising two segments, RNA1 and RNA2 (Mori *et al.*, 1992). While one study demonstrated that zebrafish were not susceptible to infection by RGNNV (Furusawa *et al.*, 2007), experimental infections with field strain of betanodavirus isolated from Malabar grouper (*Epinephelus malabaricu*s) in Taiwan caused high mortality in zebrafish larvae and established subclinical infections in adult zebrafish (Lu *et al.*, 2008). In zebrafish and goldfish procured from local ornamental fish stores in India and maintained in a laboratory for 2 weeks, clinical signs of VNN such as erratic swimming and heavy mortality (up to 32%) were observed with visible lesions in the brain and spinal cord of the samples (Binesh, 2013). Cumulative mortality was later shown to be significantly greater when infected zebrafish were housed at higher temperatures and at greater housing densities in an experiment comparing sub-clinically infected zebrafish and naïve zebrafish (Binesh, 2014). No treatment for RGNNV infection is available in zebrafish and vaccines shown to be protective for other fish species (Kai and Chi, 2008; Liu *et al.*, 2006; Pakingking *et al.*, 2009; Pakingking *et al.*, 2010; Thiéry *et al.*, 2006) have not been used on zebrafish. Thus, exclusion of new pathogens from a system remains the most effective biosecurity measure (Collymore *et al.*, 2016).

Infectious spleen and kidney necrosis virus (ISKNV). Belonging to the genus *Megalocytivirus,* ISKNV and ISKNV-like virus infect more than 50 marine fish species including not only zebrafish but also species important to commercial aquaculture and the ornamental fish trade as well as other small freshwater species that are used as research models, like the guppy (*Poecilia reticulata*) and the southern platy (*Xiphophorus maculatus*) (Rimmer *et al.*, 2015). ISKNV was the second virus reported to cause natural infections in zebrafish, causing a clinical disease but no mortality (Bermúdez *et al.*, 2018). Megalocytiviruses are very large (150-250 nm) icosahedral DNA viruses with a large linear double-stranded DNA genome (Song *et al.*, 2008). In 2015, a natural outbreak of ISKNV was detected in zebrafish from a research facility in Spain and was associated with clinical signs such as oedema, pale or hyperemic gills and petechial hemorrhage at the base of fins accompanied by loss of appetite, abnormal swimming, lethargy, and in the most severe cases, respiratory distress (Bermúdez *et al.*, 2018). Due to its extremely broad host range and prevalence in the ornamental fish trade, ISKNV poses more risk to laboratory zebrafish colonies and exclusion from entire colonies is an effective measure for

pathogen control (Collymore *et al.*, 2016). Although no treatment for ISNKV infection is available in zebrafish, this fish has been suggested as a model to develop ISKNV vaccines (Xu *et al.*, 2008). Killed vaccine preparations reported as protective for other fishes (Dong *et al.*, 2013; Fu *et al.*, 2012, 2015; Huang *et al.*, 2012; Li *et al.*, 2015b) have not been used on zebrafish.

Zebrafish picornavirus. A highly divergent novel virus in the family *Picornaviridae*, named zebrafish picornavirus-1 (ZfPV-1), was recently identified during a viral metagenomics analysis of laboratory zebrafish gut tissue. It was qualified as a member of a novel genus with a proposed name of *Cyprivirus*, as the type strain for a new species, Cyprivirus A, following phylogenetic analysis of the viral genome (Altan *et al.*, 2019). Viruses in the family *Picornaviridae* are nonenveloped, and virions consist of a single molecule of positive-sense single-stranded RNA enclosed in a small (approximately 30-32 nm in diameter) 60-protomer icosahedral viral capsid. More than 45 ICTV-recognized genera are included in the family *Picornaviridae*. *In situ* RNA hybridization studies of infected zebrafish revealed that viral RNA was confined to a subset of enterocytes and scattered subjacent cells in the lamina propria of the intestine and the intestinal mucosa (Altan *et al.*, 2019). These results support an enteric tropism and fecal-oral mode of transmission. While the virus appears to be benign, wild-type zebrafish lineage AB infected with ZfPV-1 being asymptomatic, ZfPV-1 could cause disease in immunocompromised fish, be pathogenic when occurring as a coinfection, or affect research endpoints. Furthermore, reverse transcription (RT)-PCR testing of zebrafish from North America, Europe, and Asia demonstrated that ZfPV-1 is globally distributed in zebrafish colonies from research facilities, being detected in pooled zebrafish samples from 23 (56%) of the 41 institutions tested (Altan *et al.*, 2019). A subsequent experiment using a genetically modified zebrafish strain (*isg15:GFP*) expressing GFP under an interferon-stimulated gene promoter has shown that transgenic larvae were spontaneously expressing GFP repeatedly, days after hatching (Balla *et al.*, 2020). Following RNA sequencing, it was revealed that this zebrafish picornavirus was inducing a strong canonical interferon-mediated response and hundreds of antiviral defense genes not usually observed following experimental infections with other viruses and immunostimulatory treatments, including a large set encoding GTPase of immunity-associated proteins (GIMAPs) (Balla *et al.*, 2020). It was suggested that this virus is transmitted horizontally in experimental and natural conditions, with infections first occurring upon exposure to the environment after hatching. In wild populations, the prevalence of ZfPV-1 has not been determined and its host range beyond zebrafish is currently unknown. Such as for other fish viruses, exclusion of ZfPV-1 from zebrafish colonies is the most effective control measure. As it has not yet been shown to cause any clinical disease, no treatment is available for zebrafish picornavirus.

Endogenous viral elements (EVEs). EVEs are viral sequences (DNA or CDNA) that have become integrated into the host genome in germ cells, allowing vertical transmission of the viral sequence as genomic DNA and leading possibly to the ultimate fixation of the viral sequence in the host population

(Feschotte and Gilbert, 2012). Among the numerous kinds of viruses able to be endogenized, endogenous retroviruses (ERVs) were the first viruses identified and include the great majority of known EVEs (Feschotte and Gilbert, 2012). Comprising approximately 0.89% of the zebrafish genome, at least 1514 ERV sequences have been identified in the zebrafish genome (Hayward *et al.*, 2015). Zebrafish endogenous retrovirus (ZFERV) is an Epsilon-line endogenous retrovirus which is phylogenetically related to the salmon swim bladder sarcoma virus (Naville and Volff, 2016). In both adult zebrafish and larvae, the provirus is transcribed predominantly in the thymus (Naville and Volff, 2016). With intact open reading frames for the *gag*, *pol*, and *env* genes and LTR sequences, ZFERV is the only intact endogenous retrovirus identified in teleosts (Shi *et al.*, 2015). Endogenous foamy virus (*Spumaretroviridae*) sequences are also included in the zebrafish genome, represented by *Danio rerio* Foamy Virus Type 1, 2, and 3 (Llorens *et al.*, 2009; Naville and Volff, 2016; Ruboyianes and Worobey, 2016). Finally, snakehead fish retrovirus (SnRV-like) sequences, which are primitive ERVs with an evolutionary branch close to that of the Spuma clade, have also been identified in the zebrafish genome (Hayward *et al.*, 2015).

3.3.2. Experimental susceptibility to viral infections of fish

In recent years, the use of zebrafish as a model organism for viral infections has increased with the larger number of studies mainly focusing on fish viruses. Both the developmental larvae stage and adult zebrafish can be infected and be susceptible to diseases caused by fish viruses. Many of these viruses have low optimal replication temperatures (i.e., lower than 24°C), meaning that experimental inoculations occur at temperatures below the optimal growing temperature for this fish model. Zebrafish being commonly housed at 28°C, that fact has been considered detrimental to fish and its immune response. However, zebrafish can be gradually acclimated to a wide range of temperatures, having been maintained successfully at 37°C to facilitate the study of mammalian pathogens (Sanders *et al.*, 2015). Furthermore, the ability to mount an efficient antiviral immune response even at 15°C has been demonstrated in zebrafish (Novoa *et al.*, 2006). Progress has thus been made both in the study of the zebrafish immune system and in the understanding of RNA and DNA viruses-generated pathologies. Moreover, several routes of infection have been developed for zebrafish larvae and adults, depending on the virus. These routes include injection of the virus into the duct of Cuvier, aorta, caudal vein, hindbrain, and yolk for larvae, immersion for both larvae and adults, and intraperitoneal (i.p.) injection for adults (Varela *et al.*, 2017). The experimental susceptibility of zebrafish to infection by several families of viruses has been demonstrated following infection studies designed to develop the zebrafish as a model for viral infection in commercially important fish species, suggesting that not only naturally occurring viruses can occur in this model but also that a broad range of viral families may be represented among the so-far-unidentified zebrafish viruses.

3.3.2.1. Experimental infections with RNA viruses

Rhabdoviridae – Spring viraemia of carp virus (SVCV). Members of the *Rhabdoviridae* family are important pathogens of both cultured and wild fish worldwide. Like every other member in this family, SVCV is an enveloped, bullet-shaped virus with a negative-sense, single-stranded RNA genome. It has been assigned to the genus *Sprivivirus*, which is comprised of viruses isolated predominantly from cypriniform fish. In the common carp, which is its natural fish host, SVCV is responsible for the highly contagious spring viraemia of carp (SVC) disease, leading to high mortalities in juvenile fish and causing important economic losses for carp industries (Ashraf *et al.*, 2016). In order to better understand the disease process, adult zebrafish were infected with SVCV by immersion to mimic a natural route of infection (Sanders *et al.*, 2003). Typically maintained at 28°C, zebrafish were here acclimated to lower temperatures as lethal SVCV infections mostly occur at temperatures below 15°C. Immersion in infectious water caused profound gross pathological changes resembling natural infections in zebrafish; however, typical histological changes such as edema, hemorrhage, inflammation, and necrosis were not observed (Sanders *et al.*, 2003). Those results showed that adult zebrafish were susceptible to waterborne infection by SVCV and suggested that zebrafish were not able to mount a robust immune response at 15°C or 20°C.

In larvae, infection models inducing a systemic infection have been established, using immersion (López-Muñoz *et al.*, 2010) and microinjection of the virus into the blood torrent (Varela *et al.*, 2014a). Levraud *et al.* (2007) showed that dechorionated zebrafish up to 2-3 dpf were resistant to infection by immersion. However, early swimming larvae were already susceptible, the bulk of death occurring between 48 and 65 hpi in larvae aged 5.3 dpf. This same study also used a larval zebrafish model where SVCV was injected into the systemic circulation via the caudal vein, leading to the induction of several ISGs, including *rsad2*, *mxa*, and *mxb*. Levraud *et al.* (2007) also showed that survival to SVCV infection was improved in transgenic embryos overexpressing *ifnphi1*. Following bath exposure of zebrafish larvae (3 dpf) to SVCV, the induction of the expression of several genes of the IFN type I pathway, including those encoding antiviral proteins like MxB, MxC, viperin and PKZ, was not detected when observed at 24 hpi (López-Muñoz *et al.*, 2009). These results thus revealed that zebrafish larvae were unable to mount a protective antiviral response against waterborne SVCV. López-Muñoz *et al.* (2010) showed that 3 dpf larvae exposed to SVCV at 26°C by immersion were susceptible to infection, with 50% survival happening between 3 and 4 dpi. At 2 dpi, a slight up-regulation of genes encoding pro-inflammatory cytokines (*il1b*, *tnfa*) and antiviral proteins (mostly *mxb*) was observed. In adult zebrafish i.p. injected with SVCV, the transcription of genes encoding type I IFNs and antiviral proteins was up-regulated from 10 to 1000 times when compared to uninfected control (López-Muñoz *et al.*, 2009) while in larvae, the expression of these genes was up-regulated between 2 and 10 times (Aggad *et al.*, 2009; Levraud *et al.*, 2007). Moreover, a larval SVCV immersion model was used to show that TNF-alpha can inhibit SVCV clearance by blocking the host autophagic response required for the viral eradication (Espín-Palazón *et al.*, 2016). This virus became one of the most frequently used viral infection models

in zebrafish, remarkable progress having been made in the study of the IFN system and inflammation in zebrafish with the use of SVCV (Aggad *et al.*, 2009, 2010; López-Muñoz *et al.*, 2009; Varela *et al.*, 2014a, b, 2016). Moreover, SVCV infection studies in zebrafish helped modelling the interplay between the virus and the common carp, which is genetically close to zebrafish.

Rhabdoviridae – Snakehead rhabdovirus (SHRV). SHRV is a member of the *Novirhabdovirus* genus that was used early in zebrafish to investigate the function of the virus nonvirion (NV) gene (Alonso *et al.*, 2004). Affecting warm-water fish in Southeast Asia, it is closely related to IHNV and VHSV, which are two other commercially significant viruses. Although SHRV was the first viral infection reported in zebrafish embryos, the infection induces an antiviral response also in adults following i.p. injection (Gabor *et al.*, 2013, 2015; Phelan *et al.*, 2005a, b). Phelan *et al.* (2005a) characterized the infection kinetics and gross pathology following infection with SHRV in the zebrafish. Zebrafish at 24 hpf were susceptible to infection by static immersion, showing a cumulative percent mortality (CPM) of 55%, while adult zebrafish could only be infected by i.p. injection with a CPM of 70%. Petechia, abdominal redness, and erratic swim behaviors were observed in infected zebrafish (Phelan *et al.*, 2005a). In addition, antiviral *ifnphi1* and *mxa* transcript levels were elevated following SHRV infection by immersion, although expression and intensity varied with age and route of infection. In another study, expression of the immune genes *traf6* and *tlr3* was up-regulated and the expression of *irak4* was slightly down-regulated in both embryonic and adult zebrafish infected by SHRV (Phelan *et al.*, 2005b). The overexpression of a full-length Mda5 was showed to be protective against SHRV infection, thus demonstrating that Mda5 plays an important role in virus resistance (Gabor *et al.*, 2015). In adult zebrafish, Kortum *et al.* (2014) investigated the effects of SHRV infection via i.p. injection on polymeric immunoglobulin (Ig) receptor (pIgR) expression, which is thought to be regulated by TLR3 and TLR4 signaling and to link aspects of the innate immune response to the adaptive immune response (Schneeman *et al.*, 2005). Upon infection, *pigr* and *pirl* transcripts were reduced, suggesting that the immune response is suppressed, at least partly, by SHRV through this mechanism.

Rhabdoviridae – Viral hemorrhagic septicemia virus (VSHV). Another *Novirhabdovirus* genus member is the VHSV, which is a highly contagious virus causing a prolific disease leading to high mortality in a large panel of freshwater and marine fish species, within both cultured and wild fish worldwide (Chinchilla and Gomez-Casado, 2017; Kurath and Kuhn, 2016). Also known as piscine novirhabdovirus or Egtved virus, it was used in adult zebrafish to study vaccines and adjuvants effectiveness (Kavaliauskis *et al.*, 2015; Novoa *et al.*, 2006). VHSV is one of the most threatening viral pathogens affecting finfish aquaculture. As part of this, transcriptomic and proteomic data of the infection in zebrafish can help identifying novel prevention drugs (Encinas *et al.*, 2010). For example, tannic acid administered before infection reduces the mortality rate of VHSV-infected zebrafish (Estepa and Coll, 2015). Juvenile and adult zebrafish immersion and i.p. injection models for VHSV infection

were developed by Novoa *et al.* (2006). A disease similar to that found in nature was observed in adult zebrafish infected by i.p. injection, exhibiting petechial hemorrhage, exophthalmos, distended visceral cavities, and erratic swimming behaviors. Furthermore, an increased expression of gene transcripts associated with antiviral and pro-inflammatory responses, including *tlr3*, *ifnphi1*, *mxa*, *ifng1*, and *tnfa* (Novoa *et al.*, 2006). In the same study, a recombinant salmonid novirhabdovirus (IHNV) lacking an NV gene, but expressing piscine novirhabdovirus G gene, was demonstrated to have dose-dependent protective effects for zebrafish in resisting VHSV infection, a significant reduction in mortality being observed. While the impact of VHSV has resulted in multiple studies in adult zebrafish, Dios *et al.* (2010) described a model of VHSV infection in zebrafish larvae. Larvae immersion infections were performed to compare the inflammatory and antiviral responses (i.e., the expression levels of several antiviral and inflammatory genes) against VHSV infection, after the challenge of larvae at different developmental stages (from 2 to 29 dpf) and at low temperature (15°C). Results showed that no significant changes in expression levels between control and infected fish were observed for the antiviral activity-related genes *tnfa*, *tlr3*, *ifng1*, and *mda5* (also known as *ifih1*) (Dios *et al.*, 2010).

Rhabdoviridae – Infectious hematopoietic necrosis virus (IHNV). IHNV is a member of the *Novirhabdovirus* genus that has a worldwide distribution. It is the causative agent of an acute, systemic disease in salmon and trout, called infectious hematopoietic necrosis (IHN). It is especially severe among juvenile fish where losses can be superior to 90%, with epizootics characterized by a sudden and rapid increase in mortality (LaPatra, 1998). The effects of IHNV and infectious pancreatic necrosis virus (IPNV; *Birnaviridae*) infections on hematopoietic precursors were investigated in adult zebrafish by LaPatra *et al.* (2000), as both these viruses can cause prominent hematopoietic necrosis of the major site of adult hematopoiesis in fish, i.e., the head kidney. IHNV and IPNV were both shown to trigger infections in adult zebrafish following i.p. injection, particularly affecting the hematopoietic cells of the head kidney (LaPatra *et al.*, 2000). Transient toxicity of the viruses to hematopoietic precursors was observed, however kinetics of hematopoietic defects between IHNV and IPNV infection differed. The zebrafish antiviral response was studied in larvae and adults, following intravenous injection of IHNV and immersion, respectively (Aggad *et al.*, 2009, 2010). Indeed, the expression of all four IFNs was measured in zebrafish larvae (82 hpf) infected by i.v. injection of IHNV, revealing that *ifnphi1*, *ifnphi2*, and *ifnphi3* are induced in the larvae at 24 hpi, with *ifnphi3* showing the highest levels of induction (Aggad *et al.*, 2009). In this study, IHNV bath did not result in any obvious sign of infection in adults. Furthermore, Aggad *et al.* (2010) showed that the expression levels of the type II IFN *ifng1* and *ifng2* genes did not change upon infection with IHNV performed by injection into the caudal vein of 60 hpf larvae. Importantly, the spread of a viral infection throughout the whole organism was imaged for the first time in a vertebrate using the IHNV zebrafish larvae model (Ludwig *et al.*, 2011). Larvae infected by i.v. injection died within three to four days when incubated at 24°C, which is a permissive temperature for virus replication. Furthermore, Ludwig *et al.* (2011) demonstrated the utility of the poikilothermic

zebrafish model for temperature-shift experiments, the temperature at which infected embryos or larvae are maintained possibly being shifted several degrees to block viral replication at various time points.

Nodaviridae – Nervous necrosis virus (NNV). The infection produced by one representative of the *Nodaviridae* family, the malabaricus grouper (*Epinephelus malabaricus*) nervous necrosis virus (MGNNV), has been modelled in zebrafish (Lu *et al.*, 2008). In adult zebrafish infected by i.p. injection of MGNNV, viral titers peaked at 3 dpi and histological study showed lesions in brain tissues similar to that in natural host infection. Microinjection of MGNNV in zebrafish larvae caused 98% mortality at 24 hpi, associated with a higher amount of viral RNA2 in larval stage than in adult brain samples (Lu *et al.*, 2008). The same study demonstrated that different behavior of type IFN response in larvae or in adult zebrafish can induce acute and persistent infections respectively, an elevated interferon expression in adult relative to infected larvae resulting in a higher rate of mortality in the latter. In a study investigating the susceptibility of ornamental freshwater fish to RGNNV after intramuscular injection, no apparent clinical sign or mortality was observed for adult zebrafish and viral titers were below the detection limits (Furusawa *et al.*, 2007). Following exposure of zebrafish larvae to a NNV strain 99% similar to that of the sevenband grouper nervous necrosis virus (SGNNV) by bath immersion, results were shown to be dependent on the developmental stage of the larvae during the challenge (Morick *et al.*, 2015). Indeed, developing zebrafish embryos (2 dpf) were mostly resistant to NNV (5% mortality), all 4-day old larvae succumbed to the infection and larvae at 6 and 8 dpf were much less sensitive, showing better survival rates (24% and 28% mortality, respectively).

Amnoonviridae – Tilapia lake virus (TiLV). TiLV is a recently characterized enveloped orthomyxo-like virus with a genome of 10 segments of linear negative-sense single-stranded RNA (Eyngor *et al.*, 2014). Causing many disease outbreaks in the main tilapia producing countries and massive mortality of wild and farmed tilapia species, TiLV is considered as a threat to the fast-growing tilapia aquaculture because of its spread in Asia, Africa, South and North America (Jansen *et al.*, 2019; OIE, 2019b). A zebrafish model allowing to study the mechanisms of the TiLV infection and to follow antiviral responses was developed recently by Rakus *et al.* (2020). Increased viral load was observed in liver, spleen and kidney of adult zebrafish infected with TiLV by i.p. injection at 1, 3, 6, and 14 dpi, and the expression of genes involved in antiviral immune response, such as *rig-I, ifnphi1*, *mxa*, *il1b*, *tnfa*, and *ifng1-2*, was up-regulated in spleen and kidney of adult zebrafish following TiLV infection (Rakus *et al.*, 2020). The adult zebrafish susceptibility to i.p. infection was thus demonstrated, the TiLV replicating in multiple organs with the brain showing the highest virus load at 6 dpi. Furthermore, the triggering of a strong type I IFN response and the activation of the adaptive immune response were suggested to play a role in limiting viral replication and improving fish survival, as the mortality of infected zebrafish was low in contrast to mortality of tilapia. Widziolek *et al.* (2021) showed that zebrafish larvae (2 - 2.5 dpf) were susceptible to TiLV infection upon systemic injection (i.e., microinjection in the duct of Cuvier),

TiLV replicating in larvae and causing a high rate of mortality of about 70%. Pathological abnormalities were well visible within the brain of infected larvae and the expression of immune-related genes such as *rigi*, *tlr3*, *tlr22*, *irf3*, *irf7*, *ifnphi1*, *mxa*, and *il1b*, was up-regulated. The protective effect of recombinant *ifnphi1* treatment on the survival of zebrafish larvae during TiLV infection was demonstrated, a decrease in mortality, morbidity and viral load being observed in treated larvae (Widziolek *et al.*, 2021). Finally, it was demonstrated that TiLV persists in the brain of adult zebrafish for at least 90 days (Mojzesz *et al.*, 2021). A clear sickness behavior was displayed in infected zebrafish and a strong antiviral and inflammatory response was induced in the brain of adult fish. Following injection of TiLV in the duct of Cuvier of zebrafish larvae (54 hpf), histopathological abnormalities were induced in the brain, with an activation of the microglia (Mojzesz *et al.*, 2021).

Reoviridae – Chum salmon reovirus (CSV). Belonging to the *Aquareovirus* genus, CSV is a non-enveloped virus with an icosahedral, double capsid and dsRNA genome (Winton *et al.*, 1981). CSV was isolated in 1978 from a stock of adult Chum salmon (*Onchorhynchus keta*) and its replication was demonstrated in fry of chum, chinook, and kokanee salmon, although no mortality was observed (Winton *et al.*, 1981). Recently, CSV has been reported to be able to replicate in the zebrafish cell line ZEB2J (Pinheiro and Bols, 2018). Moreover, Rakus *et al.* (2019) showed that CSV could replicate in zebrafish cell lines ZF4 and SJD.1 cell lines, inducing a cytopathic effect with formation of syncytia starting from 2 dpi. The expression of two antiviral genes, *vig-1* and *mxa*, was up-regulated in both cell lines, indicating an antiviral response of zebrafish cells to the infection. Following i.p. injection of CSV in adult zebrafish, viral RNA was detected in kidney and spleen during the whole course of the experiment until 14 dpi where a decrease in viral RNA level was observed, this being correlated to the expression of *vig-1* and *mxa* genes which was first up-regulated then decreasing (Rakus *et al.*, 2019). In larvae, no external signs of diseases and no mortalities were observed during CSV infection and no viral RNA and up-regulation of *vig-1* and *mxa* gene expression was detected following inoculation with CSV by bath infection.

Reoviridae – Grass carp reovirus (GCRV). GCRV also belongs to the *Aquareovirus* genus. It causes a serious infectious disease, called grass carp hemorrhagic disease (GCHD), mainly affecting fingerlings during rearing and leads to severe economic losses to aquaculture in China (Su and Su, 2018). Zhu *et al.* (2020b) demonstrated for the first time that zebrafish could be infected by GCRV and could display death and other pathogenic phenotypes like those displayed in infected grass carp. Protein arginine methyltransferase 7 (PRMT7), known to catalyze the formation of stable monomethylarginines of histones, was inhibited in 2-month-old zebrafish following GCRV infection by i.p. injection. The knockout of *prmt7* in zebrafish was shown to increase the resistance to GCRV infection, prmt7-null fish exhibiting enhanced expression of key antiviral genes and fewer necrotic cells in the liver and kidney. The same results were observed in a study investigating the function of zebrafish PRMT3 in response

to GCRV infection (Zhu *et al.*, 2020a), thus implicating the vital role of zebrafish *prmt3* and *prmt7* or even arginine methylation in antiviral immunity. The role of the protein Isthmin 1 (Ism1) in immunity was investigated using the GCRV-zebrafish model as well (Li *et al.*, 2021). In male zebrafish (7 months old) injected intraperitoneally with GCRV, an induction of *ism1* expression was observed, suggesting its involvement in the antiviral response. Moreover, intraperitoneal injection of recombinant Ism1 (rIsm1) into zebrafish remarkably reduced the expression of *vp5*, a gene encoding the outer capsid protein of GCRV, suggesting that Ism1 could suppress GCRV replication in this fish (Li *et al.*, 2021).

3.3.2.2. Experimental infections with DNA viruses

Iridoviridae – Infectious spleen and kidney necrosis virus (ISKNV). There is a well-established model for experimental ISKNV infection of zebrafish (Li *et al.*, 2010; Xiang *et al.*, 2010; Xiong *et al.*, 2011; Xu *et al.*, 2008). The first ISKNV adult zebrafish infection model using intraperitoneal injections of virus was developed by Xu *et al.* (2008). A wide range of clinical signs, including erratic swimming, hovering near the surface, multifocal petechial hemorrhage, and scale protrusion, were exhibited by infected zebrafish, these symptoms being reminiscent of natural infections. Using ISKNV obtained from infected mandarinfish, initial infection of adult zebrafish caused high mortality rates (80% mortality in male and 65% mortality in female); however, the mortality ranged widely (from 0% to >70%) following subsequent passages in zebrafish (Xu *et al.*, 2008). In a subsequent study comparing the course of ISKNV infection in zebrafish and in *Tetraodon nigroviridis*, a significant induction of *ifnphi1* and *tnfa* transcription was observed in zebrafish, revealing the existence of a robust antiviral and proinflammatory responses to infection (Xu *et al.*, 2010). Moreover, *ifnphi1* protection against a DNA virus was proven for the first time in fish during ISKNV infection in zebrafish (Li *et al.*, 2010).

Iridoviridae – European sheatfish virus (ESV). Although a larval infection model has not been established yet for ISKNV, another member of the *Iridoviridae*, called ESV, has been studied in larvae (Martín *et al.*, 2015). ESV is an amphibian-like ranavirus phylogenetically related to the epizootic hematopoietic necrosis virus (EHNV) of rainbow trout. This virus, which was the first ranavirus isolated in Europe, causes high mortality rates in infected sheatfish (*Silurus glanis*) and other species (Mavian *et al.*, 2012). ESV has been studied in larvae and adults after immersion challenges, results showing that experimental infections caused a lethal pathology in both life stages (Martín *et al.*, 2015).

Iridoviridae – Lymphocystis disease virus (LCDV). Belonging to the genus *Lymphocystivirus,* LCDV is the causative agent of a chronic disease characterized by the clusters of enlarged hypertrophied dermal cells on the skin and fins of sick fish (Wolf, 1988). Affecting more than 100 teleost species, including marine and freshwater fishes worldwide, it causes serious economic losses in aquaculture industries due to the unpleasant appearance of the affected fish. Relative quantities of LCDV in the tissues of zebrafish were measured following infection by injection in the peritoneal cavity of male fish

(5 months old), showing positive signals in muscle, gills, liver, intestine, spleen, and kidney sampled at 48 hpi, with higher quantities of LCDV observed in spleen and intestine than in other tissues examined (Sun *et al.*, 2013). This work also demonstrated that recombinant phosvitin (Pv), an antimicrobial agent in zebrafish, was able to reduce the virus quantities in infected fish.

Alloherpesviridae – Cyprinid herpesvirus 1 (CyHV-1) and Cyprinid herpesvirus 3 (CyHV-3). The presence of mRNA transcripts (although on a low level), and thus replication, was demonstrated for CyHV-1 and CyHV-3 even though no cytopathic effects were observed in the ZF4 and SJD.1 cell lines following infection with these viruses (Rakus *et al.*, 2019). In addition, mRNA expression of ORF71 (which encoded putative helicase-primase subunit and function in DNA replication) was also detected for CyHV-3. At 1 and 4 dpi, the up-regulation of the expression of *vig-1* and *mxa* genes was not induced in infected ZF4 cells and infection of SJD.1 cells with CyHV-1 or CyHV-3 resulted in the down-regulation of both genes' expression (Rakus *et al.*, 2019). An induction of the up-regulation of *vig-1* and *mxa* expression in kidney and spleen and the presence of viral mRNA was demonstrated in adult zebrafish after i.p. injection of CyHV-3 but not in larvae (3 dpf) infected by immersion, indicating an antiviral response of adult zebrafish to infection. It was suggested that the route of infection and the age of zebrafish could play a role in its susceptibility to CyHV-3. Finally, this study's results indicate that that CyHV-3 could be considered for being a new model of viral infection for zebrafish.

Table 6 – Fish viruses studied *in vivo* using zebrafish (adapted from Sullivan *et al.*, 2021)

Virus family	Virus	Method(s) of infection
Group I: Double stranded DNA viruses		
Alloherpesviridae	Cyprinid herpesvirus 3 (CyHV-3)	Immersion (larvae), intraperitoneal injection (adult) (Rakus *et al.*, 2019)
Iridoviridae	European sheatfish virus (ESV)	Immersion (larvae and adult) (Martín *et al.*, 2015)
	Infectious spleen and kidney necrosis virus (ISKNV)	Intraperitoneal injection, natural occurrence (adult) (Bermúdez *et al.*, 2018; Xiong *et al.*, 2011; Xu *et al.*, 2008)
	Lymphocystis disease virus (LCDV)	Intraperitoneal injection (adult) (Sun *et al.*, 2013)
Group III: Double-stranded RNA viruses		
Birnaviridae	Infectious pancreatic necrosis virus (IPNV)	Vertical transfer (female), natural occurrence, immersion, intraperitoneal injection (adult) (Langevin *et al.*, 2013; LaPatra *et al.*, 2000; Seeley *et al.*, 1977)
Reoviridae	Chum salmon reovirus (CSV)	Intraperitoneal injection (adult), immersion (larvae) (Rakus *et al.*, 2019)
	Grass carp reovirus (GCRV)	Intraperitoneal injection (adult) (Li *et al.*, 2021; Zhu *et al.*, 2020a, b)
Group IV: Positive sense single-stranded RNA viruses		
Nodaviridae	Betanodavirus (nervous necrosis virus) (NNV)	Intraperitoneal injection (adult), natural occurrence, immersion (larvae) (Binesh, 2013; Lu *et al.*, 2008; Morick *et al.*, 2015; Morick and Saragovi, 2017)
Picornaviridae	Cyprivirus (ZfPV-1)	Natural occurrence (Altan *et al.*, 2019)
Retroviridae	Zebrafish endogenous retrovirus (ZFERV)	Natural occurrence (Frazer *et al.*, 2012; Shen and Steiner, 2004)
Group V: Negative sense single-stranded RNA viruses		
Rhabdoviridae	Spring viremia of carp virus (SVCV)	Immersion (larvae and adult), intraperitoneal injection (adult), duct of Cuvier (larvae), caudal vein injection (larvae) (Aggad *et al.*, 2009; Encinas *et al.*, 2013; Langevin *et al.*, 2013; Levraud *et al.*, 2007; López-Muñoz *et al.*, 2010; Sanders *et al.*, 2003; Varela *et al.*, 2014a)
	Snakehead rhabdovirus (SHRV)	Immersion (larvae), intraperitoneal injection (adult) (Gabor *et al.*, 2015; Kortum *et al.*, 2014; Phelan *et al.*, 2005a, b)
	Piscine novirhabdovirus (VHSV)	Immersion (larvae and adult), intraperitoneal injection (adult), intramuscular injection (adult) (Chinchilla and Gomez-Casado, 2017; Clarke *et al.*, 2017; Kavaliauskis *et al.*, 2015; Langevin *et al.*, 2013; Novoa *et al.*, 2006)
	Infectious hematopoietic necrosis virus (IHNV)	Intraperitoneal injection (adult), immersion (larvae), caudal vein injection (larvae), aorta injection (larvae), yolk injection (larvae) (Aggad *et al.*, 2009, 2010; Briolat *et al.*, 2014; Langevin *et al.*, 2013; LaPatra *et al.*, 2000; Liu and Collodi, 2002; Ludwig *et al.*, 2011)
Amnoonviridae	Tilapia lake virus (TiLV)	Immersion, intraperitoneal injection (adult), duct of Cuvier (larvae) (Rakus *et al.*, 2020; Widziolek *et al.*, 2021)

Objectives

Cyprinivirus induce diseases only in their natural host species. It has been suggested that this ability may result from restrictions related to host cell susceptibility and host cell permissivity. Within the genus *Cyprinivirus*, the anguillid herpesvirus 1 (AngHV-1), cyprinid herpesvirus 2 (CyHV-2) and cyprinid herpesvirus 3 (CyHV-3) can infect, even in an ineffective manner, cells originating from species that are not their natural hosts (Boutier *et al.*, 2015a; Thangaraj *et al.*, 2021; Ueno *et al.*, 1996). Recently, Rakus *et al.* (2019) has shown that cyprinid herpesvirus 1 (CyHV-1) and CyHV-3 were able to replicate in adult and embryo zebrafish cell lines and that viral mRNA and up-regulation of antiviral gene expression were observed in adult zebrafish after CyHV-3 infection by intraperitoneal injection but not in zebrafish larvae infected by immersion. However, the ability of AngHV-1 and CyHV-2 to infect these zebrafish models has not been studied yet.

Given the issues highlighted above, the broad objective of the present thesis was to investigate the potential of the zebrafish model to study AngHV-1, CyHV-2, and CyHV-3, which are three economically important viruses in the family *Alloherpesviridae*. The aim was to compare the ability of these three viruses to infect zebrafish cells and larvae and to characterize the response to infection in the zebrafish larvae model. Accordingly, this thesis is comprised of two main objectives.

Objective 1: To compare AngHV-1, CyHV-2, and CyHV-3 in terms of their ability to infect zebrafish models both *in vitro* and *in vivo* by testing the susceptibility and permissivity of the ZF4 cell line and of zebrafish larvae to these three cyprinivirus. Presently, it has been shown that intraperitoneal inoculation of CyHV-3 induces an abortive infection in adult zebrafish and that CyHV-3 can infect zebrafish cell lines. However, up to this point, there was no existing study comparing the ability of AngHV-1, CyHV-2, and CyHV-3 to infect zebrafish cell lines or zebrafish larvae, thus we aimed to fill this knowledge gap. To meet this objective, we used several recombinant viral strains previously described by the host lab, expressing green fluorescent proteins (GFP) and luciferase as reporters. An additional strain, expressing enhanced GFP (EGFP) and referred to as the CyHV-3 EGFP strain, was constructed specifically for this study.

Objective 2: To gain further understanding of the antiviral response to cyprinivirus infection in the zebrafish larvae. This main objective of this thesis involved the first study of the zebrafish response to CyHV-3 using RNAseq analysis. The aim was to characterize the response to CyHV-3 infection in terms of the interferon stimulated genes (ISG) up-regulation, potential involvement of programmed cell death; and to establish the extent of CyHV-3 gene transcription in the zebrafish larvae model. The aim of the next part of the thesis was to test the impact of two antiviral proteins on CyHV-3 clearance using CRISPR/Cas 9 generated zebrafish mutants.

By completing these two main objectives, we aimed to (i) answer important questions regarding the susceptibility and permissivity of zebrafish models to alloherpesviruses, (ii) conduct the first in depth

characterization of zebrafish innate immune responses to a cyprinivirus, and (iii) use this information to explore the potential modulation of zebrafish permissivity to infection. The ultimate goal is to exploit this knowledge in future research projects focusing on the fundamental aspects of virus-host interaction through the use of CyHV-3-zebrafish model *versus* CyHV-3-carp models. Moreover, the zebrafish mutants generated for this study will represent useful subjects for further studies.

Experimental section

Experimental section

1st study:

Inoculation of ZF4 cells and zebrafish larvae by Cyprinid Herpesvirus 2 or Cyprinid Herpesvirus 3 leads to abortive infection

Synthèse des travaux menés

Les cyprinivirus ne provoquent des maladies que chez leurs hôtes naturels. Il a été suggéré que cette capacité pouvait résulter de restrictions liées à la sensibilité et à la permissivité des cellules hôtes. Au sein du genre *Cyprinivirus*, l'anguillid herpèsvirus 1 (AngHV-1), le cyprinid herpèsvirus 2 (CyHV-2) et le cyprinid herpèsvirus 3 (CyHV-3) peuvent infecter, même de manière inefficace, des cellules provenant d'espèces qui ne font pas partie de leurs hôtes naturels (Boutier *et al.*, 2015a; Thangaraj *et al.*, 2021; Ueno *et al.*, 1996). Récemment, il a été démontré que le CyHV-3 était capable de se répliquer dans deux lignées cellulaires dérivées respectivement d'adultes (SJD.1) et d'embryons (ZF4) de poisson-zèbre (*Danio rerio*) et qu'il pouvait également induire une infection abortive après inoculation intrapéritonéale du virus chez le poisson-zèbre adulte (Rakus *et al.*, 2019). Jusqu'à présent, il n'existait aucune étude comparant la capacité d'AngHV-1, de CyHV-2 et de CyHV-3 à infecter des modèles de poisson-zèbre, à la fois *in vitro* et *in vivo*. Cette première étude a donc pour objectif d'évaluer le potentiel du poisson-zèbre comme modèle expérimental pour étudier ces trois virus économiquement importants de la famille des *Alloherpesviridae*.

Pour atteindre cet objectif, nous avons utilisé plusieurs souches virales recombinantes précédemment décrites par le laboratoire d'accueil et exprimant des protéines fluorescentes vertes (GFP) et la luciférase (Luc2) comme rapporteurs. Une souche supplémentaire, appelée CyHV-3 EGFP, a été construite spécifiquement pour cette étude. Tout d'abord, la sensibilité et la permissivité des cellules ZF4 à l'infection par l'AngHV-1, le CyHV-2 et le CyHV-3 ont été suivies en exploitant les souches recombinantes exprimant des rapporteurs fluorescents et la microscopie à épifluorescence. Afin de caractériser davantage ces infections, nous avons utilisé le système d'imagerie cellulaire automatisée en temps réel Incucyte. Le nombre de cellules infectées, représenté par le nombre de cellules fluorescentes, a ainsi pu être relevé toutes les deux heures entre le 1er et le 11ème jour post-infection. Les données issues de l'imagerie en temps réel ont été analysées à l'aide d'un programme informatique, le plugin *Fiji Track-Mate* (v7.7.2) afin de suivre l'expression des rapporteurs fluorescents dans les cellules ZF4 infectées et d'identifier les événements d'infection et de mort cellulaire en fonction du temps. Ensuite, nous avons étudié la sensibilité et la permissivité des larves de poissons-zèbres à l'infection par l'AngHV-1, le CyHV-2 et le CyHV-3. Pour ce faire, des larves de poissons-zèbres *Wild Type* (WT) AB âgées de 3 jours post-fertilisation ont été infectées avec les recombinants exprimant les rapporteurs fluorescents et la luciférase, de deux manières différentes : par micro-injection d'une suspension virale dans le cœur et par immersion dans de l'eau contenant les virus. La progression de l'infection des larves par les souches virales recombinantes exprimant les rapporteurs fluorescents a été suivie au moyen de la microscopie à épifluorescence. Cela a facilité l'observation longitudinale de larves individuelles entre le 1er et le 4ème jour post-infection. Un système d'imagerie *in vivo* (IVIS) (IVIS Spectrum, PerkinElmer) a été utilisé pour détecter par bioluminescence chez les larves infectées, les virus recombinants exprimant la

luciférase, facilitant ainsi la quantification des niveaux viraux *in vivo*. Contrairement à l'analyse par épifluorescence, le suivi longitudinal de larves au niveau individuel, n'a pas été possible en raison des effets néfastes des injections répétées de D-luciférine chez les larves. Enfin, un microscope à nappe de lumière Zeiss Z1 a été utilisée afin d'acquérir des images de la tête et du cœur des larves vivantes infectées par le CyHV-3 EGFP entre le 2ème et le 3ème jour post-infection. Une vidéo a pu être construite à l'aide du logiciel ImageJ.

Entre le 1er et le 4ème jour post-infection, l'observation quotidienne en microscope à épifluorescence, de la lignée cellulaire ZF4 infectée par les trois cyprinivirus indépendamment a révélé que les cellules ZF4 présentaient une faible sensibilité aux cyprinivirus testés, aucune permissivité à l'infection par l'AngHV-1 et une permissivité fortement réduite à l'infection par le CyHV-2 et le CyHV-3 par rapport aux observations typiques dans les cellules dérivées de leurs hôtes naturels respectifs et couramment utilisées pour la culture de ces virus. Par ailleurs, aucune formation de syncytia, de plages de lyse ou d'autres effets cytopathiques n'a été observée dans les cellules infectées par l'AngHV-1, le CyHV-2 ou le CyHV-3.

L'analyse des données issues de l'imagerie en temps réel des cellules infectées par les trois cyprinivirus a confirmé que le nombre de cellules infectées par AngHV-1 était très faible comparé aux cellules infectées par CyHV-2 et CyHV-3 et que ce nombre n'augmentait pas au cours du temps. Nous avons observé une augmentation continue des cellules infectées par le CyHV-2 et le CyHV-3 entre 24 à 144 heures post-infection (hpi). Au cours de cette période, le taux de propagation du CyHV-2 et du CyHV-3 n'était pas exponentiel, ce qui indique une faible efficacité de réplication dans les cellules infectées et/ou une transmission réduite de nouvelles particules virales infectieuses à d'autres cellules. De 144 à 264 hpi, le nombre de cellules infectées a diminué régulièrement jusqu'à la fin de l'expérience pour les deux virus, en lien avec leur élimination progressive. Cette clairance des cellules infectées était notamment largement caractérisée par des changements morphologiques de type apoptose. En effet, les événements de mort étaient, dans la majorité des cas, associés à un rétrécissement des cellules et à la formation de proéminences cytoplasmiques membranaires (« *membrane blebbing* ») conduisant à l'apparition de débris cellulaires ressemblant à des corps apoptotiques (Orzalli and Kagan, 2017; Shlomovitz *et al.*, 2018; Zhang *et al.*, 2018). Cependant, l'apparition du mécanisme apoptotique n'a pu être définitivement confirmée par la combinaison d'autres techniques d'analyse. Par ailleurs, nous avons également observé un autre type de mort cellulaire ne correspondant pas morphologiquement à l'apoptose et qui impliquait principalement un gonflement initial suivi d'un rétrécissement des cellules et d'une absence de débris cellulaires ressemblant à des corps apoptotiques avant la disparition du signal fluorescent. Ce phénomène pourrait correspondre, d'un point de vue morphologique, à la nécrose, où la mort cellulaire est associée à une rupture de la membrane et à une fuite du contenu cytoplasmique (Orzalli and Kagan, 2017; Shlomovitz *et al.*, 2018; Zhang *et al.*, 2018). La nécrose peut également être

initiée d'une manière hautement régulée, connue sous le nom de nécroptose, et agissant comme un soutien à l'apoptose (Morgan and Kim, 2022; Verdonck *et al.*, 2022). Cependant, il n'a pas été possible de différencier la nécrose de la nécroptose sur la base de nos seules observations morphologiques, et comme pour l'apoptose, ces réponses n'ont pu être confirmées par l'utilisation d'autres techniques d'observation (notamment par l'usage de colorations cellulaires spécifiques).

Contrairement au CyHV-3, la sensibilité des cellules ZF4 à l'AngHV-1 et au CyHV-2 n'avait pas été étudiée avant cette étude. Nos résultats indiquent que si les cellules ZF4 sont également sensibles à l'infection par l'AngHV-1 et le CyHV-2, elles ne sont permissives qu'à ce dernier. Cependant, comme pour le CyHV-3, la permissivité à l'infection par le CyHV-2 était modérée et transitoire. En effet, ces deux virus sont finalement éliminés dans un processus précédé par un phénomène ressemblant morphologiquement à une mort cellulaire programmée généralisée parmi les populations de cellules infectées. Ces similitudes des réponses observées au niveau cellulaire entre le CyHV-2 et le CyHV-3 et leurs différences avec AngHV-1 dans ce contexte peuvent refléter le fait que le CyHV-2 et le CyHV-3 sont phylogénétiquement plus proches l'un de l'autre qu'ils ne le sont de AngHV-1 (Davison *et al.*, 2013; Donohoe *et al.*, 2021; Van Beurden *et al.*, 2010; Waltzek *et al.*, 2009). En outre, compte tenu de leurs hôtes naturels, il est logique que le CyHV-2 et le CyHV-3 soient intrinsèquement mieux adaptés à la croissance dans les cellules ZF4 que l'AngHV-1. Malgré l'absence de permissivité durable aux cyprinivirus, ces expériences *in vitro* indiquent que les mêmes virus recombinants peuvent être utilisés pour étudier l'infection transitoire et la clairance des cyprinivirus chez les larves de poisson-zèbre qui peuvent ainsi constituer un modèle hôte-pathogène d'intérêt.

L'inoculation de souches recombinantes exprimant des rapporteurs fluorescents et la luciférase chez les larves de poisson-zèbre par immersion ou par micro-injection a révélé que les larves ne sont sensibles à aucun des trois virus testés par immersion. Ces résultats sont similaires à ceux précédemment obtenus avec le CyHV-3 chez des larves de poisson-zèbre (Rakus *et al.*, 2019) et nous permettent de conclure que ces virus ne peuvent pas pénétrer dans les larves de poisson-zèbre par des voies naturelles *in vivo*. À l'inverse, les larves étaient sensibles à CyHV-2 et CyHV-3 lorsqu'elles étaient infectées par micro-injection dans le cœur, mais pas à AngHV-1 en utilisant cette méthode d'inoculation artificielle. Conformément aux observations antérieures *in vitro*, le CyHV-2 et le CyHV-3 s'adaptent beaucoup mieux à ce modèle que l'AngHV-1. Pour ces deux *cyprinid herpèsvirus*, un pic d'infection est atteint au 2ème jour post-infection et la clairance virale commence entre le 2 et le 3ème jour, menant à l'élimination rapide du virus. Indépendamment du mode d'inoculation utilisé ou du virus, aucune morbidité ou mortalité n'a été observée chez les larves. Compte tenu des niveaux viraux plus élevés et de la clairance plus lente observée chez les larves infectées par le CyHV-3, nous concluons que les larves de poissons-zèbres présentent une plus grande sensibilité (et peut-être une plus grande permissivité) à ce virus, ce qui indique l'utilité supérieure de ce modèle hôte-virus dans de futures études.

Nos observations sont largement cohérentes avec la description antérieure d'infections par le CyHV-3 chez le poisson-zèbre adulte (inoculation par injection intrapéritonéale) (Rakus *et al.,* 2019). Une absence notable de mortalité dans les études précédentes impliquant l'infection du poisson-zèbre par d'autres virus affectant les poissons cyprinidés avait également été observée (Rakus *et al.,* 2019). Le poisson-zèbre peut naturellement posséder des défenses robustes contre d'autres virus étroitement liés aux CyHV-2 et CyHV-3 qui peuvent avoir circulé dans leur habitat naturel au cours de leur évolution. Il est également possible que cette absence de mortalité soit liée à la dose virale ou même au site d'inoculation, qui peuvent tous deux avoir un impact sur la sévérité des infections virales chez les larves de poisson-zèbre, comme cela a été démontré dans d'autres études (Ge *et al.,* 2015; Lama *et al.,* 2022). Par ailleurs, étant donné que les souches de chaque espèce de cyprinivirus présentent une hétérogénéité naturelle en ce qui concerne la réplication *in vitro* et/ou *in vivo* (Davison *et al.,* 2013; Donohoe *et al.,* 2021), il reste possible que l'utilisation de souches de cyprinivirus alternatives puisse entraîner des résultats différents, ce qui pourrait être intéressant à explorer à l'avenir.

Les images obtenues en exploitant la microscopie à nappe de lumière ont révélé que, chez les larves de poisson-zèbre infectées par le CyHV-3, l'infection était principalement localisée autour de la région du cœur, reflétant la voie d'inoculation. Conformément aux observations précédentes, une réduction des niveaux viraux a commencé entre 2,5 et 3 jours post-infection. Les images ont notamment révélé une augmentation substantielle de la mort cellulaire de type apoptose immédiatement avant l'élimination, ce qui indique que la mort cellulaire programmée peut également jouer un rôle majeur dans ce processus *in vivo*. Bien que l'apparition de l'apoptose en réponse à l'infection par le CyHV-3 *in vivo* n'ait pas été confirmée par d'autres techniques d'analyse dans la présente étude, nos observations sont similaires à celles d'études antérieures impliquant une observation en temps réel de larves de poisson-zèbre infectées par le virus du Chikungunya (CHIKV) (Palha *et al.,* 2013). Tout au long de la période d'observation des larves, des cellules très mobiles, pouvant être des macrophages ou des neutrophiles, étaient infectées par des particules virales. Ces données n'ont pas fourni de preuves évidentes de l'apparition de nouvelles cellules infectées avant le début de la clairance. Ici, l'induction d'une réponse de mort cellulaire programmée parmi les cellules infectées *in vivo*, interrompant le cycle de réplication du CyHV-3, conduirait à une réduction de la transmission réussie du CyHV-3 à de nouvelles cellules. Toutefois, ceci implique que les cellules de larves de poisson-zèbre sont intrinsèquement permissives à la réplication du CyHV-3. Pour ce faire, le suivi de l'expression de tous les gènes codant pour les protéines essentielles du CyHV-3 *in vivo* serait nécessaire. Nous proposons donc d'utiliser une approche RNA-Seq afin d'établir l'étendue de la transcription des gènes du CyHV-3 *in viv*o et d'étudier la nature des paramètres de l'immunité innée impliqués dans la réponse contre ce virus.

Experimental section

1st study:

Inoculation of ZF4 cells and zebrafish larvae by Cyprinid Herpesvirus 2 or Cyprinid Herpesvirus 3 leads to abortive infection

In Susceptibility and permissivity of zebrafish (*Danio rerio*) larvae to cypriniviruses

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Abstract

The zebrafish (*Danio rerio*) represents an increasingly important model organism in virology. For instance, it has acted as a useful model to study the Human Herpesvirus 1 (HHV−1) and viruses infecting reared fish such as the spring viraemia of carp virus (SVCV). It has recently been shown that Cyprinid Herpesvirus 1 (CyHV−1) and Cyprinid Herpesvirus 3 (CyHV−3) can replicate in a zebrafish cell line established from 1−day−old embryos (ZF4 cell line) and that CyHV-3 can replicate in adult zebrafish, however this is followed by rapid clearance of the viral infection. We evaluated the utility of the zebrafish model in the study of economically important viruses from the genus *Cyprinivirus*, namely Cyprinid Herpesvirus 2 (CyHV−2, affecting goldfish and Gibel carp), the CyHV−3 (affecting common and koi carp) and the Anguillid Herpesvirus 1 (AngHV−1, affecting eel species). This revealed that zebrafish larvae were not susceptible to these viruses after immersion in contaminated water, but that infections with CyHV−2 and CyHV−3 could be established, using artificial infection models *in vitro* (zebrafish cell lines) and *in vivo* (microinjection of larvae). However, infections were transient, with rapid viral clearance associated with apoptosis−like death of infected cells.

Keywords: anguillid herpesvirus 1; cyprinid herpesvirus 2; cyprinid herpesvirus 3; alloherpesvirus; cyprinivirus; zebrafish

1. Introduction

The zebrafish (*Danio rerio*) is a member of the family *Cyprinidae*. It is an extremely useful experimental subject due to its high fecundity and short generation time and is currently one of the most widely used laboratory animal model organisms. Also, its transparent larval stage is highly suited to *in vivo* imaging, making it particularly well suited to studying host−pathogen interaction, including during viral infection [1]. Furthermore, the availability of a well−annotated zebrafish reference genome [2] and large range of recombinant and mutant zebrafish lines [3] greatly facilitates investigations into gene function in various biological contexts. The zebrafish is known to possess a well−developed immune system, composed of both innate and adaptive immune responses [4,5]. Despite some notable differences and although sites of maturation differ [6], many mammalian immune system cell types have zebrafish counterparts [7,8]. Also, zebrafish orthologs of many (but not all) mammalian pathogen recognition receptors (PRRs), cytokines, adaptor proteins for signal transduction and other important components have been identified [6,9,10], indicating that zebrafish represent a relatively useful model for studying the mechanisms that vertebrates use to detect and respond to pathogen−associated molecular patterns (PAMPs).

Although juvenile and adult zebrafish utilize both the innate and the adaptive branches of the immune system, the embryonic and larval stages rely solely on innate immunity, which is detectable and active on the first day of zebrafish embryogenesis, whereas the adaptive system is fully matured by 4–6 weeks post−fertilization [11,12]. During these early life stages, cellular immunity is mediated by myeloid cells only, with macrophages and neutrophils acting as the main effector cells [13,14]. As in mammals, the zebrafish antiviral response is orchestrated by type I pathogen induced interferons (IFNs). These are named IFNϕ1, IFNϕ2, IFNϕ3, and IFNϕ4 [15] (referred to hereafter by the respective gene symbols *ifnphi1*, *ifnphi2*, *ifnphi3,* and *ifnphi4*) and are structurally similar to mammalian type I (α and β) IFNs. As in all vertebrates, type I IFNs in zebrafish induce the expression of antiviral genes broadly referred to as interferon stimulated genes (ISGs). However, the IFN response in zebrafish larvae is mediated solely by *ifnphi1* and *ifnphi3*, with *ifnphi2* being expressed only in adults and with *ifnphi4* having little activity [16,17]. The zebrafish type II IFN family consists of two members, IFNγ1 and IFNγ2 which are also responsible for the induction of ISGs induced by type I IFNs [18].

Taken together, this indicates that the zebrafish represents a relevant and useful model for studying viral pathogenicity, vertebrate host immune response, and viral host−interactions. Strikingly, very few viruses are known to infect zebrafish naturally [19–22]. Moreover, despite the lower host temperature, several mammalian viruses can infect zebrafish under experimental conditions, with these hosts exhibiting varying degrees of susceptibility and permissivity to infection. This property has also been exploited to study human viruses such as influenza A virus (IAV), Chikungunya virus (CHIKV), human herpesvirus 1 (HHV−1) and human norovirus [23–26]. Moreover, infection of zebrafish has been

explored in studying severe acute respiratory syndrome coronavirus 2 (SARS−CoV−2) [27,28]. Zebrafish can also be infected with several important fish viruses [29–35]. One of these, the spring viraemia of carp virus (SVCV), which is a rhabdovirus responsible for a highly contagious disease of the common carp (*Cyprinus carpio*), has become one of the viruses most frequently used in infection models for studying the antiviral immune response in zebrafish larvae and adults [16,17,35–37]. Recent work by Rakus *et al.* [38] demonstrated that cyprinid herpesvirus 3 (CyHV−3) induces an abortive infection after intraperitoneal inoculation of adult zebrafish. CyHV−3 causes mass mortality in common carp and koi carp (*Cyprinus rubrofuscus* var. "*koi*"), resulting in massive economic losses [39]. CyHV−3 is a member of the genus *Cyprinivirus* in the family *Alloherpesviridae*, which consists of herpesviruses that infect fish and amphibians.

In addition to CyHV−3, the genus *Cyprinivirus* contains two other economically important viruses: anguillid herpesvirus 1 (AngHV−1) and cyprinid herpesvirus 2 (CyHV−2) [40]. AngHV−1 infects the European eel (*Anguilla Anguilla*), Japanese eel (*Anguilla japonica*), and American eel (*Anguilla rostrata*) [41]; CyHV−2 also infects goldfish (*Carassius auratus*) and the closely related Prussian carp (*Carassius gibelio*) and crucian carp (*Carassius carassius*) [42]. Like zebrafish, the natural hosts of CyHV−2 and CyHV−3 are also members of the family *Cyprinidae*. Cypriniviruses cause diseases only in their natural host species, which suggests the existence of restrictions related to host cell susceptibility (i.e., the ability to support virus entry) and host cell permissivity (i.e., the ability to support viral replication and the transmission of viable viral progeny to new cells, although the former may occur without the latter). Notably, experiments relying on infection of cell lines have demonstrated the ability of cypriniviruses to infect, even if inefficiently, cells originating from non−natural host species. Indeed, both CyHV−2 and CyHV−3 are capable of infecting cell lines derived from species within the family *Cyprinidae* that are not their natural hosts [39,42], with CyHV−3 already known to infect zebrafish cell lines [38]. Similarly, despite not naturally infecting species outside of the family *Anguillidae*, it has been demonstrated that AngHV−1 can infect at least one cell line derived from a member of the family *Cyprinidae* [43]. These data suggest that the ability of cypriniviruses to induce diseases only in their natural host species may be related to complex host−virus interactions downstream of host cell susceptibility.

In the present study, we conducted an in−depth evaluation and comparison of AngHV−1, CyHV−2, and CyHV−3 in terms of their ability to infect zebrafish models both *in vitro* and *in vivo*. These experiments involved the exploitation of recombinant viruses expressing reporters, timelapse epifluorescence microscopy *in vitro* and live imaging. Our results suggest that the zebrafish ZF4 cell line and zebrafish larvae are moderately susceptible to CyHV−2 and CyHV−3 infection and exhibit transient permissiveness to these cypriniviruses. However, the infection is ultimately cleared both *in vitro* and *in vivo*, in a process which is preceded by the apoptosis-like death of infected cells.

2. Materials and methods

2.1 Cells and viruses

The zebrafish embryonic fibroblast cells line (ZF4) [44] was kindly provided by Dr K. Rakus (Department of Evolutionary Immunology, Jagiellonian University, Poland) and cultured in advanced Dulbecco's modified Eagle's Medium/Ham's F−12 (Gibco), supplemented with 10% fetal calf serum (FCS), 2% penicillin–streptomycin (Sigma−Aldrich) and 1% L-glutamine (Lonza). Cells were cultured at 25 °C in a humid atmosphere containing 5% CO_2. Eel kidney (EK−1) [45], Ryukin goldfish fin (RyuF−2) [46], and common carp brain (CCB) [47] cell lines were used to produce stocks of AngHV−1, CyHV−2, and CyHV−3, respectively. These cells were cultured as described previously [46,48,49].

Three previously described recombinant viral strains were utilized. The CyHV−3 FL BAC revertant ORF136 Luc strain (referred to as CyHV−3 Luc; GenBank accession KP343683.1) was derived from the CyHV−3 FL BAC plasmid and encodes a firefly (*Photinus pyralis*) luciferase (Luc2) reporter cassette driven by a human cytomegalovirus (CMV) promoter inserted between ORF136 and ORF137 [50]. The AngHV−1 Luc−copGFP and the CyHV−2 Luc−copGFP recombinant strains both encode the same reporter genes consisting of the Luc2 cassette and a copepod (*Pontellina plumata*) GFP (copGFP) cassette linked by a T2A sequence, driven by a eukaryotic translation elongation factor 1 alpha (EF-1α) promoter. To generate these recombinants, the dual Luc2/copGFP cassette was inserted in the region between ORF32 and ORF33 in the AngHV−1 UK parental strain genome (GenBank accession MW580855.1) [40] (Delrez *et al.*, unpublished data) and in the intergenic region between ORF64 and ORF66 in CyHV−2 YC−01 parental strain genome (GenBank accession no. MN593216.1) [51] using homologous recombination in eucaryotic cells, as described previously [50].

2.2 Production of CyHV−3 ORF136 EGFP recombinant strain

In addition to the three recombinant strains described above, a fourth strain, expressing enhanced (EGFP), referred to as the CyHV−3 EGFP strain, was derived from the CyHV−3 Luc strain and constructed specifically for this study. In all experiments, CCB cells and CyHV−3 were both cultured at 25°C as described previously [50,52].

2.2.1. Production of a ORF136−ORF137−EGFP recombinant plasmid

A modified pcDNA3−LUC vector, kindly provided by M. Bremont (INRA, France), previously used by our lab [50], was subjected to XbaI digestion, resulting in the excision of a luciferase ORF. The luciferase ORF was replaced with an EGFP ORF. This was done by PCR amplification of an EGFP ORF using Phusion High−Fidelity PCR Master Mix (NEB) from a p3E−EGFPpA plasmid (tol2kit plasmid), kindly provided by Dr Isabelle Manfroid (GIGA Stem Cells − Zebrafish Development and Disease Model, University of Liège, Belgium). The primers used for this (Fw−EGFP−linkDH59 and Rev-Fw−EGFP−linkDH59) bore homology to regions (15 bp length) adjacent to their respective XbaI cut−sites in the pcDNA3−LUC vector facilitating insertion using NEBuilder, Hifi DNA Assembly

Master Mix (NEB). This resulted in pcDNA3−EGFP vector containing an EGFP gene expression cassette, flanked by a human cytomegalovirus (CMV) promoter and poly 3'UTR/Polyadenylation signal. The pGEMT−136NheI137 vector, containing an insertion partially encompassing CyHV−3 ORF135, ORF136 and ORF137 (nucleotides 234511−235330 of the CyHV−3 Luc genome, GenBank accession no. KP343683.1) with an NheI restriction site inserted into the intergenic region between CyHV−3 ORF 136 and ORF 137 (previously used by our lab [50]), was digested by NheI. The EGFP expression cassette was amplified by PCR from the pcDNA3−EGFP vector as described previously. The primers used for this (Fw−CMV−EGFP−link PGEMT136/137 and Fw−CMV−EGFP− link PGEMT136/137) bore homology to regions (15 bp length) adjacent to the NheI cut site (with forward and reverse primers corresponding to nucleotides 231,413−231,422 and 231,423−231,432 of the CyHV−3 FL Luc genome respectively). This homology was used to guide insertion of the amplified EGFP expression cassette into the NheI−digested pGEMT−136NheI137 vector. This resulted in the creation of the pGEMT−136NheI137−EGFP vector expressing the EGFP reporter gene, in which the EGFP cassette is flanked by CyHV−3 sequences (Figure 1a). All primer sequences are provided in Table S1.

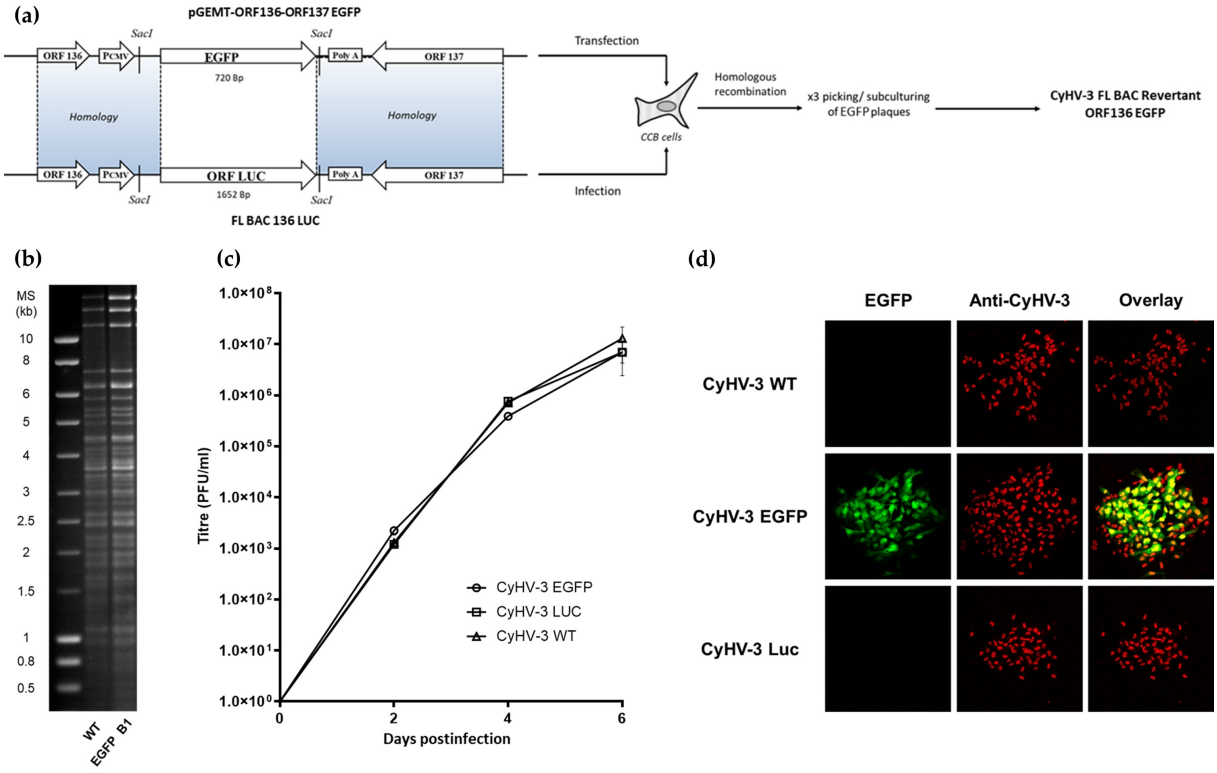

Figure 1. Production and characterization of the CyHV−3 EGFP recombinant strain. (**a**) Schematic representation of the strategy used to produce the CyHV−3 EGFP strain. The EGFP expression cassette was inserted into the intergenic region between ORF136 and ORF137 of the CyHV−3 Luc strain by homologous recombination in CCB cells, resulting in the CyHV-3−EGFP recombinant strain. (**b**) Comparison of SacI genome restriction between the CyHV−3 WT and EGFP recombinant strain genomes. (**c**) The replication kinetics of CyHV−3 EGFP strain was compared to those of the parental Luc and WT strains. Data presented are the mean ±

standard errors of replicate measurements. No significant differences were found between the strains. (**d**) *In vitro* expression of reporter protein by the CyHV−3 EGFP recombinant strain. CCB cells were infected with serial dilutions of the WT, Luc or EGFP strains and then overlaid with medium containing CMC. Four days post-infection, the cells were analyzed by immunofluorescent staining Anti−CyHV−3 (Mab 8G12) as the primary antibody and GAM 568 IgG2a as the secondary antibody and individual plaques were observed by confocal microscopy.

2.2.2. Generation of CyHV−3 EGFP by homologous recombination between CyHV−3 Luc and pGEMT ORF136−ORF137−EGFP vector

Freshly cultured CCB cells were transfected with the newly engineered pGEMT ORF136−ORF137−EGFP using Lipofectamine Plus as per manufacturers recommendation (Invitrogen) and incubated at 25°C for 24 hours (h). The same cell monolayers were then infected with parental CyHV3 Luc (GenBank accession no. KP343683.1) at the multiplicity of infection (MOI) of 1 plaque forming unit (PFU)/cell. Following an additional 5 days of incubation at the same temperature, single EGFP plaques were picked and sub−cultured. This was repeated three times until complete EGFP fluorescence was observed in the absence of luciferase signal over the entire cell monolayer.

2.2.3. Genetic characterization of the CyHV−3 EGFP recombinant strain

The sequence of the CyHV−3 EGFP recombinant strain was confirmed by monitoring SacI restriction fragment length polymorphism (RFLP) by agarose gel electrophoresis and full−length genome sequencing as described previously [49] (Figure 1b).

2.2.4. Viral growth assay

Triplicate cultures of CCB cells were infected with each CyHV−3 strain (CyHV−3 WT, CyHV−3 Luc and CyHV−3 EGFP) at a MOI of 0.05 PFU/cell. After an incubation period of 2 h, the cells were washed with PBS and overlaid with culture medium. The infected cells were scraped off and collected with the supernatant at successive intervals (0, 2, 4 and 6 days postinfection (dpi)) and stored at −80°C. Titers of infectious viral particles were determined by triplicate plaque assays in CCB cells as described previously [50,52] (Figure 1c).

2.2.5. Viral plaque area assay

Triplicate cultures of CCB cells grown in 6−well plates were inoculated with three different isolates (CyHV−3 WT, CyHV−3 Luc and CyHV−3 EGFP) at an MOI of 200 PFU/well. After incubating for 2 h, the cells were washed with PBS and overlaid with culture medium supplemented with 1.2% (w/v) carboxymethylcellulose (CMC; medium viscosity, Sigma), in order to obtain isolated plaques [53]. At various times postinfection (pi), individual plaques were visualized by indirect immunofluorescence staining and imaged using a confocal microscope (Nikon A1R or Leica SP5), and areas were measured using ImageJ software [54].

Cells were fixed in phosphate buffered saline (PBS) containing 4% (w/v) paraformaldehyde (PAF) at 4°C for 15 min and then at 20°C for 10 min. After washing with PBS, samples were permeabilized in PBS containing 0.1% (v/v) Nonidet P−40 at 37°C for 15 min. Immunofluorescent staining (incubation and washes) was performed in PBS containing 10% (v/v) fetal calf serum (FCS). The mouse monoclonal antibody 8G12 raised against an unidentified CyHV−3 nuclear protein (diluted 1:500, v/v) was used as a primary antibody and was incubated at 37°C for 1 h. After washing with 10% v/v PBS−FCS, Alexa Fluor 546 goat anti−mouse (GAM) IgG2a (Invitrogen) was used as the secondary antibody (diluted 1:600) in 10% (v/v) PBS−FCS. The secondary antibodies were incubated at 37 °C for 30 min. After washing, the cells were mounted using Prolong Gold antifade reagent (Invitrogen) and analyzed by confocal microscopy (Figure 1d).

2.3 In vitro experiments

2.3.1. Virus infections

ZF4 cells cultured in 24−well plates were mock-infected or infected at 24 hours (h) after seeding. Virus was diluted in 0.5 mL serum−free cell culture medium to provide a MOI of 3 plaque forming units (PFU)/cell. After incubating for 2 h, 1 mL fresh cell culture medium was added without removing the inoculum, and the cells were incubated at 25 °C with 5% CO_2.

2.3.2. Timelapse imaging of infected cells

At 24 h post infection (hpi), virus−infected ZF4 cells in 24−well plates were placed in an IncuCyte Zoom HD/2CLR microscopy system (Sartorius), which was maintained at 25 °C with 5% CO_2. Each well was imaged at 9 different fields of view every 2 h from 1–11 days post infection (dpi). Images were collected in phase contrast and in the green (GFP) channels. Each infection was done in triplicate wells.

2.3.3. Image analysis

Data from timelapse imaging of infected cells was analyzed using the Fiji plugin TrackMate (v7.7.2) [55] to track fluorescent reporter expression from individual ZF4 cells infected with CyHV−2 or CyHV−3, and by extension, to identify cell infection and cell death events with respect to time. Image sequences containing 123 frames/field of view/well were generated using a series of images acquired from 1 to 11 dpi. Analysis was performed using the default settings with LoG Detector and Simple LAP Tracker. Additional parameters were adjusted empirically in order to adequately detect and monitor fluorescence from infected cells within frames (estimated object diameter: 28.6 pixels; quality threshold: 1). Data were exported in .csv format and imported into GraphPad Prism (v8.0.1) for further analysis and visualization.

2.4 Experiments using zebrafish

2.4.1. Zebrafish larvae maintenance

Wild−type (WT, +/+) AB strain adult zebrafish (*Danio rerio*) were obtained by natural spawning and maintained at 27 °C, on a 14/10 h light/dark cycle. They were housed in the GIGA Zebrafish facility in Liège (Belgium) according to animal research guidelines and with the approval of the local ethical commission for animal care and use. Larvae were obtained by pairwise mating of adults in mating cages and maintained in petri dishes with standard embryo medium (E3) and incubated at 25 °C prior to use in experiments.

2.4.2. Inoculation of larvae by immersion

Zebrafish larvae (3 days post −fertilization (dpf)) were placed in 24−well plates containing 1 ml E3 medium and either mock-infected or infected by immersion. For infection, virus suspensions were added to each well and mixed gently (final concentration: 4000 PFU/mL), and plates were incubated at 25 °C.

2.4.3. Inoculation of larvae by microinjection

Borosilicate glass capillaries were loaded with 10 µL of medium containing virus suspensions (1.2×10^6 PFU/ml) and then connected to a FemtoJet microinjector (Eppendorf, Framingham, MA, USA) as described elsewhere [56]. After breaking the capillary tip, the pressure was adjusted to obtain droplets with a diameter of ~0.13 mm. Larvae (3 dpf) were anesthetized in a bath containing tricaine (0.2 mg/mL). The fish were positioned on a petri dish, and the surface of the dish was dried entirely in order to avoid drifting of the larvae during viral injections. In order to visualize the hearts of the larvae, the petri dish was placed under a binocular magnifier (LEICA MZ6) at 4x magnification and illuminated by an external light source (LEICA CLS 50X). The capillary was then manually inserted into the pericardial cavity and three pulses were performed to inject approximately 3 nL of virus suspension (infected fish) or 3 nL of PBS (mock−infected fish). After microinjection, the larvae were transferred into individual wells in a 24−well plate containing 1 mL E3 medium and incubated at 25 °C.

2.4.4. Epifluorescence microscopy

The progression of infection with recombinant viruses expressing fluorescent reporters was monitored using epifluorescence microscopy. This facilitated longitudinal observation of the same larvae at multiple timepoints. Prior to observation, larvae were anesthetized in a bath of E3 medium containing tricaine (0.2 mg/ml) and methylcellulose (2% w/v) in order to avoid drifting of larvae. Imaging of larvae was performed using a Leica DM2000 epifluorescence microscope at 5x and 10x magnification. After imaging, larvae were immediately transferred back to their individual wells and returned to the incubator. After the final observation timepoint, larvae were euthanized using an overdose of tricaine in E3 media (400 mg/L).

2.4.5. *In vivo* bioluminescent imaging

An *in vivo* imaging (IVIS) system (IVIS Spectrum, PerkinElmer) was used to detect bioluminescence in larvae infected with Luc2−expressing recombinant viruses, thus facilitating the

monitoring and quantification of viral levels *in vivo*. At the time of imaging, larvae were anesthetized (as described for epifluorescence microscopy analysis), injected with ~3 nL of D−luciferin (15 mg/mL), and imaged 5 minutes (min) after injection. Images were acquired using the following settings: field of view A, small binning, automatic exposure time with a maximum of 1 min and a subject height of 0.30 cm. Unlike epifluorescence analysis, longitudinal monitoring of individual larvae was not possible due to the harmful effects of repeated D−luciferin injections in the same larvae. Relative bioluminescence intensities were analyzed using Living Image software (v4.7.3). Regions of interest (ROIs) were drawn by manually outlining the larval body, and bioluminescence within the ROI was recorded in terms of mean radiance (photons/s/cm²/sr).

2.4.6. *In vivo* timelapse imaging

For time−lapse imaging, live larvae infected with CyHV−3 EGFP were imaged using a Zeiss Z1 light sheet microscope according to the protocol described elsewhere [57]. Briefly, larvae were embedded inside FEP tubes containing 0.1% low melting point agarose and tricaine (55 µg/mL) and maintained at 27 °C. Z−stacks encompassing the entire head and heart regions were acquired every 10 min from 2 to 3 dpi and were used to generate a maximum−intensity projection video with ImageJ.

2.4.7. Ethics statement

The experiments performed in the present study did not require a bioethical permit as they involved the use of larvae before implementation of feeding. However, all experiments were designed and conducted in accord with the 3R rules and other bioethics standards.

2.5 Statistical analysis

Each dataset was first tested for normality using the Shapiro–Wilk test, which was conducted as a stand−alone test or as part of a two−way ANOVA analysis of residuals implemented in GraphPad Prism (v8.0.1). The omnibus tests used were dependent on the outcome of the Shapiro−Wilk tests. For datasets exhibiting normal distribution, One−way ANOVA, Two−way ANOVA, or Two−way repeated measures (RM) ANOVA were used and implemented in GraphPad Prism. For datasets not exhibiting normal distribution, the Durbin test was used (PMCMR package v4.4 [58]), implemented in R (v4.2.0) [59]. The variables of interest relating to each of these tests and their significance are described in the text. Survival curves were compared using Logrank tests implemented in GraphPad Prism. Post−hoc multiple comparisons between groups of interest were made using either the Sidak test (two groups) or the Tukey test (more than two groups) implemented in Graphpad Prism (in conjunction with ANOVA tests), for data exhibiting normal distribution. Multiple comparisons were made using Dunn's pairwise test (FSA package v0.9.3 [60]) with Benjamini−Hochberg *p*−value adjustment done using the p.adjust function in R (in conjunction with the Durbin Test), for datasets not exhibiting normal distribution. For the purposes of visual clarity, only significant results from post−hoc multiple comparisons are indicated

in each corresponding figure. The results of multiple comparisons tests are represented using the following symbols, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3. Results and Discussion

3.1 ZF4 cells express low susceptibility and reduced or even no permissivity to Cyprinivirus infection leading to abortive infection of cell monolayers

In this experiment we tested the susceptibility and permissivity of the ZF4 cell line to infection with AngHV−1, CyHV−2 and CyHV−3, using recombinant strains expressing green fluorescent proteins as reporters. Cells were monitored from 1 dpi onwards using epifluorescence microscopy. At 1 dpi, infected cells were observed, with much less AngHV−1 infected cells relative to CyHV−2 and CyHV−3. The amount of CyHV−2 and CyHV−3−infected cells increased from 1–4 dpi, while the amount of AngHV−1−infected cells decreased after 2 dpi (Figure 2).

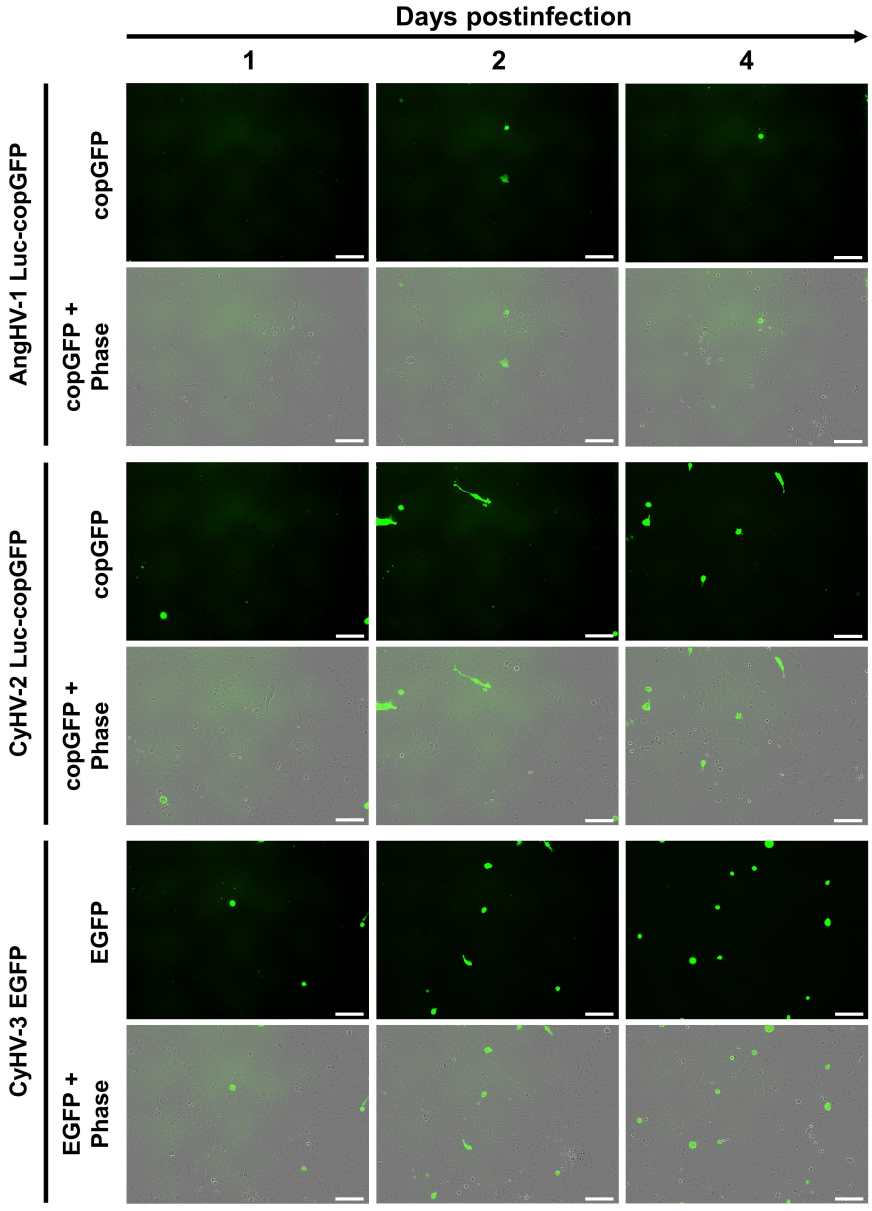

Figure 2. Infection of ZF4 cells by cyprinivirus. ZF4 cells were infected with the AngHV−1 Luc−copGFP, CyHV−2 Luc−copGFP and CyHV−3 EGFP recombinant strains. Infection progression was imaged by epifluorescence microscopy. Infected cells were identified based on green fluorescence expression at the indicated timepoints of infection. Scale bars = 100 µm.

Syncytia formation, lysis plaques, or other cytopathic effects (CPE), were not observed in monolayers infected with CyHV−2 or CyHV−3. Together, these data revealed that ZF4 cells expressed some level of susceptibility to the cyprinivirus tested, no permissivity to AngHV−1 infection, and greatly reduced permissivity to CyHV−2 and CyHV−3 infection relative to typical observations in cells derived from their respective natural hosts that are routinely used for culture of these viruses.

To further characterize the infection of ZF4 cells by the three cyprinivirus in a more quantitative manner, we utilized timelapse microscopy (Figure 3). ZF4 cells were infected, and the numbers of infected cells present with respect to time were tracked from 1–11 dpi as illustrated in Figure 4. Again, the number of AngHV−1 infected cells were low relative to CyHV−2 and CyHV−3 infections and did not increase over time (data not shown). Consequently, AngHV−1 was excluded from further quantification analysis *in vitro*. None of the infections led to the formation of detectible CPE. We observed a steady increase in CyHV−2− and CyHV−3−infected cells from ~24 −144 hpi, followed by a rapid clearance of both viruses from ZF4 monolayers (Figure 3). As evident in Figure 2, during the most rapid period of virus propagation within the monolayer (from ~24–144 hpi) the rate of CyHV−2 and CyHV−3 spread was not exponential (Figure 3), indicating poor replication efficiency within infected cells and/or reduced transmission of progeny virus to additional cells.

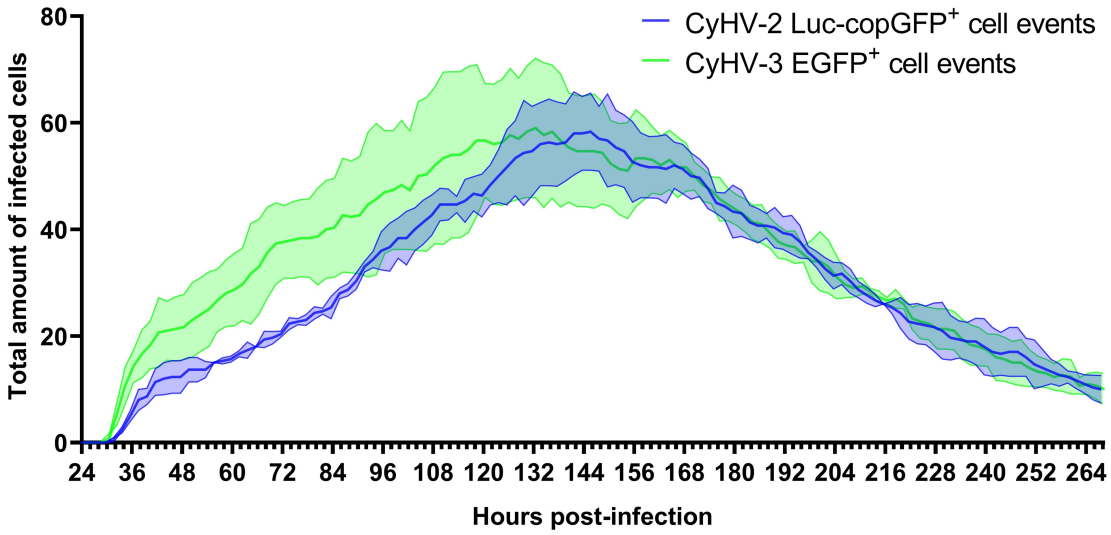

Figure 3. Quantification of CyHV−2 and CyHV−3-infected cells in ZF4 monolayer over time. This data was acquired via time−lapse fluorescent microscopy (IncuCyte). Cells were cultured in a 24−well plate and infected with CyHV−2 Luc−copGFP or CyHV−3 EGFP recombinants (1.2×10^6 PFU/mL for each recombinant). At 24 hpi, cells were imaged every 2 h for 11 days. Data represent the mean ± standard errors from three replicates/wells. Data from each replicate at each timepoint represent the sum of fluorescent cells observed in nine separate locations of each well.

In the CyHV−2−infected monolayers, the peak of infected cells occurred at 146 ± 4 hpi with a mean of 58 ± 7 infected cells observed per well (sum of nine different fields of view in each well, sums from three replicate wells used to derive mean). This peak occurred earlier in CyHV−3−infected monolayers at 124 ± 11 hpi with a mean of 59 ± 13 infected cells at this point. Overall, time postinfection was shown to have a significant effect on the number of CyHV−2 and CyHV−3−infected cells observed (Two−way RM ANOVA, *p* value < 0.0001), but there was no significant difference between the two viruses in this respect (Two−way RM ANOVA, *p* value = 0.3164) (Figure 3).

However, from ~24–144 hpi, the mean number of infected cells tended to be higher in the CyHV−3 infected monolayer. For example, at 48 hpi there was a mean of 21 ± 6 CyHV−3−infected cells observed per well compared to a mean of 12 ± 3 CyHV−2−infected cells. From ~144–264 hpi, the number of infected cells evolved similarly for both viruses, with infected cell numbers decreasing steadily until the end of the experiment, representing the gradual clearance of infected cells from the monolayer (Figure 3). Notably this clearance was largely characterized by apoptosis−like morphological changes.

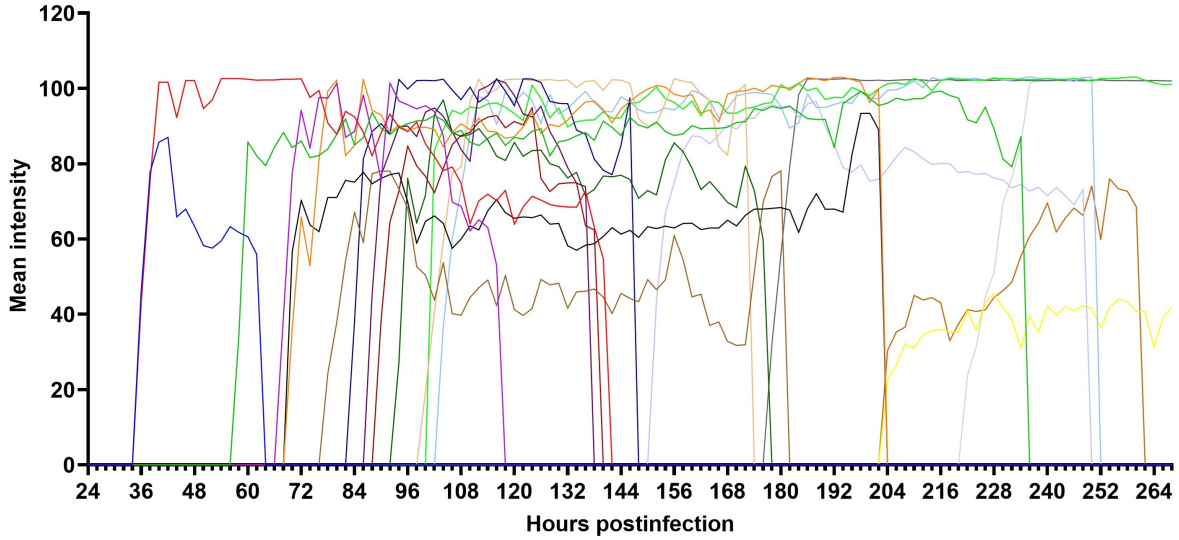

Figure 4. Evolution of fluorescence intensity in infected ZF4 cells. Using an IncuCyte and TrackMate, the evolution of fluorescence intensity in each infected cell with respect to time post infection was recorded. In this example, 19 independent EGFP+ cells infected with CyHV−3 were identified and monitored in a single field of view in a well from a 24−well plate (in total there were 9 fields of view per well). Each color represents an independent cell. Mean intensity measured with ImageJ (FIJI).

These two time−ranges, i.e., ~ 24–144 hpi and ~144–264 hpi, corresponded to periods approximately before and after the peak of infected cells, respectively. Thus, we further scrutinized these two distinct periods separately in order to determine the extent of any differences between CyHV−2 and CyHV−3. After defining the timepoints corresponding to the latest infection peak in each replicate, we examined the two distinct periods of infection, comprised of viral propagation (pre−peak) and clearance (post−peak), by quantifying the appearance (beginning of infection) and disappearance (cell death) of

infected cells (Figure 5). This revealed that infected cells appeared at a mean rate of 0.64 ± 0.05 cells per hour for CyHV−2 and 0.78 ± 0.13 cells per hour for CyHV−3 before the peak, with no significant differences between the two viruses in this respect (Two−way ANOVA, *p* value = 0.1704). It also revealed that a mean of 75 ± 4.16% and 72 ± 8.89% of newly infected CyHV−2 and CyHV−3 cells appeared before the peak of infection, respectively, in what appears to have been several waves of infection (Figure 5). For both viruses, in all replicates, an initial peak of infected cell appearance occurred at ~36 hpi, followed by a period of particularly low appearance of newly infected cells until after ~48 hpi. This may represent the transmission of the first generation of viral progeny to the second generation of infected cells (from ~24–36 hpi), and subsequent progeny to the next generation of infected cells (occurring after ~48 hpi). However, the low numbers of newly infected cells yielded from this transmission provides more evidence to support the possible inefficient replication and/or transmission of CyHV−2 and CyHV−3 between ZF4 cells (as observed in Figure 2).

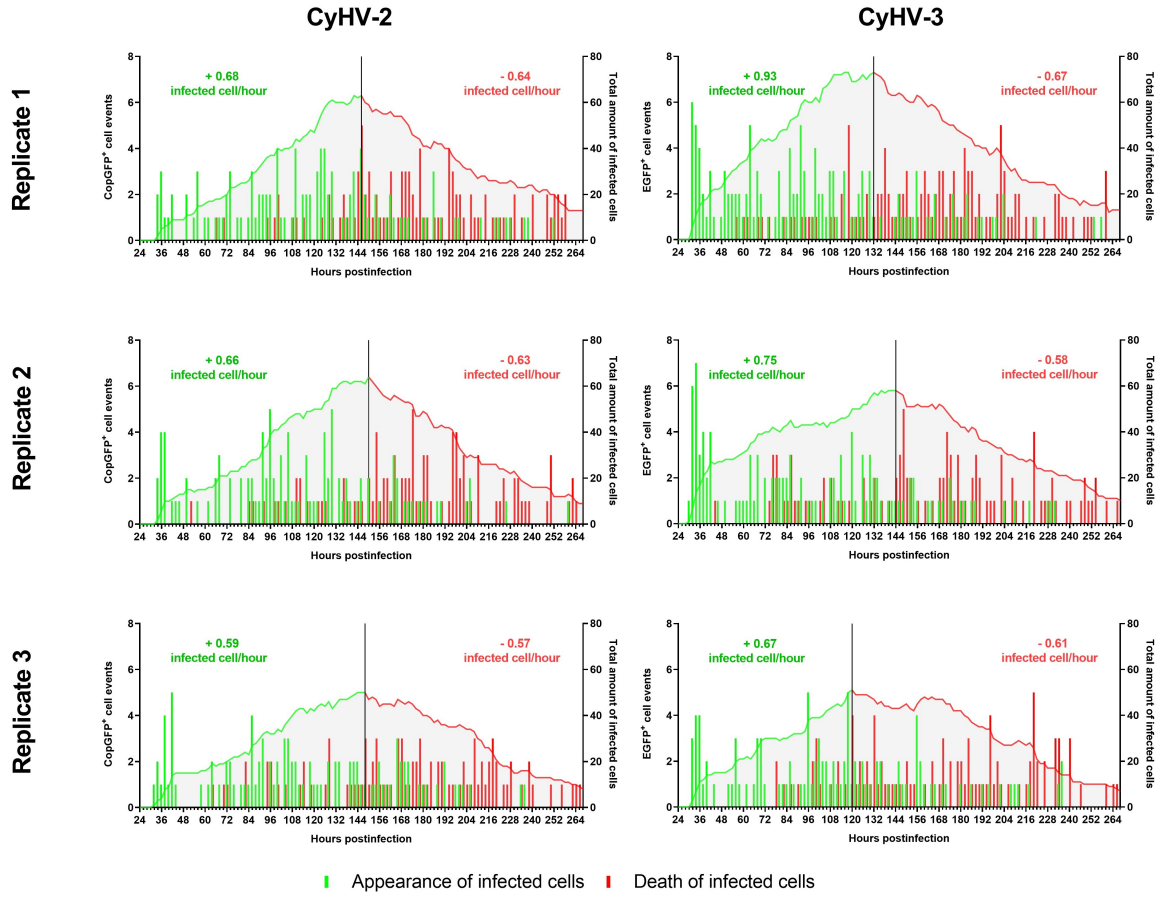

Figure 5. Kinetics of appearance and death of CyHV−2 and CyHV−3−infected cells before and after infection peak. The bars relate to the temporal pattern of appearance and disappearance of CyHV−2−infected or CyHV−3−infected cells (based on fluorescent reporter expression). The quantities are based on the total amount of observations made in 9 different locations in each well/replicate. The green and red curves show the total amount of infected cells up until the peak of infection (represented by the black vertical line) and after the peak, respectively. The values on top of the curves represent the average rate of appearance of infected

cells per hour (green) and the average rate of death per hour (red). Analyzing the rate of appearance/hour before the peak for CyHV−2 and CyHV−3 revealed no differences between the viruses.

For both CyHV−2 and CyHV−3, a substantial amount of newly infected cells (25–30%) appeared after the peak, even beyond 10 dpi, indicating that transmission of viral progeny was sustained into the later stages of the experiment. We have recently demonstrated that CyHV−3 virions lose infectivity rapidly in cell culture media (>95% by 24 h) [61], thus excluding the possibility that newly infected cells, particularly beyond 10 dpi, could have originated from the initial inoculum due to delayed viral entry into cells. It is also unlikely that we are observing delayed expression of viral genes, as all fluorescent reporters used in this experiment were driven by highly active constitutive promoters (CMV and EF−1α). Also, we reasoned that because cells expressing fluorescent reporters are actively cleared at increased rates and appear at decreased rates as the experiment continued, the outcome is distinct from that of spurious reporter expression (i.e., without expression of other viral genes, owing to integration of the expression cassette into the host cell genome), which should persist for longer without triggering cell death. Together, these observations indicated the occurrence of at least some viral progeny transmission to non−infected cells after an initial round of viral replication. However, as increase in the numbers of newly infected cells was not exponential, but linear, it indicated that efficient replication and/or transmission of CyHV−2 and CyHV−3 was very rare in ZF4 cells. Nonetheless, it provided evidence that ZF4 cells are transiently permissive to CyHV−2 and CyHV−3 infection. The observation of isolated infected cells without plaque formation indicated the absence of transmission via cell−cell contact. This may indicate a high degree of heterogeneity within ZF4 monolayers regarding susceptibility to these viruses, or very strong or fast innate responses in neighboring cells. Within at least one permissive cell line, CyHV−3 cell−cell transmission may be greatly enhanced by syncytia formation (in particular with CyHV−3 FL strain derived recombinants, which we recently described [61]). However, we observed a notable lack of syncytia formation among CyHV−3−infected ZF4s, which may also contribute to reduced transmission via cell−cell contact.

It is important to note that for all viruses used in this study, the use of a high MOI of 3 (although calculated in the context of permissive cell lines used for viral production), did not result in many initial infected ZF4 cells, indicating a general lack of cyprinivirus susceptibility among ZF4 populations. This may happen for many reasons, for example, a lack of optimum cell surface receptors, resulting in inefficient viral entry. Conversely, entry may occur, but the viral replication may not commence due to a lack of crucial cellular factors. The exact reasons for this remain speculative and are beyond the scope of this present study, but it provides an opportunity for further investigation via single cell sequencing analysis in the future.

Notably, cell death before the infection peak was low with 74 ± 0.03% and 74 ± 0.07% of CyHV−2 and CyHV−3−infected cells dying after the peak, respectively. Therefore, the higher transmission, prior

to the peak, was not reliant on the release of virions via infected cell lysis/death but rather on the normal mechanism of herpesvirus egress [62,63]. Programmed cell death prior to completion of the viral replication cycle in particular acts as an innate defense mechanism which infected cells can employ to reduce virus replication [64]. Indeed, this is what was observed post infection peak, with an increase in cell death correlating with a reduction in newly infected cells (Figure 5). We propose that relative to cells at the earlier stages of the experiment, both infected and uninfected cells present at later stages would have been subject to cytokine stimulation as part of the innate immune response. Even if such stimulation was transient, these cells (many of which may exhibit limited susceptibility to begin with) may have adopted a stronger antiviral−state at later stages of the experiment.

In order to compare the virulence of CyHV−2 and CyHV−3 in ZF4 cells, we returned to the data displayed in Figure 3 and monitored all positive cells present at 120 hpi until their death, using this information to generate survival curves (Figure 6). This 120 hpi timepoint was selected, as it represented the earliest peak of infection out of the six that were defined in Figure 5, thus maximizing infected cell sample size while using a common timepoint for all groups. The median survival time for infected cells was 61 ± 3 h and 53 ± 12 for CyHV−2 and CyHV−3−infected groups, respectively. Although CyHV−2−infected cells tended to survive longer, there was no significant difference survival between the two groups (Log−rank Mantel−Cox test, *p* value = 0.0822).

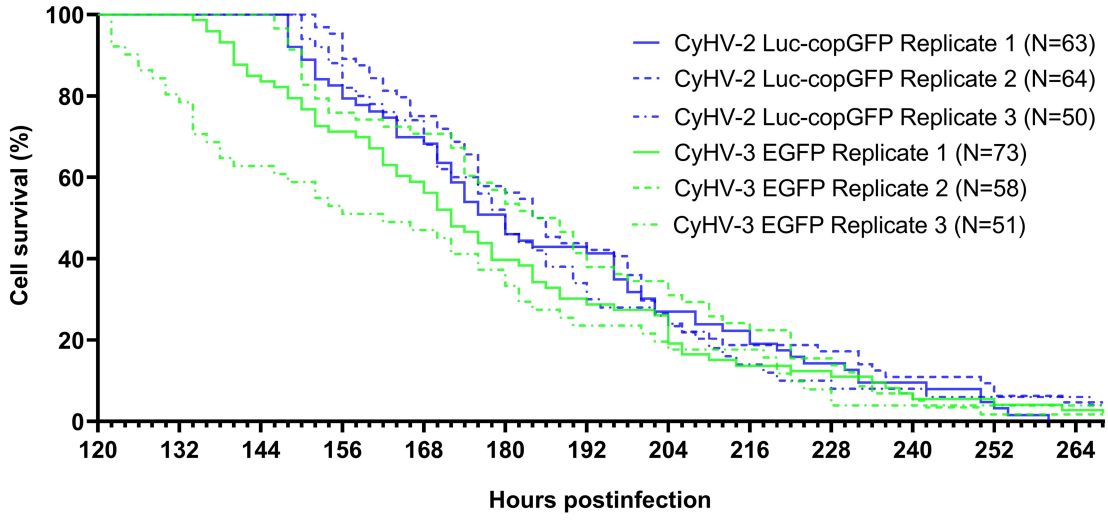

Figure 6. Survival kinetics for CyHV−2 and CyHV−3−infected cells displayed as Kaplan−Meier plots. CyHV−2 and CyHV−3−infected cells observed at 120 hpi were monitored until the end of the experiment. Cell death events and times were identified based on the disappearance of fluorescent signals (Figure 4). N= Number of cells followed.

In the majority of cases, death events were morphologically consistent with apoptosis, i.e., cell shrinkage, membrane blebbing leading to the appearance of cell debris resembling apoptotic bodies [65–67]) (Figure 7a, top panel). However, the occurrence of apoptosis was not definitively confirmed as cell staining was not performed. We also observed another distinct type of cell death that was not

morphologically consistent with apoptosis. In these cases, morphological features mostly included initial cell swelling, followed by cell shrinkage and an absence of cell debris resembling apoptotic bodies prior to disappearance of fluorescent signal (Figure 7a, bottom panel). This is somewhat morphologically consistent with necrosis, where cell−death is associated with membrane rupture and leaking of cytoplasmic contents [65–67].

Notably, necrosis can also be initiated in a highly regulated manner known as necroptosis, which acts as a back−up for apoptosis [68,69]. However, it was not possible to differentiate between necrosis and necroptosis based on our morphological observations alone, and as with apoptosis, neither were definitively confirmed, cell staining not being performed.

In any case, the apoptosis−like form of cell death was observed to be the dominant form of death among infected cells (Figure 7b). However, there were differences between the two viruses in this respect (Two−way ANOVA, *p*−value = <0.0001), with the proportion of CyHV−3−infected cells undergoing apoptosis−like cell death being significantly lower (Figure 7b), possibly indicating that CyHV−3 may be more efficient at blocking this apoptosis−like death in ZF4 cells. There was no significant difference between the two viruses in terms of survival times (Two−way ANOVA, *p*−value = 0.1112). However, among CyHV−3−infected cells, those undergoing non−apoptosis−like death exhibited significantly longer survival times than those undergoing apoptosis−like death (Figure 7c).

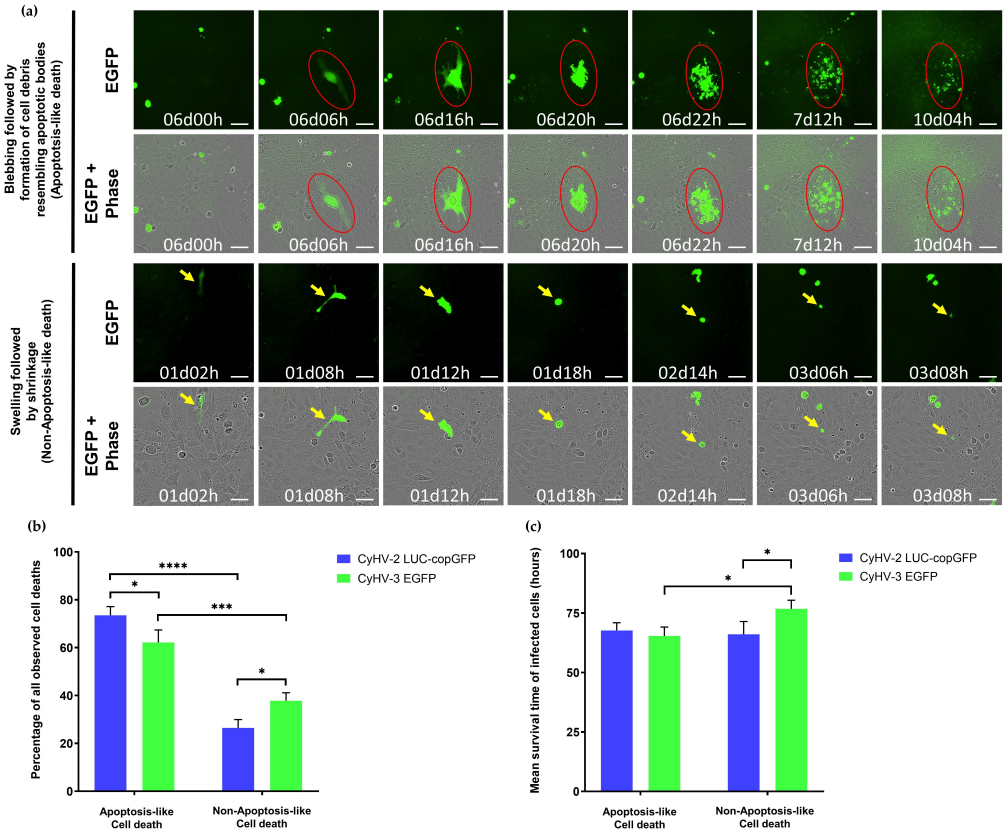

Figure 7. Cell death characteristics observed in CyHV−2 and CyHV−3 infections. (**a**) Representative morphological observations among populations of infected cells (those exhibiting fluorescence) in the periods leading up to cell death (disappearance of fluorescence). Top panel: Morphological features consistent with apoptosis (cell shrinkage, membrane blebbing followed by the appearance of cell debris resembling apoptotic bodies, and progressive decrease of fluorescent signal). Bottom panel: Morphological features not consistent with apoptosis (cell swelling, followed by cell shrinkage, and absence of cell debris resembling apoptotic bodies prior to disappearance of fluorescent signal). Key examples of individual cells undergoing apoptosis−like and non−apoptosis−like death in each panel are highlighted by red circle and yellow arrows, respectively, which track the progression of morphology in a single cell with respect to time. Time postinfection (in days and hours) is indicated in images. Scale bars = 100 µm. (**b**) Percentage of infected cells exhibiting features of apoptosis−like or non−apoptosis−like cell death among those that died during the observation period (**c**) Mean survival time of infected cells undergoing cell death during the observation period according to the type of death observed. Data represents mean ± standard error from 3 replicates. **** $p < 0.0001$; *** $p < 0.001$; *$p < 0.05$.

Previously, a separate study demonstrated that CyHV−3 could indeed infect ZF4 cells, with increasing viral RNA levels observed from 1–4 dpi, and an absence of CPE was also noted [38]. However, the viral dosages used were not directly comparable with this present study, and the possibility of viral clearance after 4 dpi was not investigated. In this present study, we monitored the progression of CyHV−3 infections for much longer (up to 11 dpi). Crucially, through the exploitation of reporter genes, in addition to demonstrating viral gene expression, we were also able to identify and quantify new cell infection events. This revealed continuous CyHV−3 transmission right up until the clearance of infection, albeit with increasingly reduced rates of newly infected cells. While we demonstrated that ZF4s are certainly susceptible to CyHV−3 infection, any initial productive infections leading to transmission of viable progeny were not sustained. Thus, ZF4 cells are transiently permissive to CyHV−3 with inefficient viral replication/transmission unable to overcome the innate immune response among infected and non−infected cells. This may be similar to previous observations with snakehead fish vesiculovirus (SHVV) infections in ZF4 cells where initial increases in virus levels were followed by a decrease, corresponding to ISG up−regulation [70].

Unlike CyHV−3, prior to this study, the susceptibility ZF4 cells to AngHV−1 and CyHV−2 had not been investigated. Our results indicate that while ZF4 cells are also susceptible to both AngHV−1 and CyHV−2 infection, they are only permissive to the latter. However, as with CyHV−3, permissiveness to CyHV−2 infection was moderate and transient. These similarities between CyHV−2 and CyHV−3, and their differences to AngHV−1 in this context may reflect the fact that CyHV−2 and CyHV−3 are phylogenetically closer to each other, than each are to AngHV−1 [40,71–73]. Furthermore, given their natural host species, it stands to reason that CyHV−2 and CyHV−3 may also be inherently better adapted to growing in ZF4 cells relative to AngHV−1. Despite the lack of sustained permissivity to cyprinivirus, these *in vitro* experiments with ZF4 cells did provide some indication that the same recombinant viruses may be used to study transient cyprinivirus infection and clearance in zebrafish larvae, which, for many reasons (outlined earlier), may represent a valuable virus−host model.

3.2 Zebrafish larvae are susceptible to CyHV-2 and CyHV-3 but not to AngHV-1 infection. Inoculation by the two cyprinid herpesviruses leads to an abortive infection.

We next investigated the susceptibility and permissivity of zebrafish larvae to the same three cypriniviruses. To investigate this, we used WT AB zebrafish larvae at 3 dpf. In the first experiment, larvae were infected with the same recombinants previously used (Figures 1–7). Larvae were inoculated by pericardial microinjection with 1.2×10^6 PFU/mL of each recombinant or PBS. In parallel, larvae were also infected by immersion with a final concentration of 4000 PFU/mL of each recombinant or PBS. The susceptibility of larvae to these viruses was assessed using epifluorescence microscopy to detect reporter expression from each recombinant. Independently of the mode of inoculation used or the virus, no morbidity or mortality was observed among larvae. Epifluorescence microscopy indicated no infection in larvae inoculated by immersion. Conversely, viral infection was detected from 1 dpi in larvae inoculated with CyHV−2 and CyHV−3 by microinjection (Figure 8a) with no fluorescence detected in the AngHV−1 inoculated group (data not shown). Fluorescence intensity in CyHV−2 and CyHV−3−infected larvae increased from 1–2 dpi. However, as per earlier *in vitro* observations, these infections were transient, with fluorescence intensity (Figure 8a) and the numbers of infected larvae (Figure 8b) decreasing by 4 dpi.

While the pattern was similar for both viruses, the CyHV−3 group exhibited greater fluorescence intensity and significantly higher rates of infected larvae (Two−way RM ANOVA *p*−value = 0.0214). Infection clearance was most pronounced in the CyHV−2−infected group, with a significantly higher proportion of larvae infected at earlier timepoints exhibiting viral clearance by 4 dpi relative to CyHV−3 (Figure 8b). The differences between these three cypriniviruses were investigated further by measuring Luc2 expression from recombinants, representing a more quantitative comparison of viral levels *in vivo*. This involved the same AngHV−1 and CyHV−2 recombinants used in Figure 7a, with CyHV−3 EGFP replaced with CyHV−3 Luc. Larvae were inoculated or mock−inoculated as per Figure 8a. Again, no mortality was observed in any groups and no infection was detected in the AngHV−1 group. The CyHV−3−infected group exhibited significantly higher viral levels relative to CyHV−2 (Durbin Test, *p*−value = 0.0008), indicating that CyHV−3 replicates better in this model. Also, for both CyHV−2 and CyVH−3, a reduction in virus levels occurred at 3 dpi, coinciding with a reduction in the numbers of infected fish, indicating the initiation of viral clearance. However, as per Figure 8b, clearance was significantly greater within the CyHV−2-infected group by 4 dpi (Figure 8c).

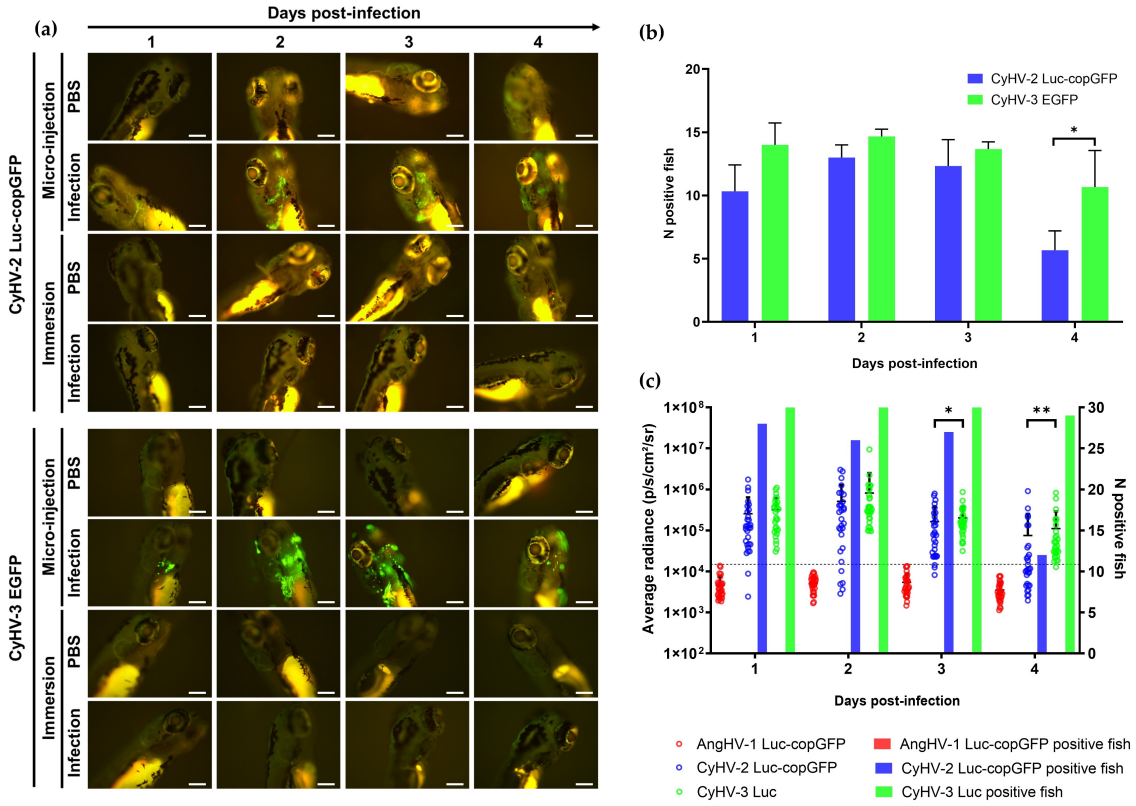

Figure 8. Susceptibility and permissivity of zebrafish larvae to infection with cyprinivirus after inoculation by microinjection. (**a**) Epifluorescence microscopy images representative of larvae inoculated with CyHV−2 and CyHV−3 according to time postinfection (longitudinal observation of the same larvae over all timepoints) Scale bars = 200 µm. (**b**) Numbers of CyHV−2 and CyHV−3−infected larvae among groups inoculated by microinjection (n = 15). Data represents mean ± standard errors from 3 independent experiments (longitudinal observation of the same larvae over all timepoints). (**c**) Levels of AngHV−1, CyHV−2 and CyHV−3 detected in infected larvae according to time postinfection based on Luc2 signal expressed by viral recombinants. The data points represent the mean radiance per larvae according to time postinfection with mean ± standard error represented for each group at each timepoint (n = 30). The discontinuous line represents the cut−off for positivity and represents the mean + 3×SD of the values obtained for mock−infected larvae. The number of positive larvae at each timepoint is represented by bars. *$p < 0.05$; **$p < 0.01$.

These experiments revealed that zebrafish larvae are not susceptible to any of these viruses via immersion, which may be considered a more natural route. This is similar to previous findings with CyHV−3 in Tübingen zebrafish larvae [38]. Conversely, larvae were susceptible to CyHV−2 and CyHV−3 when inoculated via pericardial microinjection, but not to AngHV−1 via the same route. In line with earlier observations *in vitro*, CyHV−2 and CyHV−3, which naturally infect members of the family *Cyprinidae*, are much more fit in this zebrafish model relative to AngHV−1. For CyHV−2 and CyHV−3, a peak of infection was reached at 2 dpi, with viral clearance initiating from 2–3 dpi. Notably, this is the first report of cyprinivirus infection in zebrafish larvae. Our observations are largely consistent with previous description of CyHV−3 infections in adult zebrafish (inoculation by intraperitoneal injection) [38]. There was also a notable lack of mortality in previous studies involving the challenge of zebrafish with other viruses of cyprinid fish [38]. One explanation is that zebrafish may naturally possess

robust defences against other viruses that are closely related to CyHV−2 and CyHV−3 which may have circulated in their natural habitat during their evolution. However, few viruses are known to naturally infect zebrafish [1,19], thus it would be useful to determine if any extant uncharacterized members of the family *Alloherpesviridae* naturally infect zebrafish as a primary host, as it would open up new avenues of investigation with a valuable homologous herpesvirus−host model in zebrafish. It is also possible that this lack of mortality is related to the viral dose or even inoculation site, both of which can impact the severity of viral infections in zebrafish larvae, as exemplified elsewhere [74,75].

Our observations indicated that CyHV−3 exhibits greater fitness in these zebrafish models relative to CyHV−2. Thus, in addition to CyHV−3 being the most studied and the archetype fish alloherpesvirus [39], it also represented a more valuable model to utilize in the further study of alloherpesvirus infections in zebrafish larvae. Thus, CyHV−3 was selected for all further *in vivo* investigations in this study.

3.3 Pericardial inoculation of zebrafish larvae with CyHV−3 leads to infection of resident and motile cells around the inoculation site followed by their apoptosis−like death and viral clearance

Earlier experiments revealed that the levels of CyHV−3 signal increased from 1–2 dpi with clearance commencing from 2–3 dpi (Figure 8 a, c). However, it remained unclear if increases in viral signal were merely due to increasing levels of viral gene expression or the numbers of infected cells. We chose to investigate this using light sheet microscopy to capture epifluorescence and brightfield images at regular intervals in live CyHV−3−infected larvae from 2–3 dpi and subsequently generated a timelapse video with this data (Video S1). This timepoint was selected as it overlapped with the highest viral signals and the beginning of the viral clearance process (Figure 8), and because no viable virus from the original inoculum should have persisted to this timepoint [61].

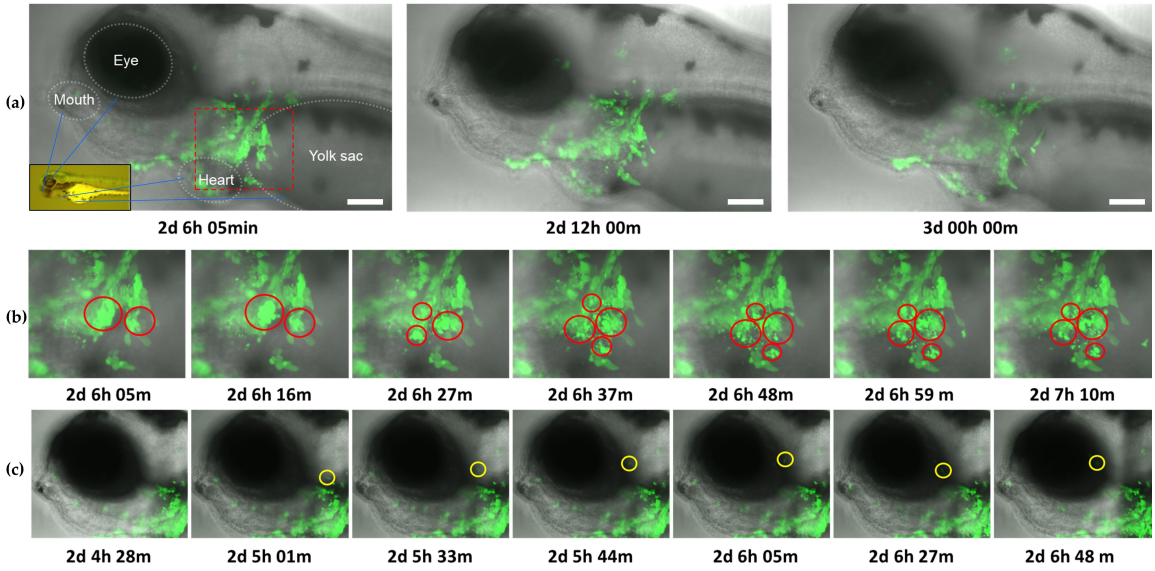

Figure 9. Frames from timelapse video of CyHV−3 EGFP infection in zebrafish larvae from 2–3 dpi (Video S1). The video represents overlay of brightfield/transmission and EGFP fluorescence (green). Time

postinfection (in days, hours, and minutes) is indicated under each frame. (**a**) Entire field of view from light−sheet microscopy. For the purposes of visual orientation, identifiable anatomical features, and corresponding locations within larvae body (inset image) are indicated in the first panel. Images show that the infection is primarily localized around the inoculation site (red square), and a decrease in viral levels from 2.5–3 dpi. Scale bars = 100 µm. (**b**) Enlarged images of the area within red square in (a), representing key examples of apoptosis−like death occurring among large numbers of infected cells (red circles) around the inoculation site, with such events primarily characterized by blebbing followed by the appearance of cell debris resembling apoptotic bodies (**c**) Key example of highly motile infected cell (highlighted with yellow circle), migrating away from the site of inoculation.

As per Figure 8a, the infection was mainly localized around the heart area, reflecting the inoculation route. In line with earlier observations, a reduction in viral levels commenced between 2.5–3 dpi (Figure 9a and Video S1). Notably, the data revealed a substantial upsurge in apoptosis−like cell death immediately prior to clearance, indicating that programmed cell death may also play a major role in this process *in vivo* (Figure 9b and Video S1). Although the occurrence of apoptosis in response to CyHV−3 infection *in vivo* was not confirmed by staining in this present study, our observations are similar to previous studies involving timelapse analysis of CHIKV−infected zebrafish larvae [23]. Throughout the monitoring period, highly motile cells, possibly macrophages or neutrophils, were also observed to be infected. These did not remain localized around the inoculation site. However, they were not observed to establish secondary infection sites elsewhere (Figure 9c and Video S1). Furthermore, some of these motile cells appeared also to undergo apoptosis−like and non−apoptosis−like cell death consistent with necroptosis (Video S1). Unlike earlier *in vitro* observations, this data did not provide unambiguous evidence of newly infected cells appearing before clearance commenced. Indeed, the induction of a programmed cell death response among infected cells *in vivo*, thus interrupting the CyHV−3 replication cycle, would lead to a reduction in successful CyHV−3 transmission to new cells. Consequently, CyHV−3 propagation *in vivo* may be sufficiently restricted to facilitate its clearance via the innate immune response alone. This hypothesis still implies that zebrafish cells are inherently permissive to CyHV−3 replication. However, this would, at the very least, require expression of all essential CyHV−3 protein coding genes *in vivo*. Thus, we propose that it would be interesting to investigate this and the nature of the innate immune response via transcriptomic analysis of infected larvae.

4. Conclusions

The aim of this present study was to investigate the potential of the zebrafish model to study AngHV−1, CyHV−2, and CyHV−3, which are three economically important viruses in the family *Alloherpesviridae*. We conclude that while the zebrafish ZF4 cell line is moderately susceptible to these three viruses, it is less susceptible and not permissive to AngHV−1 (Figure 2). ZF4 cells do exhibit transient permissiveness to CyHV−2 and CyHV−3 infection. These cells are more permissive to CyHV−3, but both viruses exhibit inefficient cell to cell viral transmission in this *in vitro* model (Figures 3 and 5). These viruses are ultimately cleared from ZF4 monolayers, in a process which is preceded by what resembles widespread programmed cell death among infected cell populations (Figures 3, 5 and 7). As zebrafish larvae were not susceptible to these viruses via inoculation by immersion, we conclude

that these viruses may not be capable of entering zebrafish larvae through natural routes *in vivo* (Figure 8). However, zebrafish larvae are susceptible to infections with CyHV−2 and CyHV−3 via microinjection, an artificial inoculation route (Figure 8). Conversely, we conclude that zebrafish larvae are not susceptible to AngHV−1 via both inoculation methods used in this study (Figure 8). This lower susceptibility to AngHV−1 *in vitro* and *in vivo*, may reflect the fact that, unlike CyHV−2 and CyHV−3, AngHV−1 does not naturally infect host species from the family *Cyprinidae*. Even though larvae exhibit greater susceptibility to CyHV−2 and CyHV−3, we conclude that these infections are rapidly cleared (Figure 8). We also conclude that zebrafish larvae exhibit more susceptibility (and possibly more permissivity) to CyHV−3, given higher viral levels and slower clearance, indicating the superior utility of this virus−host model in future studies. Unlike infections *in vitro*, we observed no clear evidence of CyHV−3 transmission to new cells prior to clearance *in vivo* (Figure 9, Video S1). Thus, the extent to which this permissiveness leads to successful CyHV−3 transmission between cells *in vivo* remains unclear. As per observations *in vitro*, CyHV−3 clearance in zebrafish larvae is also preceded by apoptosis−like death among infected cells (Figure 9, Video S1). Interestingly, given that strains within each cyprinivirus species clad exhibit natural heterogeneity regarding replication *in vitro* and/or *in vivo* (at least with AngHV−1 and CyHV−3 [40,71]), it remains possible that the use of alternative cyprinivirus strains with the same zebrafish models may result in different outcomes, and is something which remains to be explored in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v15030768/s1, Table S1. Primers used in this study; Video S1.

Author Contributions: Conceptualization, I.M. and A.V.; Data curation, C.S., N.M.S., A.J.D. and O.D.; Formal analysis, L.M., A.J.D. and O.D.; Funding acquisition, I.M., S.B., A.J.D., O.D. and A.V.; Investigation, C.S., L.M., O.D. and A.V.; Methodology, C.S., B.H., L.M., H.Z., N.D., M.F., I.M., N.M.S., O.D. and A.V.; Project administration, S.B. and A.V.; Supervision, I.M., S.B., O.D. and A.V.; Validation, C.S., O.D. and A.V.; Visualization, C.S., O.D. and A.V.; Writing—original draft, C.S., O.D. and A.V.; Writing—review & editing, C.S., O.D. and A.V. All authors have read and agreed to the published version of the manuscript.

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Experimental section

2nd study:

Transcriptomic analysis of zebrafish larvae infected with Cyprinid Herpesvirus 3 reveals upregulation of interferon-stimulated genes encoding programmed cell death mediators

Synthèse des travaux menés

Les résultats obtenus dans l'étude précédente (chapitre I de la section expérimentale) ont révélé que, bien que des infections par CyHV-3 pouvaient être établies en utilisant des modèles d'infection artificiels *in vitro* (lignées cellulaires de poisson-zèbre) et *in vivo* (micro-injection de larves), les infections étaient transitoires, avec une clairance virale rapide associée à une mort de type apoptose au sein des cellules infectées. Afin d'étudier la nature de la réponse immunitaire innée impliquée dans la réponse antivirale contre le CyHV-3, une analyse transcriptomique des larves entières de poisson-zèbre infectées par ce virus a été réalisée en exploitant la technologie de séquençage de l'ARN (RNA-Seq). En vue de réaliser cette étude transcriptomique, des larves de poisson-zèbre de souche WT AB ont été infectées par micro-injection intra-cardiaque, avec une souche recombinante virale exprimant l'EGFP, appelée souche CyHV-3 EGFP ou, dans le cas du groupe témoin, avec du PBS. Les larves infectées et non infectées ont été échantillonnées à 1, 2 et 4 jours après l'infection. Une fois les larves lysées, l'ARN total a été isolé et sa qualité vérifiée pour chaque échantillon à l'aide d'un bio-analyseur Agilent. Les échantillons ont été utilisés pour la préparation de librairies RNA-Seq, lesquelles ont été séquencées avec le système Illumina NextSeq 500. Les lectures de séquences ont été alignées sur le génome de référence du poisson-zèbre GRCz11 (Ref Seq : GCF_000002035.6) afin de générer des données d'expression génique. Les données ont été utilisées pour identifier les gènes exprimés différentiellement (DEGs) dans les échantillons infectés par rapport aux échantillons non infectés. Les DEGs ont été analysés de façon approfondie afin d'évaluer les éventuelles relations fonctionnelles entre ces gènes et d'identifier les principales réponses biologiques à l'infection.

Conformément aux niveaux viraux observés dans l'étude précédente (chapitre I de la section expérimentale), les niveaux d'ARN viral ont atteint un pic lors du 2ème jour post-infection, chutant considérablement au 4ème jour. La transcription des 155 gènes codant pour les protéines du CyHV-3 a été détectée dans les larves de poisson-zèbre infectées lors du 2ème jour post-infection, indiquant que les cellules de poisson-zèbre peuvent effectivement être permissives à la réplication du CyHV-3 dans ce modèle *in vivo*. L'expression différentielle des gènes de l'hôte en réponse à l'infection a également atteint son maximum lors du 2ème jour post-infection, avec 7,4% des gènes exprimés classés comme DEG.

Avant cette étude, la réponse des larves de poisson-zèbre à l'infection par le CyHV-3 en termes d'expression génique de l'interféron (IFN) de type I était inconnue. Conformément à d'autres études (Aggad *et al.*, 2009), nous avons constaté que *ifnphi2* n'était pas exprimé à ce stade du développement. La réponse IFN chez les larves de poisson-zèbre repose sur l'expression des gènes *ifnphi1* et/ou *ifnphi3* (Aggad *et al.*, 2009). Cependant, nous n'avons pas observé d'expression convaincante de l'un ou l'autre de ces gènes. Nos points d'échantillonnage se situent entre 1 et 4 jours post-infection, ce qui équivaut à

96-168 heures post-fertilisation. Des études antérieures ont indiqué que les larves de poisson-zèbre WT AB étaient capables d'exprimer *ifnphi1* et *ifnphi3* à ce stade de développement (Aggad *et al.*, 2009; Palha *et al.*, 2013). Notamment, ces études antérieures, impliquant des infections avec le virus de la virémie printanière de la carpe (VPC) et le virus du Chikungunya (CHIKV), ont détecté la transcription de ces gènes en utilisant la RT-qPCR qui peut être plus sensible que la technologie RNA-Seq dans certaines situations.

Alors que le CyHV-3 est connu pour inhiber la réponse de l'IFN *in vitro* (Adamek *et al.*, 2012; Zhang *et al.*, 2022), nos observations n'indiquent pas nécessairement une inhibition de cette réponse chez le poisson-zèbre. En effet, dans la présente étude, le classement des 250 gènes ayant l'expression différentielle la plus significative, basée sur la *p*-value ajustée par FDR (taux de fausses découvertes), lors du 2ème jour post-infection est dominée par les gènes dits stimulés par l'interféron (ISG). Cette induction d'ISG en l'absence de détection de l'IFN est cohérente avec des études précédentes sur des larves de poisson-zèbre WT infectées par le virus causant l'encéphalopathie et la rétinopathie virale (VER) (Lama *et al.*, 2022). Dans les deux études, il est probable que l'augmentation de la réponse IFN se soit produite avant le point d'échantillonnage le plus précoce. Le rôle de *ifnphi1* et *ifnphi3* dans l'induction des ISG dans notre modèle d'infection n'est pas encore établi, et cette question pourra faire l'objet d'études futures, avec un échantillonnage à des moments plus précoces.

Nous avons également procédé à une caractérisation plus poussée des principaux types de gènes exprimés de manière différentielle en réponse à l'infection par le CyHV-3 chez les larves de poisson-zèbre. En utilisant la fonction STRING du logiciel d'analyse et de visualisation de données de réseaux *Cytoscape*, nous avons généré un réseau représentant les relations fonctionnelles entre les gènes ayant l'expression différentielle la plus significative lors du 2ème jour post-infection. L'analyse de ce réseau a révélé que ces DEGs étaient principalement associés aux réponses immunitaires et au stress. Trois groupes principaux se sont formés au sein de ce réseau.

Le groupe le plus important représentait principalement des gènes impliqués dans l'infection virale et les réponses de cytokines, incluant des gènes codant pour des protéines GTPases antivirales telles que *mxa*, *mxb*, *mxc* et *mxe*, ainsi que *rsad2* (ou *vig-1*, *viperin*). En ce qui concerne la réponse cytokinique, les gènes codant pour les facteurs de régulation de l'IFN *irf7* et *irf9* faisaient également partie de ce groupe principal. En outre, les gènes codant pour d'autres éléments importants de la réponse IFN, tels que *stat1a*, *stat1b*, *stat2*, ainsi que pour l'augmentation et la régulation de cette réponse, tels que *isg15* figuraient également dans ce groupe. Les acides nucléiques viraux représentent les principaux motifs moléculaires associés aux pathogènes (PAMPs, acronyme de Pathogen-associated molecular patterns) au cours des infections, et les gènes codant pour les récepteurs de reconnaissance de motifs moléculaires (PRRs, acronyme de Pattern recognition receptors) qui détectent ces PAMPs figuraient parmi les gènes ayant l'expression différentielle la plus significative dans notre modèle expérimental. Par exemple, les

gènes codant pour d'importants orthologues du récepteur RIG-I-like (RLR) du poisson-zèbre, tels que *ifih1* (codant pour l'orthologue de MDA-5) et *dhx58* (codant pour l'orthologue de *LGP2*) étaient situés au centre de ce grand groupe. Outre les RLR, d'autres gènes codant pour des protéines de liaison à l'ARN, comme *adar, eif2ak2* (codant pour un orthologue de PKR), *pkz* et *ifit10* (orthologue de l'IFIT5 humain), se trouvaient également dans le même grand groupe. Finalement, le deuxième groupe principal formé au sein du réseau comportait des gènes principalement impliqués dans l'apprêtement des antigènes et les réponses des phagosomes et les gènes du troisième groupe étaient principalement liés au système du complément.

En outre, nous notons que bon nombre des gènes ayant l'expression différentielle la plus significative en réponse à l'infection par le CyHV-3 chez les larves de poisson-zèbre n'ont pas été caractérisés et que certains n'avaient pas été signalés auparavant comme étant impliqués dans la réponse immunitaire. Il s'agit notamment de cinq transcrits non codants, dont l'un était le 6ème gène le plus régulé positivement à 2 jours post-infection. Nous proposons d'appeler provisoirement ces cinq transcrits « *Zebrafish Non-coding Infection Response Element* » 1-5 (ou ZNIRE 1-5). Cette observation est particulièrement intrigante, et leur importance au cours de la réponse immunitaire pourrait faire l'objet de futures recherches. Nous avons également observé la régulation positive de trois rétrotransposons. Il est possible que cette réactivation/régulation des rétrotransposons en réponse à l'infection soit bénéfique pour l'hôte. Leurs intermédiaires génomiques d'ARN cytoplasmique et/ou d'ADN peuvent potentiellement agir en tant que ligands pour les PRR (Chernyavskaya *et al.*, 2017), renforçant ainsi la réponse immunitaire à l'infection virale et présentant une hypothèse intéressante pour une étude plus approfondie avec notre modèle.

Dans une analyse plus poussée, nous avons étendu notre recherche à tous les gènes inclus dans l'analyse à 2 jours post-infection, en explorant la réponse à l'infection au niveau des « ensembles de gènes ». À l'aide d'une analyse d'enrichissement d'ensembles génétiques (GSEA) et des bases de données Gene Ontology (GO) et KEGG PATHWAY, nous avons identifié les ensembles de gènes « GO » et « KEGG pathway » qui étaient enrichis ou déplétés dans les larves infectées par le CyHV-3 à 2 jours post-infection. Le logiciel *Cytoscape* a été utilisé pour générer un réseau de ces ensembles de gènes significativement enrichis sur la base des relations fonctionnelles entre eux, permettant de mieux déterminer quels processus biologiques pourraient être impliqués dans la réponse à l'infection par le CyHV-3 chez les larves de poisson-zèbre, mais également leurs possibles interactions. Notamment, un seul ensemble de gènes, la voie KEGG du "Ribosome" (DRE03010), s'est avéré être régulé négativement de manière significative, toutes les autres réponses significatives d'ensembles de gènes impliquant une régulation positive.

Au cours du processus de création du réseau, les ensembles de gènes ont été regroupés en fonction de leur coefficient de similarité. Ce processus a abouti à la formation de plusieurs grands groupes, que

nous avons numérotés. Le groupe n°1 est le plus grand d'entre eux et présente le plus grand nombre de connexions fonctionnelles avec les groupes environnants ; à ce titre, il représente un aspect majeur de la réponse à l'infection par le CyHV-3. Au sein de ce groupe, il existe deux sous-groupes principaux. L'un d'eux est dominé par des ensembles de gènes liés à la mort cellulaire programmée, tandis que l'autre est dominé par des ensembles de gènes liés aux voies de signalisation médiées par les PRR et aux réponses inflammatoires et contre les pathogènes. Dans le groupe n°1, la voie KEGG de la nécroptose (DRE04217) est l'ensemble de gènes le plus significativement enrichi. Notamment, cet ensemble de gènes est fonctionnellement lié à d'autres ensembles de gènes dans les sous-ensembles « apoptose » et « PRR/réponse inflammatoire/pathogène ». Cela reflète l'importante interaction qui existe entre la mort cellulaire programmée et les voies de signalisation activées par les PRR en réponse à l'infection.

La proéminence des ensembles de gènes liés à la nécroptose et à l'apoptose enrichis positivement dans le groupe n°1 soutient les hypothèses dérivées de nos précédentes observations, à la fois *in vitro* et *in vivo*, selon lesquelles la mort cellulaire programmée de type apoptotique et non apoptotique joue un rôle important dans la réponse du poisson-zèbre à l'infection par le CyHV-3. L'un des gènes importants de la voie de la nécroptose est *eif2ak2* (ou *pkr*). Il a été identifié comme l'un des principaux gènes contribuant au signal d'enrichissement de l'ensemble de gènes de la nécroptose. Il représente un lien important entre la réponse immunitaire innée et l'initiation de la nécroptose (Thapa *et al.*, 2013). Ce gène code pour une protéine appelée "protéine kinase activée par l'ARN double brin induite par l'interféron" ou, plus communément, "protéine kinase R" (ci-après dénommée PKR). PKR fonctionne à la fois comme un capteur de stress cellulaire général et comme un PRR. Cette protéine joue donc un rôle diversifié dans la réponse immunitaire innée aux infections virales et dans de nombreux processus cellulaires fondamentaux, y compris la mort cellulaire programmée (Gal-Ben-Ari *et al.*, 2019). Par ailleurs, le gène *eif2ak2* codant pour PKR figurait parmi les 250 gènes ayant l'expression différentielle la plus significative lors du 2ème jour post-infection.

Contrairement aux autres vertébrés, les membres des familles de poissons téléostéens *Salmonidae* et *Cyprinidae* produisent également une protéine supplémentaire semblable à PKR, appelée "protéine kinase contenant des domaines de liaison à l'ADN Z" (ou PKZ). Dans cette étude, le gène *pkz* (codant pour PKZ) était le 23ème gène ayant l'expression différentielle la plus significative à 2 jours post-infection dans notre modèle, mieux classé que le gène *eif2ak2* (codant pour PKR, 250ème). Étant donné que leurs fonctions peuvent se chevaucher, nous avons proposé de générer des souches mutantes de poisson-zèbre PKR-KO (dépourvu d'*eif2ak2*) et un mutant distinct, PKZ-KO (dépourvu de *pkz*) afin d'étudier l'importance de ces deux kinases dans la clairance du CyHV-3 chez les larves de poisson-zèbre.

Experimental section

2nd study:

Transcriptomic analysis of zebrafish larvae infected with Cyprinid Herpesvirus 3 reveals upregulation of interferon-stimulated genes encoding programmed cell death mediators

In Susceptibility and permissivity of zebrafish (*Danio rerio*) larvae to cypriniviruses

Viruses ***15(3)*** *(2023), 768*

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Abstract

Cyprinid herpesvirus 3 (CyHV−3) is the etiological agent of a highly contagious disease which causes widespread mortalities and important substantial economic losses in both common carp (*Cyprinus carpio*) and ornamental Koi (*Cyprinus rubrofuscus* var. "*koi*") aquaculture sectors, thus representing an important threat to long-term sustainability of these activities within some regions. Notably, several studies have demonstrated that feral strains are more resistant to CyHV−3 infection relative to cultured strains. Transcriptomic analyses investigating the existence of a mechanism limiting CyHV−3 replication revealed that susceptible and resistant fish exhibited different immune responses to CyHV−3 infection. Similar to this observation of resistance in some host populations, in the previous study (first chapter), we demonstrated that CyHV−3 infections in ZF4 cells and zebrafish larvae were quickly cleared. Therefore, we sought to identify what aspects of the innate immune response are important for successful CyHV-3 clearance in zebrafish, on the basis that some of these highly conserved processes may also represent an important feature of resistance in common carp, albeit easier to study in zebrafish due to possibility of genetic manipulation. Specifically, we studied the response to infection in terms of gene expression changes via transcriptomic analysis of whole zebrafish larvae infected with CyHV−3. This analysis revealed up−regulation of interferon−stimulated genes, particularly those encoding nucleic acid sensors, but also genes encoding mediators of programmed cell death and related genes. The later supported our observations from the previous study (first chapter), that regulated cell death played prominent role in CyHV-3 clearance. Notably, we also identified uncharacterized non−coding RNA genes and retrotransposons that were also among those most up−regulated in response to CyHV-3 infection. Collectively these observations strongly support the importance of cyprinivirus adaptation to the immune response of their natural host in order to overcome rapid viral clearance and provides further insight into the kinds of evolutionarily innate immune responses that are important for clearance of CyHV-3 infection.

Keywords: cyprinid herpesvirus 3; alloherpesvirus; cyprinivirus; zebrafish; RNA sequencing; innate immunity

1. Introduction

Aquaculture production and further growth of this industry are severely hampered by infectious disease outbreaks. Cyprinid Herpesvirus 3 (CyHV−3) represent a major threat to common carp (*Cyprinus carpio*) and ornamental koi (*Cyprinus rubrofuscus* var. "*koi*") aquaculture in many countries, resulting in widespread mortalities and economic loss in both food production and ornamental koi sectors [1]. CyHV−3 is a large dsDNA virus within the genus *Cyprinivirus*, belonging to the family *Alloherpesviridae* and the order *Herpesvirales* [2]. First reported in the late 1990s [3,4], CyHV−3 outbreaks have been persisting ever since, reaching most carp aquaculture areas, and leading to substantial population losses of up to 100% [5]. CyHV-3 is the etiological agent of a highly transmissible disease affecting carp, both in aquaculture settings and in the wild, with outbreaks occurring mainly when water temperatures range between 18 and 28°C [6,7].

In common carp, the disease can cause morbidity and mortality within 6 to 10 days after infection [5]. In affected fish, the progress of the CyHV−3 infection is associated with lethargy, loss of appetite, gradual hyperemia at the base of the fins, increase in mucus production, erosion of fin extremities and skin lesions [4,7–9]. Histopathological changes include erosion of skin epidermis [10,11], epithelial hyperplasia, hypertrophy, and necrosis in the gills [12–14] as well as necrosis and inflammation in the kidneys, lamina propria of the intestine, liver, and the spleen [4]. CyHV−3 infection is thought to cause mortality by impairing the host osmoregulatory function and immune system, leading to increased susceptibility to infection by other pathogenic microbes [7,15]. Several studies have reported that cultured strains are susceptible to CyHV−3 infection while feral strains are more resistant to this virus [16–21]. Compared to susceptible fish, infected resistant fish exhibit less severe symptoms and can recover from the disease. It was suggested that resistant fish have a mechanism to limit CyHV−3 replication within their body, as a significantly lower viral load was observed in resistant fish relative to susceptible ones [20].

Transcriptomic analysis has become an essential and efficient research tool in common carp to study the development of the fish immune response to CyHV−3 and the mechanisms of carp resistance. High-throughput sequencing of the transcriptome, which is the complete set of transcripts in a cell, tissue, organ or entire organism under a specific developmental stage or physiological condition [22], provides a snapshot of the cellular processes that are active or inactive under specific settings. Nowadays, RNA sequencing (RNA−seq) technology is widely used in the aquaculture research field to understand the immune system of fish and the molecular mechanisms of immune defense against pathogenic infection [23]. Transcriptomic analysis of koi spleen tissue during CyHV−3 infection showed that several immune−related pathways, including the mitogen−activated protein kinase (MAPK) signaling pathway, the innate immune response, involving the toll-like receptor (TLR) signaling pathway and the up−regulation of Mx and IFN−gamma2, and the cytokine-mediated signaling

pathway, were strongly implicated in the infection [24]. The first transcriptional analysis of CyHV−3−infected common carp anterior kidney showed that the humoral immune system was stimulated during the acute phase of infection, associated with the up−regulation of IL−10 and C7 components of the complement cascade and the down−regulation of almost all major histocompatibility class II (MHC II) antigens and various perforin genes [25]. Moreover, *nitric oxide synthase*, *microfibril-associated glycoprotein 4*, *myeloperoxidase* and *cytoglobin 1* were highly expressed in the kidney of carp during replicative CyHV−3 infection. The immune responses of susceptible and resistant common carp were compared via spleen transcriptomic analyses [26]. Results demonstrated that a typical antiviral response, including interferon and interferon responsive genes, was elicited earlier in susceptible fish than in resistant fish. While susceptible fish up−regulated more *ccl19* chemokines, attracting T−cells and macrophages, resistant fish up−regulated more *cxcl8/il8* chemokines, attracting neutrophils. Elsewhere, using quantitative trait locus mapping and genome-wide association study, Jia *et al.* revealed that *galectin−8* (*lgals8b*), *hypoxia inducible factor 1 subunit alpha* (*hif1a*), *paladin*, *rootletin*, and *tumor necrosis factor−alpha* (*tnfa*), were also associated to resistance against CyHV−3 [27]. Using transcriptomic data, an integrative analysis of the protein−coding genes and long non-coding RNAs (lncRNAs) in the head kidney of CyHV−3−infected susceptible and resistant common carp recently showed that several biological processes, namely autophagy, phagocytosis, cytotoxicity, and virus blockage by lectins and MUC3 were involved in the resistance to CyHV-3 infection [28]. In addition, expression of *itgb1b* and *tlr18* were identified as risk factors that increase susceptibility to CyHV−3, and it was suggested that Nramp and PAI regulated by lncRNA could both promote virus infection and proliferation in infected cells. However, it is difficult to investigate any of these observations further in the absence of processes that facilitate easy and rapid manipulation of carp genomes, long generation time of carp may not make this practical.

In contrast to other fish species, zebrafish can be easily and rapidly genetically manipulated thus facilitating investigations into gene function or contribution to various biological processes, including response to viral infection. In recent years, the zebrafish model has become increasingly popular in virology and a powerful tool to study economically important fish viruses [29–34]. In the context of CyHV-3, Rakus *et al.* recently demonstrated that CyHV−3 could replicate in zebrafish cell lines and that it induces an abortive infection in adult zebrafish following intraperitoneal inoculation, associated with the expression of antiviral genes, suggesting an innate antiviral response against CyHV−3 [35]. Moreover, we demonstrated in the previous study (first chapter) that infections with CyHV−3 can be established in zebrafish larvae, however the infection is rapidly cleared, infected cells undergoing apoptosis-like death. In order to investigate the potential involvement of programmed cell death and other immune processes in this antiviral response to CyHV−3, we performed transcriptomic analysis of zebrafish larvae inoculated with CyHV−3 at various timepoints to examine the gene expression dynamics of infected zebrafish larvae. By comparing gene expression from mock-infected and

CyHV−3-infected zebrafish larvae, we observed the up−regulation of interferon−stimulated genes in infected larvae, more specifically those coding for nucleic acid sensors, mediators of programmed cell death and related genes. Importantly this also indicated that all CyHV−3 protein coding genes were expressed, indicating that the virus is capable of replication in this model. Notably this analysis also revealed a strong up−regulation of host uncharacterized non−coding RNA genes and retrotransposons in response to infection, providing plenty of scope for further investigations.

2. Material and methods

2.1 Zebrafish larvae maintenance

Wild−type (WT, +/+) AB strain adult zebrafish (*Danio rerio*) were obtained by natural spawning and maintained at 27 °C, on a 14/10 h light/dark cycle. They were housed in the GIGA Zebrafish facility in Liège (Belgium) according to animal research guidelines and with the approval of the local ethical commission for animal care and use. Larvae were obtained by pairwise mating of adults in mating cages and maintained in petri dishes with standard embryo medium (E3) and incubated at 25 °C prior to use in experiments.

2.2 Zebrafish larvae infection, sampling, and lysis

WT AB zebrafish larvae were inoculated with a recombinant strain expressing enhanced (EGFP), referred to as the CyHV−3 EGFP strain (1.2×10^6 PFU/mL) or mock−infected with PBS via pericardial microinjection. The larvae were placed in 24−well plates with 1 mL E3 medium per well and incubated at 25 °C. Infected and mock−infected larvae were sampled at 1, 2 and 4 days postinfection (dpi) (triplicates at each timepoint with 5 larvae pooled per replicate). Prior to sampling, larvae were euthanized using an overdose of buffered tricaine in E3 media (400 mg/L). Each replicate group of euthanized larvae was transferred immediately to 1.5 mL tubes, excess E3 medium was removed, and 700 µL QIAzol lysis reagent (Qiagen) was added. Whole larvae were then completely homogenized in lysis reagent by passing the lysate through a 21 G needle 20 times using a 2 mL syringe. After homogenization, lysates were stored at −80 °C until RNA isolation.

2.3 RNA isolation, library construction and RNA sequencing

Larvae lysates were thawed and incubated at room temperature for at least 5 min and 140 µL chloroform was added to each sample. Lysates were then vortexed for 15 seconds, incubated at room temperature for 3 min and centrifuged for 15 min at 12,000 x g at 4 °C. After centrifugation, 240 µL of the aqueous layer was removed and 360 µL of 100% ethanol was added with immediate mixing by pipetting. Samples were then added to RNeasy spin columns, and RNA was isolated using an RNeasy Mini Kit (Qiagen) with on−column DNase treatment. RNA was eluted in 100 µL RNase−free water using two 50 µL elution steps, and split into smaller aliquots for storage at −80 °C. For each sample, a single aliquot was used to check the quality of RNA using an Agilent Bioanalyzer, ensuring that RNA integrity (RIN) values were at least 9.5 before proceeding. Samples were used as input for barcoded

RNA−Seq library preparation using the TruSeq Stranded mRNA kit (Illumina), and libraries were sequenced using the Illumina NextSeq 500 System.

2.4 Bioinformatics analysis

Before alignment, raw reads (in Fastq format) were processed using BBduk (v38.26) [36] facilitating adaptor sequence removal and quality trimming. This was followed by assessment of processed Fastq files using FastQC (v0.11.8) [37]. Processed reads were then aligned to the zebrafish reference genome GRCz11 (Ref Seq: GCF_000002035.6) using HISAT2 (v2.1.0) [38,39]. The resulting SAM files were converted to BAM format using SAMTools (v1.9) [40]. This mapping data was used as input for StringTie (v1.3.5) [38] which was used to conduct reference−guided transcript expression estimation.

Differential gene expression analysis between infected and mock samples at each timepoint was then conducted using DESseq2 (v1.36.0) [41]. Taking each time point (1, 2 and 4 dpi) separately, first, the python script PrepDE.py [42] was used to derive gene expression data from the StringTie output files and parse it into a format compatible with DESeq2. In the resulting CSV file, using a combination of Excel and awk (Linux) processing**,** the default StringTie gene identifiers were converted to Entrez Gene IDs using information from the corresponding genome GFF file. For non−unique Entrez Gene IDs (for example where the same genes on defined chromosomes also exist in alternate loci groups, or unplaced scaffolds), only data relating to the chromosome loci were retained. In instances where Entrez Gene IDs were non−unique but were only annotated on unplaced scaffolds, genes were eliminated from further analysis. After import of CSV files into R (v4.2.0) [43], in order to minimize false positives within differential expression output (caused by genes with extremely low or inconsistent expression i.e. absent in some replicates and detected at low levels in others), genes with read counts ≤10 in ≤3 samples were eliminated from DESeq2 analysis. Global, differential gene expression data from DESeq2 output (infected relative to mock) was visualized using volcano plots, generated using EnhancedVolcanoPlot (v1.4.0) [44].

In order to ascertain the functions and biological processes associated with the most significantly differentially expressed genes (DEGs), the top 250 ranked DEGs (based on FDR adjusted *p*−value) were further analysed in Cytoscape (v3.8.0) [45]. This was done using the STRING Cytoscape application (v1.6.0) [46] which identifies known functional associations between genes, which is subsequently used to generate protein interaction/functional association networks, facilitating the identification of the main biological responses to infection. This was also used to infer putative novel associations via node clustering patterns. Network layout was generated using GeneMania Force Directed Layout available as part of the GeneMania Cytoscape application (v3.5.2) [47]. Cytoscape was also used to analyse each of these networks using a Maximal Clique Centrality algorithm via the CytoHubba Cytoscape

application (v0.1) [48], inferring the relative importance of each node within the functional association network.

Using the entire gene expression dataset, GSEA (v4.1.0) [49,50] was then used to identify enriched gene-sets. As expression data input for GSEA, normalized DESeq2 expression data was exported to tab delimited format, parsed to Gene Cluster Text file format (*.gct) as per Broad Institute definitions [51] and imported in the GSEA. As gene–set input for GSEA, lists of *Danio rerio* Gene Ontology (GO) [52,53] and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway [54–56] gene–sets (based on Entrez/NCBI/GenBank Gene IDs) were both retrieved and converted to Gene Matrix Transposed file format (*.gmt) using EnrichmentBrowser (v2.20.7) [57]. After running GSEA on expression data, EnrichmentMap (v3.3.1) [58] was used to generate a network of significantly enriched/depleted GO and KEGG pathway gene–sets (FDR adjusted $p-$value <0.25), based on similarity coefficients between gene–sets. These networks were viewed and analysed in Cytoscape. Gene–sets were grouped into clusters, with the MCL cluster algorithm implemented via the Cytoscape app AutoAnnotate (v1.3.3) [59]. KEGG pathways of interest were analysed further by mapping DEG data to pathway maps. This was done with Pathview (v1.36.0) [60] using Log_2-Fold change data from DESeq2 as input. Gene symbols and descriptions were retrieved using myGene (v1.34.0) [61]. Zebrafish orthologs of proteins of interest in this study were identified using the KEGG database. Statistical analysis associated with all bioinformatics tools that were used in this study are described in the respective publications associated with each tool.

Read counts for each CyHV−3 ORF were also generated using HISAT2, SAMTools, StringTie and PrepDE as described above, except reads were mapped to the CyHV−3 RefSeq genome (GenBank Accession Number: NC_009127.1).

2.5 Ethics statement

The experiments performed in the present study did not require a bioethical permit as they involved the use of larvae before implementation of feeding. However, all experiments were designed and conducted in accord with the 3R rules and other bioethics standards.

3. Results and Discussion

In order to characterise the response to CyHV−3 infection in this zebrafish larvae model in terms of the ISG up−regulation, the potential involvement of programmed cell death and to establish the extent of CyHV−3 gene transcription in this model, we conducted transcriptomic analysis of infected zebrafish larvae. CyHV−3−infected and mock−infected larvae were sampled at 1, 2, and 4 dpi for RNA extraction and sequencing. RNA−Seq, yielded ~15–20 million reads per sample with data publicly available under BioProject Accession number PRJNA929940. Gene expression was compared between infected and mock−infected samples at each timepoint to identify DEGs. In line with viral levels observed in earlier experiments, viral RNA levels reached a peak at 2 dpi (0.34% of total transcriptome), falling considerably by 4 dpi (Table S2). Notably, transcription from all 155 CyHV−3 ORFs was detected by 2 dpi (Figure 1 and Table S3), indicating that indeed, in this model, cells may be permissive to CyHV−3 replication. Host differential gene expression in response to infection also peaked at 2 dpi, with 7.4% of expressed genes classified as DEGs (Table S2 and Figure 2).

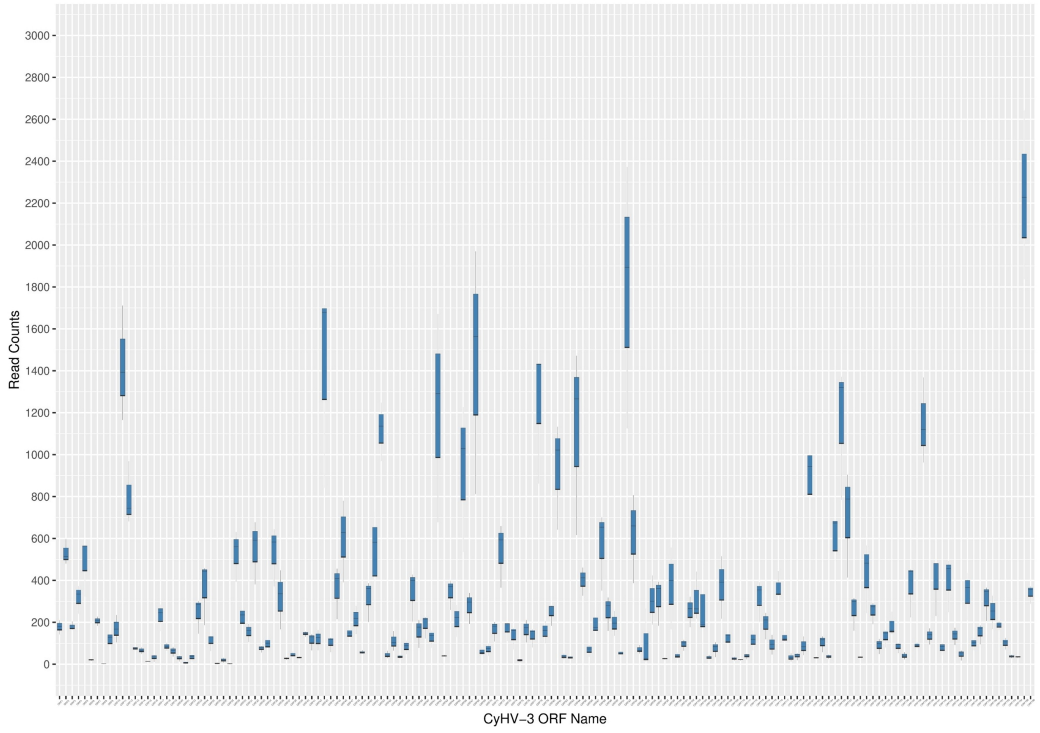

Figure 1. Box Plots of read counts mapping to each known CyHV−3 open reading frame (ORF) at 2 dpi. Transcription was detected from all known 155 CyHV−3 ORFs, including transcription from 4 disrupted ORFs in the CyHV−3 FL strain genome (GenBank Accession Number: MG925487.1). Read counts are based on mapping to CyHV−3 Refseq genome (GenBank Accession Number: NC 009127.1) due to more complete and standardized annotation. Box plots represent the distribution across three replicates i.e., the median, two hinges (the 25th and 75th percentiles) and two whiskers (minimum and maximum values). Read counts corresponding to each ORF in each replicate are provided in Table S3.

Prior to this study, it was unknown how zebrafish larvae respond to CyHV−3 challenge in terms of type I IFN gene expression. Consistent with other reports [62], we found that *ifnphi2* was not expressed

at this developmental stage. The IFN response in zebrafish larvae relies on expression of *ifnphi1* and/or *ifnphi3* genes [62]. However, we did not observe convincing expression from either gene at any timepoint. Our sampling points range from at 1–4 dpi, which equate to 96–168 hpf, with previous studies indicating that WT AB zebrafish larvae are capable of expressing *infphi1* and *infphi3* by this developmental stage [62,63]. Notably, these previous studies, involving SVCV and CHIKV challenge, utilized RT−qPCR to detect IFN gene transcription, which may be more sensitive than RNA−Seq in some situations.

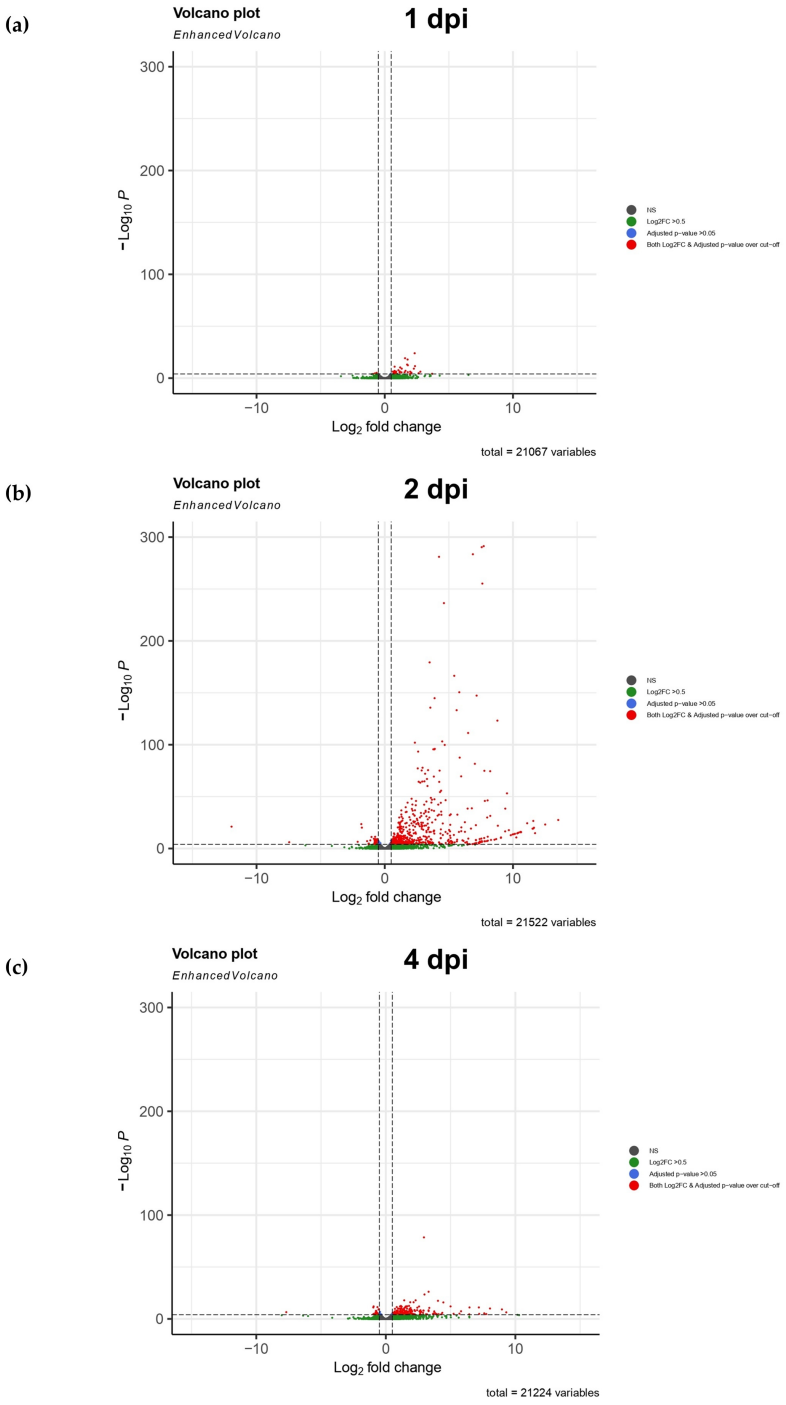

Figure 2. Volcano plots summarizing DEGs at each time point. All genes included in differential gene expression analysis are represented in these plots. For each gene, log_2fold change (Log2FC) is represented on the

x-axis and significance (−$\log_{10}$ of the FDR adjusted p−value) is represented on the y-axis. These represent changes in expression in CyHV−3 infected relative to mock infected zebrafish larvae at 1 (**a**), 2 (**b**) and 4 (**c**) dpi. Genes exhibiting the most pronounced changes in response to infection (with Log2FC >|0.5| threshold and FDR adjusted p−values >0.5 threshold) are highlighted in red. Time points were compared in terms of the amount of DEGs exceeding both these thresholds. Based on these criteria, the 2 dpi timepoint was identified as exhibiting the most pronounced changes in expression in response to infection. Subsequently all further analysis was based on data from this timepoint.

While CyHV−3 is known to inhibit the IFN−response *in vitro* [64,65], our observations do not necessarily indicate inhibition of the IFN−response in zebrafish. It is possible that the up−regulation of IFN genes occurs very early after infection, returning to basal levels rapidly, prior to the first sampling point. The effects of this rapid and short−lived IFN response should be still observed in the form of subsequent ISG induction. Indeed, in this present study, the list of the 250 most significant DEGs at 2 dpi is dominated by typical ISGs (Table S4). This ISG induction in the absence of IFN detection is similar to previous studies with WT zebrafish larvae infected with nervous necrosis virus (NNV) [66]. In both studies, it is likely that IFN up−regulation occurred prior to the earliest sampling point. However, the kinetics of type I IFN induction in WT AB zebrafish may depend on the nature of the viral challenge (virus, dosage, and inoculation site/route). For example, in previous studies in which WT AB larvae were inoculated with HSV−I and CHIKV (72 hpf), *ifnphi1* up−regulation peaked at 36 hpi [67] and 24hpi [63], respectively, with further differences in sustained up−regulation after these timepoints. Furthermore, the expression of *infphi1* and *ifnphi3* may be model−specific. For example, Tübingen strain zebrafish larvae inoculated with Tilapia Lake Virus (TiLV) (48–60 hpf) were only observed to exhibit significant *ifnphi1* up−regulation but not insignificant *infphi3* up−regulation by 48 hpi [68]. It remains unclear if only one or both IFN genes are responsible for this ISG induction (Table S4) in our infection model, and this will be the subject of future studies, involving sampling at earlier timepoints.

We also conducted further characterisation of the main types of genes that were differentially expressed in response to CyHV−3 infection in zebrafish larvae. Using STRING, we generated a network (Figure 3) representing the functional relationships between the top 250 most significant DEGs at 2 dpi (Table S4). As expected, functional enrichment analysis of this network revealed that these DEGs were mainly associated with the immune and stress responses (Table S6). Three main clusters formed within this network. The largest cluster (Figure 3a) mainly represented genes involved in viral infection and cytokine responses. These include genes encoding the antiviral GTPase proteins such as *mxa*, *mxb*, *mxc*, and *mxe*, as well as *rsad2* (or *vig−1*, *viperin*). This is consistent with previous observations in zebrafish larvae infected with NNV [66], Zebrafish Picornavirus (ZfPV) [69], and CyHV−3−infected adult zebrafish [35]. In terms of the cytokine response, genes encoding IFN regulatory factors *irf7* and *irf9* were also part of this main cluster. Notably, zebrafish *irf3* was also among the top 250 most significant DEGs (Table S4), however as STRING returned no results for this gene, it was not included in the network in Figure 3. In addition, genes encoding other important elements of the IFN response, *stat1a*,

stat1b, *stat2*, and augmentation and regulation of this response such as *isg15* [70] were also featured in this cluster, consistent with zebrafish larvae responses to HHV−1 [67] and NNV [66].

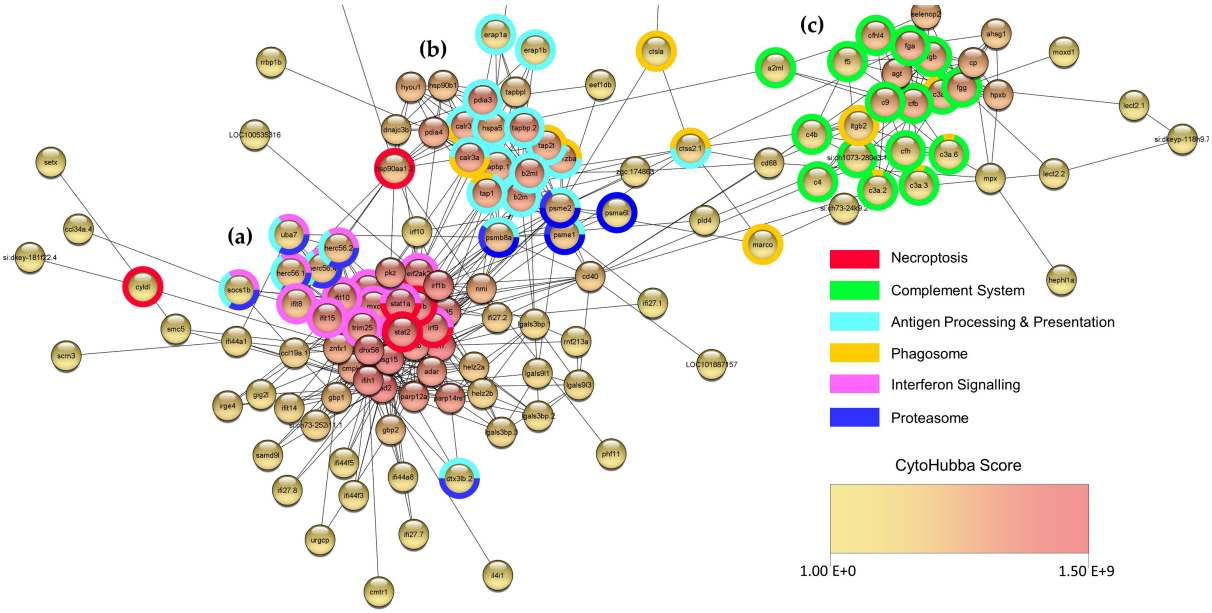

Figure 3. Network representing the functional associations between some of the top 250 most significant DEGs at 2 dpi. Using STRING protein query function in Cytoscape, 208 of the top 250 most significant DEGs were identified and scored based on functional association with each other. These data were used to generate a network in Cytoscape, which was then arranged based on GeneMania force directed layout. Each DEG is represented by a node, with edges (connecting lines) representing functional association. The largest contiguous network resulting from this analysis (136 nodes and 696 edges) is displayed. For visualization purposes, nodes in the peripheral regions of the network (representing DEGs *LOC100006895*, *rnasel3*, *ndrg1b*, *pde6ha*, and *serpinb1l1*) were omitted. This resulted in one large cluster (**a**), and two smaller clusters (**b**) and (**c**). STRING functional enrichment analysis indicated that most DEGs in this network were related to the immune response to infection (Table S6), and genes were labelled based on the main types of gene−set categories enriched in each of their respective clusters. This revealed distinct functions associated with each gene cluster, for example (**a**) interferon and PRR signalling, (**b**) antigen processing and presentation, and (**c**) complement response. The network was also analysed by CytoHubba, which was used to identify the potentially most important hub nodes within the network, with each node scored and coloured based on maximal clique centrality within the network, according to the CytoHubba score colour scale provided; however, this is better represented in Figure 4, with corresponding CytoHubba scores in Table S7.

The detection of "non−self" material in cells via PRRs is an important part of the innate immune response. Viral nucleic acids represent major PAMPs during infections, and genes encoding PRRs to detect these PAMPs were among the most significant DEGs in our experimental model. For example, genes encoding important zebrafish RIG−I−like receptor (RLR) orthologs, such as *ifih1* (encoding *MDA−5* ortholog) [71], and *dhx58* (encoding *LGP2* ortholog) [72] were centrally located within this large cluster (Figure 3a). An additional gene, *rigi*, encoding the zebrafish ortholog of RIG−I, the most−studied RLR [73], was also significantly up−regulated in response to infection, but not among the top 250 most significant DEGs used to generate this network (274th most significant DEG, Table S5).

Genes encoding other important components of the RLR viral RNA sensing apparatus such as *trim25* [74,75] were also centrally located in this large cluster (Figure 3a). In addition to RLRs, other genes encoding RNA binding proteins are important actors in the innate immune response such as *adar* [76], *eif2ak2* (encoding PKR ortholog) [77], *pkz* [77–80], and *ifit10* (human *IFIT5* ortholog) [81–83] also co−locate within the same large cluster. Interestingly, we noted that two additional genes, *helz2*a and *helz2*b, encoding proteins that may act as evolutionarily conserved RNA sensors [84], can be observed at the peripheral regions of this main cluster. Many known vertebrate dsDNA sensing PRRs are absent in teleost fish [84,85]. Of the few known genes encoding dsDNA sensing PRRs in zebrafish, which include *ddx41* [67,86], *cgasa* [87], *dhx9* [67], and *dhx36* (the latter of which, may act as a conserved RNA and DNA PRR [88]), only *cgasa* was significantly up−regulated, but not featured in the top 250 DEGs (623rd most significant DEG, Table S5). This may indicate that RNA sensing as opposed to DNA sensing PRRs represent an important part of the response to CyHV−3 infection in zebrafish larvae, even though it is a dsDNA virus. This is consistent with growing evidence for the role of RLRs in the detection of dsDNA viruses, such as members of the family *Herpesviridae* or *Adenoviridae* [89–93].

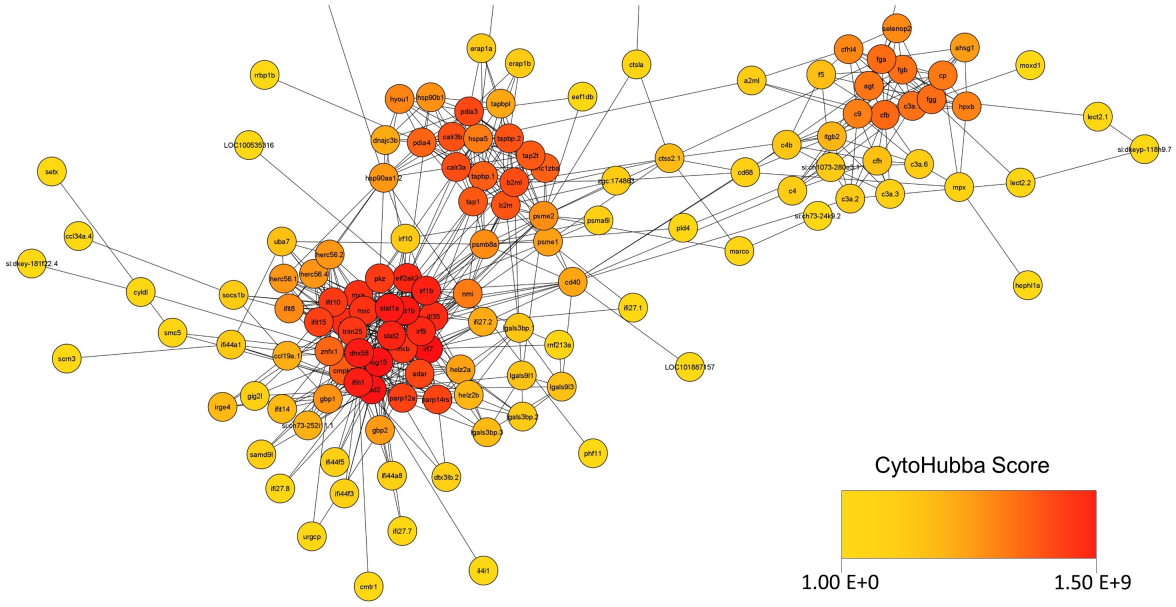

Figure 4. CytoHubba Analysis of STRING protein query network. After generating the STING network in Fig.3, CytoHubba was used to identify the potentially most important hub nodes within the network. Each node is scored and coloured (according to CytoHubba colour scale) based on maximal clique centrality within the network. Corresponding CytoHubba scores for all genes/nodes in this network are available in Table S7. As per Fig. 3, for visualization purposes, nodes in the peripheral regions of the network (representing DEGs *LOC100006895*, *rnasel3*, *ndrg1b*, *pde6ha* and *serpinb1l1*) are omitted.

Within the largest cluster, in addition to genes being generally involved in antiviral responses, functional enrichment analysis identified a subset of clusters representing genes belonging to IFN signalling and necroptosis gene−sets (Figure 3a). The same functional enrichment analysis indicated that genes in the smaller central cluster were mainly involved in antigen processing and phagosome

responses (Figure 3b), with genes in the smaller cluster on the right mainly related to the complement system (Figure 3c). Furthermore, the identification of the potentially most important hub nodes within the network in Figure 3 (based on maximal clique centrality) revealed that nodes representing RNA PRRs *ifih1* (MDA5 ortholog) and *dhx58* (LGP2 ortholog) were ranked highest, along with *rsad2* (or *vig−1*, *viperin* ortholog), *stat1a*, *irf7*, *isg15* and *stat1b* (Figure 4 and Table S7). Notably, all the top ten ranked hub nodes (twenty in total) represent genes located in the largest cluster (Figure 3a), most of which are described above.

Interestingly, in addition to many commonly studied ISGs, we also observed up−regulation of genes encoding NACHT−domain and leucine−rich−repeat−containing (NLR) proteins, for example, *loc100535428* (Table S4). These represent a protein−class that is now increasingly recognised as representing important elements of the innate immune response in teleost fish [69,94]. We also note the up−regulation of many genes encoding uncharacterized products in response to CyHV−3 infection, some of which were >1000–5000−fold up−regulated (Table S4). Focusing on those within the top 250 significant DEGs that were >100 fold up−regulated, we noted that four of these were not previously described as being up−regulated in response to infection or immune stimulation (Table S4). We also noted the up−regulation of five non−coding RNA genes in response to CyHV−3 infection, one of which was >3,000 fold up−regulated (Table S4), representing the 6th most up−regulated gene in the dataset. All other uncharacterized genes occurring within the group of top 250 most significant DEGs were further cross−referenced with existing GenBank entry information on predicted protein domains (Table S4). This revealed that three of these genes potentially encode additional NLR proteins, three encode RNA binding domains, and three encode proteins containing retrotransposon derived reverse transcriptase−like (RT−like) domains (Table S4). In the case of the latter, the three genes encoding RT−like domains are all paralogs of each other (KEGG Database) and similarly up−regulated (>29–35−fold, Table S4). Further inspection of corresponding entries for these gene products in UniProt and InterPro revealed predicted retrotransposon gag, aspartic proteinase, RT, RNase H, and integrase domains, indicating they may indeed encode retrotransposon polyproteins. The domain organization and motifs are consistent with retrotransposons within the family *Belpaoviridae* [95] (also referred to as Bel/Pao, Class I retrotransposons based on previous classification systems [96]). It should be noted that the up−regulation of retrotransposons and other transposable elements in response to infection has been observed in other organisms [97–99], and to the best of our knowledge this is the first description of this in a zebrafish model. Interestingly, up−regulation of class I retrotransposons in zebrafish has also been observed in response to genome demethylation, leading to the induction of antiviral responses [100].

In further analysis, we expanded our investigation to all genes included in differential expression analysis at 2 dpi (Table S5), exploring the response to infection at a “gene−set level”. Using GSEA, we

identified GO and KEGG pathway gene−sets that were to a significant extent positively or negatively enriched in CyHV−3−infected larvae at 2 dpi (Table S8, Table S9, Figure 5).

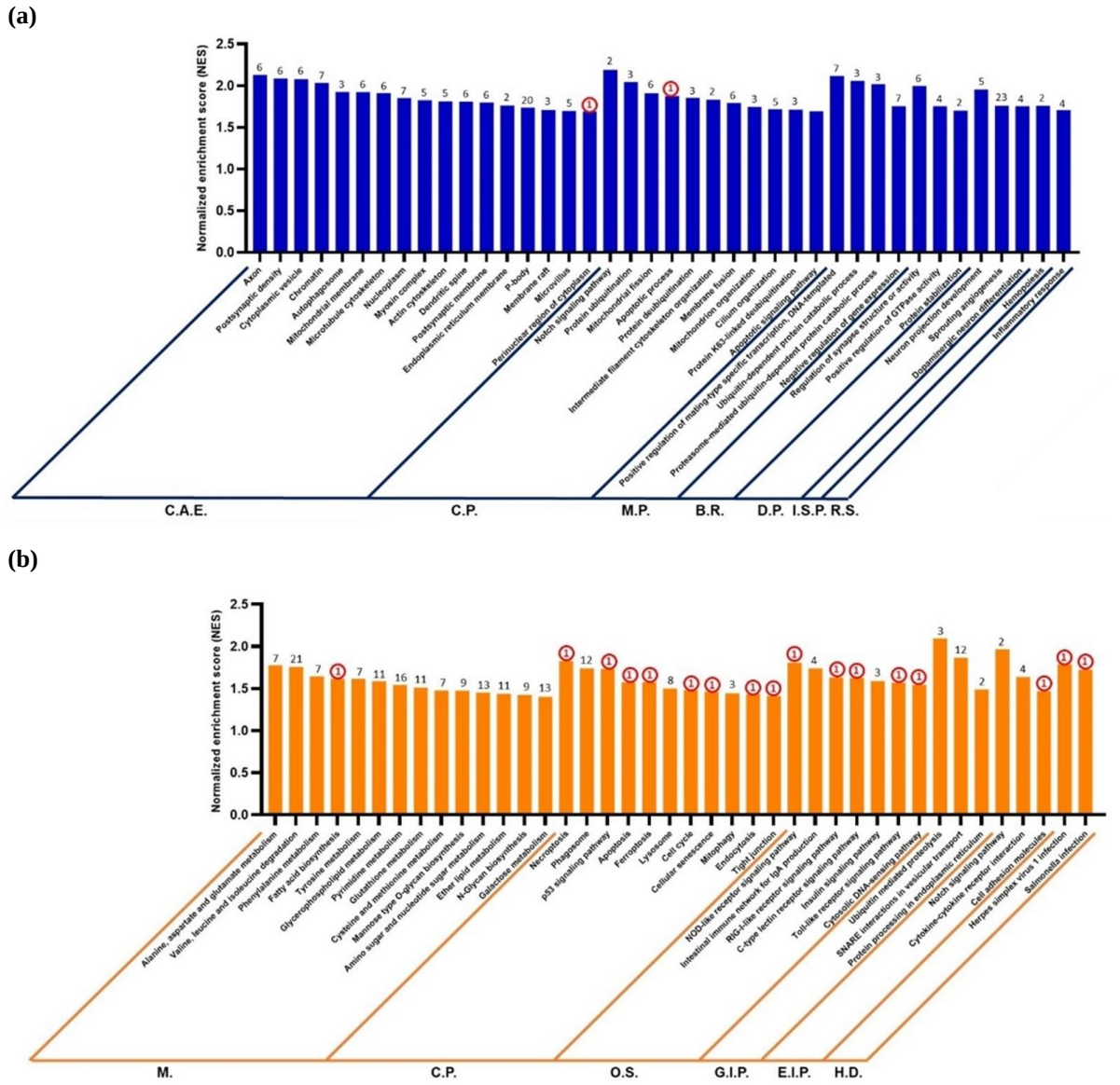

Figure 5. Summary of the top 40 positively enriched GO and KEGG gene sets. (**a**) Top 40 positively enriched GO gene sets (FDR adjusted p−value <0.25) based on NES. Gene-set names are on the x−axis with corresponding normalized enrichment score (NES) on the y-axis. Within the x−axis, gene sets are grouped by sub-category and then arranged by NES within sub-category. The sub−categories include cellular anatomical entity (C.A.E.), cellular process (C.P.), metabolic process (M.P.), biological regulation (B.R.), developmental process (D.P.), immune system process (I.S.P.) and response to stimulus (R.S.). Numbers above each bar correspond to clusters from the Cytoscape network displayed in Fig. 6, with gene-sets corresponding to cluster−1 (programmed cell death and PRR signalling responses) circled and coloured in red. Details of all significantly enriched GO gene sets are available in Table S8. (**b**) Summary of the top 40 positively enriched KEGG pathway gene sets (FDR adjusted p-value <0.25) based on NES presented as described for (a). The sub−categories include metabolism (M), cellular processes (C.P.), organismal systems (O.S.), genetic information processing (G.I.P.), environmental information processing (E.I.P.) and heart diseases (H.D.). Details of all significantly enriched KEGG pathway gene sets are available in Table S9.

Cytoscape was used to generate a network of these significantly enriched gene−sets based on the functional relationships between them (Figure 6), providing a greater insight into what biological processes are implicated in the response to CyHV−3 infection in zebrafish larvae, and how they are related. Notably, only one gene−set, “Ribosome” (DRE03010), was found to be significantly negatively enriched, with all other significant gene−set responses involving positive enrichment. During the process of generating the network presented in Figure 6, nodes (i.e., gene−sets) were clustered together based on their similarity coefficient (related to gene−set/functional overlap). This process resulted in the formation of several large clusters, which we numbered. Cluster−1 is the largest of these and exhibits the highest quantity of functional connections with surrounding clusters, and as such, it represents a major aspect of the response to CyHV−3 infection. Within Cluster−1, there are two main sub−clusters. One of these is dominated by gene-sets related to programmed cell death, the other is dominated by PRR signalling, pathogen and inflammatory response gene−sets. Notably, enrichment of the RIG−I−like signalling pathway, the Toll−like receptor signalling pathway, and the Herpes simplex virus 1 gene−sets are consistent with zebrafish larvae response to NNV infection [66]. In Cluster−1, the KEGG Necroptosis pathway (DRE04217) is the most significant positively enriched gene−set, and joint most significantly enriched gene−set overall (Table S8 and Table S9). Notably, this pathway gene−set is functionally related to other gene−sets in the apoptosis and PRR/inflammatory/pathogen response sub−clusters (manually isolated from these two sub−clusters in Cluster−1, Figure 6), exhibiting gene overlap with 15/19 of these gene−sets, with eight of these resulting in similarity coefficients >0.2 and thus displayed in Figure 6. This reflects the substantial crosstalk that exists between programmed cell death and PRR signalling in response to infection [101,102].

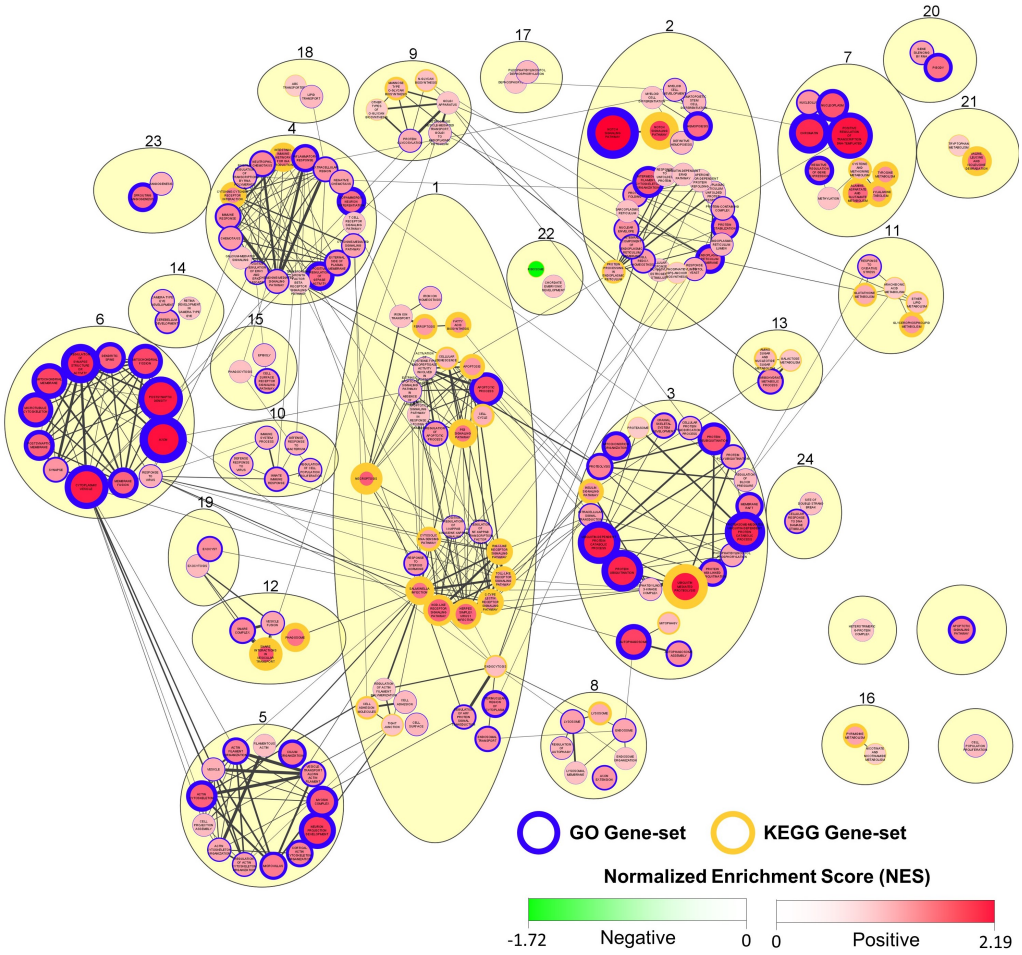

Figure 6. Summary of GSEA output indicating gene−set enrichment based on gene expression in CyHV−3−infected relative to mock−infected zebrafish larvae at 2 dpi. Cytoscape Network representing functional relationships between all significantly enriched gene-sets (positive or negative) identified in GSEA output (FDR adjusted p−value <0.25). Nodes in the network represent GO (blue border) and KEGG Pathway (gold border) gene−sets. Edges (connecting lines) between nodes represent the similarity coefficient (measuring the functional/gene overlap between pairs of gene-sets). Edge thickness corresponds to magnitude of similarity coefficient (only edges with coefficient ≥0.2 are displayed). Each gene set exhibits either a positive or negative normalized enrichment score (NES), indicating predominant up−regulation or down−regulation of constituent genes, respectively. Accordingly, node colour and size both represent NES magnitude (exponentially transformed scale), with positive and negative enrichment represented by red and green, respectively, according to the colour scale provided. The node border thickness indicates the significance of enrichment (inverse of FDR adjusted p−values, thus the lower the FDR adjusted p−value, the greater the thickness). Using the MCL cluster algorithm, GO and KEGG gene−sets were clustered together based on their functional similarity as indicated by similarity coefficients (beige ovals), and numbers were assigned to each cluster. For the purposes of visual clarity, clusters were manually repositioned, and within some clusters, sub−clusters were manually grouped based on functional similarity. Clusters that are overlapping or touching in the absence of any visible edges between their respective nodes have shared edges below the 0.2 coefficient cut−off for display. Clusters that do not exhibit edges between their respective nodes and are also not touching or overlapping either have no common edges or have common edges with similarity coefficient <0.2.

The prominence of positively enriched necroptosis and apoptosis related gene−sets in Cluster−1 supports the hypotheses derived from earlier observations *in vitro* and *in vivo*, that apoptosis−like and non−apoptosis−like programmed cell death feature heavily in the zebrafish response to CyHV−3

infection. One of the important genes in the necroptosis pathway is *eif2ak2* (or *pkr*). It was identified as one of the main genes contributing to the enrichment signal for the necroptosis gene−set (Figure 7). It represents an important link between the innate immune response and the initiation of necroptosis [103]. This gene encodes a protein referred to as "interferon−induced, double−stranded RNA−activated protein kinase", or more commonly, "Protein Kinase R" (referred to as PKR hereafter). PKR functions as both a general cellular stress sensor and PRR. Thus, it plays a diverse role in the innate immune response to viral infections and many fundamental cellular processes including programmed cell death [104].

NCBI Entrez Gene ID	Gene Symbol	GSEA Ranking	Raw Normalized Expression Values 2 dpi					
			Infected Replicate 1	Infected Replicate 2	Infected Replicate 3	Control Replicate 1	Control Replicate 2	Control Replicate 3
368481	stat1b	4.27						
565200	stat2	3.62						
403013	irf9	3.17						
30768	stat1a	2.75						
768248	fas	2.73						
100001092	eif2ak2	2.37						
557302	casp10	2.24						
561108	cyldl	2.24						
561021	casp22	2.11						
393386	cybb	1.78						
565155	hsp90aa1.2	1.69						
403126	tlr3	1.69						
431715	rbck1	1.68						
798864	pla2g4f.1	1.68						
405770	il1b	1.53						
373114	cflara	1.46						
58022	casp8	1.45						
406471	tnfrsf1a	1.43						
562317	fadd	1.3						
30591	hsp90aa1.1	1.29						
553353	capn1	1.24						
561370	jak3	1.13						
57923	pycard	1.07						

Figure 7. Leading edge analysis of the necroptosis pathway. This table, exported from the EnrichmentMap Cytoscape app, shows the subset of genes within the KEGG Necroptosis pathway gene−set (DRE04217) that contributed the most to the enrichment signal of this gene set in CyHV−3 infected vs Non−infected zebrafish larvae at 2 dpi. The corresponding heatmap on the right represents raw normalized expression values for each gene in each replicate in both infected and mock-infected zebrafish larvae groups. Expression values are represented as colours ranging from green to magenta, corresponding to low and high raw normalized expression values respectively.

PKR−mediated programmed cell death is important for the clearance of viral infections [103,105,106]; however, the antiviral roles of PKR are diverse. It also contributes to the antiviral actions of other enriched gene−sets within Cluster−1 (Figure 6). For example, in the "Herpes simplex virus 1" response gene−set (DRE05168), PKR is activated by dsRNA formed during infection, and subsequently phosphorylates eIF2α (its main substrate), resulting in the stalling of mRNA translation [104,105,107] (Figure 8a). However, some mRNA species are less affected by this [108–110]. This translational

stalling also leads to the formation of stress granules (SGs) [111–113], which in some cases are important for detection of viral RNA via PRRs as in the "RIG−I−like receptor signalling pathway" (DRE04622) [114,115]. Furthermore, PKR also facilitates/promotes the NF−κB pathway, indirectly [104,108]. While this induces a pro−inflammatory response which may be useful in terms of counteracting infection, the accompanying pro−survival response (although helpful to some aspects of immune−response [116]) is counter to the pro−apoptotic function of PKR, but may act to only temporarily delay cell death [117]. Notably, expression from the zebrafish *nfkb1* gene, which encodes the zebrafish NF−κB ortholog, was not significantly up−regulated at 2 dpi in our model (Table S5 and Figure 8b).

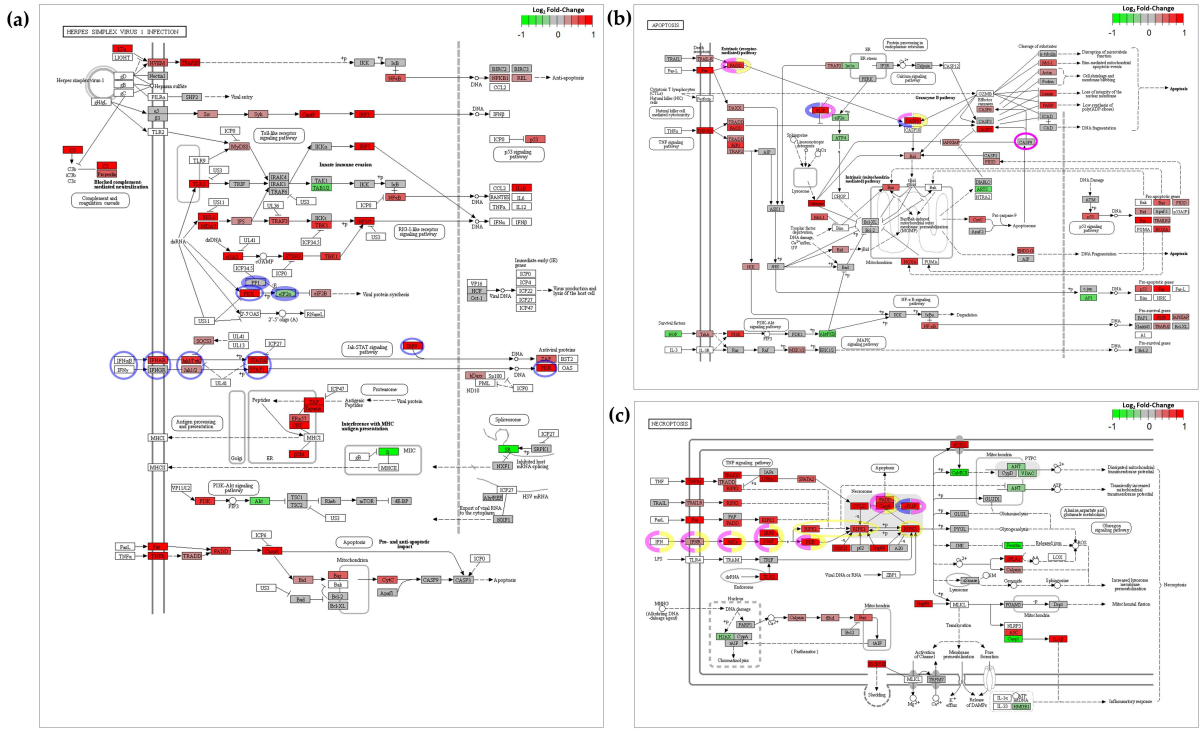

Figure 8. Visualization of differential gene expression in CyHV−3−infected zebrafish larvae (2 dpi) within KEGG pathway maps. Using the R package Pathview, gene expression data from our experiment was mapped to corresponding nodes in KEGG pathways (**a**) Herpes simplex virus 1 infection (**b**) Apoptosis and (**c**) Necroptosis pathways. Nodes represent zebrafish homologs of genes known to be involved in each pathway, with colour representing the $\log_2$−fold−change in gene expression in CyHV−3−infected relative to mock infected zebrafish larvae. Up−regulated and down−regulated genes are represented by red and green shades respectively, according to scale in the top right of each pathway. For visual clarity (due to large differences in fold change between genes) the maximum and minimum values in the colour scale is set at –1 and 1 $\log_2$−fold−change (corresponding to a two−fold change). It should be noted that many nodes represent combined differential expression from several zebrafish paralogs, thus the generic KEGG gene symbols are used as node names, which relate to the common names used to refer to protein products at each node. Not all the paralogs represented by each node are significantly differentially regulated. The list of zebrafish orthologs/paralogs corresponding to each node in these pathways can be accessed in the KEGG database using the corresponding gene-set references (Herpes simplex virus 1 infection (DRE05168), Apoptosis (DRE04210) and Necroptosis (DRE04217)), which can then be cross−referenced with data in Table S5 (using NCBI/Entrez/GenBank Gene IDs or Gene Symbols). Key genes involved in IFN−stimulated PKR−mediated programmed cell death, i.e., translational inhibition [104,106,118] leading to apoptosis [102] (blue), IFN−stimulated PKR−mediated apoptosis [119,120] (pink), and IFN−stimulated

PKR−mediated necroptosis [103] (yellow) are highlighted. Genes with dashed line borders indicate instances where down−regulation, translational inhibition or post−translational inactivation of protein products promote the processes in question (see main text and references provided within this caption for details). White nodes represent instances where zebrafish homologs have not been assigned thus far, or where gene expression from zebrafish homologs have not been detected.

PKR−mediated apoptosis can occur via the "extrinsic" FADD−caspase−8 mediated pathway [121]. The circumstances under which this occurs are quite diverse. For example, PKR−mediated translational inhibition leads to apoptosis [105,106] via depletion of cFLIP protein [102] which acts as an important inhibitor of caspase−8 (Figure 8b) [122,123]. PKR phosphorylation by PACT (in response to stress) can also lead to translational inhibition leading to caspase−8 dependent apoptosis [124], as can overexpression of PKR [125–127]. In addition to IFN stimulation leading to up−regulation of PKR, IFN−stimulated PKR−mediated apoptosis can also occur via JAK/TYK−mediated phosphorylation of PKR [119]. Notably, along with *eif2ak2* (encoding PKR), many other zebrafish genes encoding orthologs of ISGs involved in IFN−stimulated PKR−dependent apoptosis are also up−regulated at 2 dpi in our model (Figure 8b, c). In parallel, PKR may also promote caspase−9 mediated apoptosis via the "intrinsic" apoptosis pathway. However, unlike caspase−8, caspase−9 was not up−regulated at 2 dpi in our experiment (Figure 8b), indicating, as with other viral−host models [108,120,121], that caspase−8 mediated apoptosis also plays a more dominant role in response to infection in the CyHV−3−zebrafish larvae model.

Many viruses have evolved ways to interfere with apoptosis by disrupting elements of the FADD−caspase−8 pathway [104,128–130]. To counteract this, necroptosis may have evolved as a back−up mechanism of programmed cell death [128], which can occur via compromising of the cell membrane though action of MLKL [131] and/or production of reactive oxygen species [132]. This relies on the interaction of RIPK1 and RIPK3 for necrosome formation, a process that is inhibited by the FADD−caspase−8 complex [128,132,133]. Like apoptosis, PKR−mediated necroptosis can occur in response to IFNs, possibly requiring PKR interaction with RIPK1 [103]. While other groups have also observed a physical association between PKR and RIPK1 [134], the exact role that PKR plays in initiating necroptosis in response to IFN stimulation remains unclear [135]. Notably it has been proposed that IFN−stimulated PKR−mediated necroptosis is restricted to the G2M stage of the cell cycle, when FADD is disabled, preventing capase−8 inhibition of necrosome formation [103]. Given that in zebrafish larvae, and to lesser extent, in ZF4 monolayers, we expect widespread, frequent occurrence of mitosis, our models may be particularly predisposed to this type of PKR−mediated necroptosis. Notably, in addition to PKR itself, genes encoding zebrafish orthologs of ISGs involved in PKR−mediated necroptosis are also up−regulated at 2 dpi (Figure 8c).

The *eif2ak2* gene encoding PKR was also among the top 250 most significant DEGs in this study (Table S4) and identified as an important hub gene in functional network in Figure 9, being ranked 3rd

overall (Table S7). Given the importance of this ISG in terms of antiviral defence [102,105,136], particularly regarding programmed cell death, we hypothesized that the knock−out (KO) of the *eif2ak2* gene may impact CyHV−3 clearance in zebrafish larvae.

Unlike other vertebrates, members of the teleost fish families *Salmonidae* and *Cyprinidae* also encode an additional PKR−like protein referred to as "protein kinase containing Z−DNA binding domains" (or PKZ) [77,78,80]. PKZ genes may have evolved through duplication of the PKR encoding genes in these teleost fish families, after divergence from tetrapods [77]. Consequently, PKZ exhibits a high degree of sequence similarity to PKR proteins encoded in the same genomes, predominantly to the C−terminal kinase domain, which is responsible for eIF2α phosphorylation by PKR [78,137].

However, unlike PKR, PKZ contains Zalpha (Zα) domains instead of dsRNA binding domains in the N−terminal [137]. These domains are capable of binding to Z−DNA/RNA, which exist in the left−handed double helix conformation as opposed to the more common right−handed conformation of dsDNA/RNA (referred to as A and B−DNA/RNA) [79].These two features indicate that: (1) Like PKR, PKZ acts as an eIF2α kinase and mediates translational stalling, and induction of apoptosis via eIF2α phosphorylation [77,78,138,139], and (2) Like PKR, PKZ acts as a cytosolic PRR, but is activated by a greater diversity of nucleic acids than PKR. PKZ nucleic acid binding, B−to−Z conversion, and PKZ−mediated translational stalling have been best demonstrated using B and Z−DNA [78,140–143], indicating co−operative antiviral roles for PKZ and PKR. However, given that the Zα domains of PKZ do bind to RNA analogues [144] and that some Zα domains exhibit A−to−Z RNA conversion (as we recently demonstrated [79]), like PKR, PKZ may also detect and be activated by dsRNA. Thus, PKZ may provide at least some degree of back−up for PKR, leading to some redundancy among zebrafish IFN induced eIF2α kinases.

Notably, the *pkz* gene (encoding PKZ) was the 23rd most significantly up−regulated gene at 2 dpi in our model, up−regulated more than the *eif2ak2* gene (encoding PKR, ranked 250th, Table S4), and the *pkz* expression levels were >3 fold higher. In addition, *pkz* was ranked 9th in hub gene analysis in Figure 9 (see also Table S7). Given their potentially overlapping functions, we propose that generating a PKR−KO mutant (lacking *eif2ak2*) and a separate mutant PKZ−KO (lacking *pkz*) would be interesting to investigate the importance of both these multifunctional eIF2α kinases in the clearance of CyHV−3 in zebrafish larvae.

4. Conclusions

The aim of this present study was to investigate the nature of the innate immune response to CyHV−3 infection in the zebrafish larvae model and to establish the extent of CyHV−3 gene transcription *in vivo* by conducting a transcriptomic analysis of whole zebrafish larvae infected with CyHV−3. As we observed transcription of all 155 known CyHV−3 protein coding genes in infected zebrafish larvae (Figure 1, Table S3), we conclude that zebrafish cells may be permissive to CyHV−3 replication *in vivo*. CyHV-3 infections stimulate the up−regulation of many ISGs (Figure 3, Tables S4 and S5). The up−regulation of genes involved in programmed cell death and nucleic acid sensing PRR pathways represent a core part of the immune response (Figures 6 and 8), which is consistent with our previous observations, both *in vitro* and *in vivo*, of apoptosis−like death among infected cells. PKR and PKZ are also up−regulated in response to infection (Figure 3, Table S4) and may significantly contribute to both programmed cell death and nucleic acid sensing PRR pathways during infection (Figures 6 and 8). We propose that the contribution of these two genes to viral clearance may be investigated via by generating KO zebrafish strains, and to determine the impact on CyHV-3 clearance.

Furthermore, we note that many of the most significantly up−regulated genes in response to CyHV−3 infection in zebrafish larvae were uncharacterized, and some were previously unreported as being involved in the immune response (Table S4). These include five non−coding transcripts (one of which was >3000−fold up−regulated and the 6th most up−regulated gene at 2 dpi). We propose to provisionally refer to these five transcripts as “Zebrafish Non−coding Infection Response Element” 1–5 (or ZNIRE 1−5, complete details in Table S4). This observation was particularly intriguing, and we propose that further research into their importance during the immune response will be necessary. We also observed the up−regulation of three retrotransposons (all ~30−fold up−regulated, Table S4). It is possible that this retrotransposon re−activation/up−regulation in response to infection may be beneficial. Their cytoplasmic RNA and/or DNA genome intermediates may potentially act as ligands for PRRs [100], thus enhancing the innate immune response to viral infection and presenting an interesting hypothesis for further study with our model.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v15030768/s1, Table S2. Summary of DEGs from each timepoint; Table S3. Read Counts mapping to each known CyHV−3 Open Reading frame (ORF) at 2 dpi; Table S4. Top 250 significant DEGs in zebrafish larvae infected with CyHV−3 at 2 dpi; Table S5. Raw output of DESeq2 in zebrafish larvae infected with CyHV−3 at 2 dpi; Table S6. Summary of STRING enrichment analysis; Table S7. STRING CytoHubba Ranking; Table S8. Summary of Gene Ontology (GO) enrichment analysis; Table S9. Summary of Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway gene set enrichment analysis.

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Experimental section

3rd study:

Disruption of PKR and/or PKZ does not impair the clearance of Cyprinid Herpesvirus 3 infection in zebrafish larvae

Synthèse des travaux menés

Dans les cellules de poisson, les infections virales provoquent l'induction de nombreux gènes stimulés par l'interféron, notamment la protéine kinase activée par l'ARN double brin et induite par l'interféron (PKR) et la protéine kinase activée par l'ADN Z (PKZ). Nos précédentes expériences *in vitro* et *in vivo* (chapitre I de la section expérimentale) ont indiqué que l'infection par le Cyprinid Herpèsvirus 3 (CyHV-3) était rapidement éliminée dans les modèles de poisson-zèbre par le biais de la mort cellulaire programmée. Ceci a été confirmé par l'analyse transcriptomique des larves infectées avec le CyHV-3 (chapitre II de la section expérimentale), qui a également confirmé le rôle potentiellement important des kinases PKR et PKZ dans ce processus.

Dans cette étude, l'impact de PKR et PKZ sur la clairance du CyHV-3 par les cellules de poisson-zèbre a été étudié en générant des lignées mutantes de poisson-zèbre *mut eif2ak2 (pkr)*^{ulg025}, *mut pkz*^{ulg027} et *mut eif2ak2 (pkr) L15-1* knockout (KO), ci-après dénommées PKR-KO, PKZ-KO et PKR-KO, par le biais de la technologie CRISPR/Cas9. La réplication du CyHV-3 au cours du temps a été mesurée à l'aide de la microscopie à fluorescence, de l'imagerie par bioluminescence in vivo (IVIS) et de la quantification relative par qPCR.

Les lignées mutantes de poisson-zèbre ont été générées par la technologie CRISPR/Cas9 comme décrit précédemment (Gagnon *et al.*, 2014; Jao *et al.*, 2013; Varshney *et al.*, 2015). L'ARNm nls-zCas9-nls a été synthétisé par transcription du plasmide pT3TS-nCas9n (Addgene #46757). Tout d'abord, le poisson-zèbre de souche WT AB a été utilisé pour générer les souches mutantes PKR-KO et PKZ-KO. L'outil CHOPCHOP a été utilisé pour concevoir deux ARN guide (appelé aussi single guide RNA ou sgRNA) GAGCACTCACAGTGATGAACCGG et CCACCGTGAACAGGCATCT (les motifs PAM sont soulignés) pour cibler l'exon 2 du gène WT *eif2ak2* (ou *pkr*) (NCBI/Entrez/GenBank Gene ID : 100001092) et l'exon 1 du gène WT *pkz* (NCBI/Entrez/GenBank Gene ID : 503703), respectivement. Environ 1 nL d'une solution comportant 50 ng d'ARN guide et 300 ng d'ARNm nls-zCas9-nls a été injecté dans des embryons de poisson-zèbre au stade 1-cellule. Les embryons fondateurs (génération F0) porteurs d'une mutation germinale dans *eif2ak2* ou *pkz* ont été élevés jusqu'à l'âge adulte et croisés avec des poissons WT pour générer des poissons F1 hétérozygotes. Les poissons porteurs de mutations *frameshift* ont été conservés et utilisés pour élever des lignées F2 homozygotes KO stables. Par la suite, les poissons-zèbres de la souche mutante PKZ-KO ont été utilisés pour générer la souche mutante double KO PKR-PKZ-KO en répétant le processus utilisé pour générer la souche mutante PKR-KO. Ces mutations ont toutes abouti à des gènes produisant des protéines tronquées et ont été vérifiées en génotypant par PCR deux larves de poissons-zèbres âgées de 4 jours post-fertilisation par lignée mutante.

L'inoculation de la souche CyHV-3 EGFP ou de la souche CyHV-3 Luc a été réalisée par micro-injection intra-cardiaque chez des larves à l'âge de 3 jours post-fertilisation. La progression de l'infection par le CyHV-3 EGFP a été suivie à l'aide de la microscopie à épifluorescence. Un système d'imagerie *in vivo* (IVIS) a été utilisé pour détecter la bioluminescence dans les larves infectées par le CyHV-3 Luc. Ayant émis l'hypothèse que le début de la clairance de l'infection pourrait prendre plus de temps à se produire chez les mutants PKR-KO ou PKZ-KO, nous avons également prolongé la période de surveillance de 4 jours post-infection (jpi) (dans les expériences précédentes) à 5 jpi.

Pour la quantification du génome virale par PCR TaqMan, l'ADN a été extrait des larves entières à l'aide du kit DNeasy Tissue (Qiagen), et environ 1 ng d'ADN génomique a été utilisé pour chaque réaction TaqMan PCR. Chaque échantillon a été analysé en triplicatas. Les copies du génome viral ont été normalisées par rapport aux copies du génome du poisson-zèbre (contrôle interne) en amplifiant également l'ADN génomique du poisson-zèbre comme décrit précédemment (Ji *et al.,* 2005). Les PCR du virus et du poisson-zèbre (contrôle interne) ont été réalisées dans des puits séparés, mais toujours sur les mêmes plaques. Des contrôles négatifs et positifs ont été inclus sur chaque plaque. Les données ont été exportées vers Excel à l'aide du logiciel CFX Manager v3.0 (Bio-Rad). Les niveaux relatifs de copies du génome viral ont été calculés à l'aide de la méthode $2-\triangle\triangle CT$ comme décrit précédemment (Livak and Schmittgen, 2001).

Les résultats obtenus en exploitant la microscopie à épifluorescence suggèrent que les mutants PKR-KO et PKZ-KO éliminent l'infection virale aussi efficacement que les larves WT. Il n'y avait pas non plus de différence entre les souches de poisson-zèbre en termes de nombre de larves infectées à chaque point dans le temps (Two-way ANOVA RM, *p*-value = 0,6440), tous les groupes présentant une diminution spectaculaire du nombre de poissons positifs à 5 jpi. La comparaison de la réplication virale par l'exploitation du système IVIS n'a révélé aucune différence significative entre les trois lignées de poisson-zèbre (test de Durbin, *p*-value = 0,6500). Les différences relatives de signaux entre les lignées WT et PKR-KO ont été irrégulières au cours de la période de surveillance, aucune tendance claire n'indiquant une différence entre les deux souches. En revanche, les niveaux de virus dans la souche PKZ-KO étaient systématiquement plus élevés que dans les souches WT et PKR-KO de 1 à 4 jpi, avec des différences significatives à 3 jpi. Cependant, les niveaux de virus dans les larves PKZ-KO étaient significativement plus bas que les autres souches à 5 jpi, indiquant une plus grande clairance, malgré des niveaux de virus plus élevés de 1 à 4 jpi.

Conscients de la redondance possible de la fonction « kinase eIF2α », qui pourrait avoir permis à PKZ de compenser l'absence de PKR dans le mutant PKR-KO, et *vice versa*, nous avons généré un troisième mutant, PKR-PKZ-KO, dépourvu des deux gènes *pkz* et *eif2ak2*. Cette souche a été incluse dans une expérience supplémentaire, exploitant la microscopie à fluorescence et la quantification relative du génome virale par qPCR. Cependant, de manière surprenante, les charges virales observées

chez les mutants PKR-PKZ-KO n'étaient pas significativement différentes de celles des poissons-zèbres WT. Dans l'ensemble, les résultats de ces deux expériences indiquent que 1) PKR et PKZ ne sont pas essentielles pour la clairance de l'infection par le CyHV-3 chez les larves de poisson-zèbre et 2) même à ce stade précoce du développement, le système immunitaire du poisson-zèbre présente une redondance suffisante pour permettre la clairance de l'infection par le CyHV-3 en l'absence de PKZ et/ou de PKR.

Si la mort cellulaire programmée joue également un rôle important dans la réponse à l'infection par le CyHV-3 dans ces souches mutantes de poisson-zèbre, comme l'ont suggéré des observations antérieures dans la lignée WT, ces processus devraient être médiés par d'autres mécanismes. Notamment, en plus de la mort cellulaire programmée médiée par PKR/PKZ stimulé par l'IFN (Su *et al.*, 2007; Thapa *et al.*, 2013; Zuo *et al.*, 2022), ces processus peuvent être stimulés par d'autres cytokines telles que FAS, TNFα et TRAIL (Berghe *et al.*, 2014; Land, 2018; Nailwal and Chan, 2019; Zhou *et al.*, 2017) (les orthologues de ces protéines chez le poisson-zèbre sont codés par les gènes *faslg*, *tnfa* et *tnfsf10*, respectivement). Comme l'IFN, ces cytokines agissent également en se liant à leurs récepteurs respectifs de la membrane cellulaire et les interactions entre ces protéines et diverses autres sont nécessaires pour déclencher l'apoptose et/ou la nécroptose. Dans notre modèle, aucune expression des gènes *faslg* et *tnfa* n'a été observée lors de notre étude transcriptomique (chapitre II de la section expérimentale) et l'expression du gène *tnfsf10*, bien qu'observée, n'a pas été positivement régulée en réponse à l'infection. Par conséquent, il est possible que, dans ce modèle, la régulation positive de ces trois cytokines soit également extrêmement brève, se produisant très tôt après l'infection et revenant rapidement aux niveaux de base par la suite. D'autres études seront nécessaires pour établir la cinétique d'expression de ces cytokines en réponse à l'infection par le CyHV-3 dans ce modèle d'hôte et pour déterminer dans quelle mesure, le cas échéant, elles contribuent à la mort cellulaire programmée et à la clairance de l'infection par le CyHV-3.

Dans les deux expériences, le mutant PKZ-KO présentait une charge virale plus élevée que les autres souches aux premiers stades de l'infection. Les niveaux plus élevés de CyHV-3 en l'absence de PKZ peuvent indiquer l'importance des récepteurs de reconnaissance de motifs moléculaires (PRRs) contenant un domaine Zα, tels que PKZ, dans la restriction de CyHV-3 aux premiers stades de l'infection. Ceci est cohérent avec une étude récente du laboratoire d'accueil dans laquelle sont fournies des preuves solides que la protéine CyHV-3 ORF112, qui contient également un domaine Zα, agit comme un antagoniste essentiel des PRRs détectant l'ARN au cours de l'infection par CyHV-3 (Diallo *et al.*, 2023). Cependant, l'absence de PKZ conduit à une clairance virale plus importante à 5 jpi par rapport aux lignées mutantes possédant PKZ. Nous émettons l'hypothèse qu'une réplication virale plus importante, de 1 à 4 jpi, peut avoir conduit à une réponse immunitaire innée plus importante, amorçant une clairance plus spectaculaire à 5 jours. Même si l'absence de PKZ n'empêche pas la clairance virale, les niveaux plus élevés de réplication virale à des stades plus précoces peuvent entraîner une augmentation des

dommages tissulaires par le biais d'une réponse inflammatoire potentielle, ce qui peut être préjudiciable à l'hôte. Par conséquent, il peut être important de disposer du répertoire complet des PRRs nécessaires à une restriction efficace de la réplication du CyHV-3 avant la clairance. De manière surprenante, nous n'avons pas observé de charges virales plus élevées à des stades précoces de l'infection chez le mutant PKR-PKZ-KO (également dépourvu de PKZ), qui présentait au contraire un phénotype similaire à celui des souches WT et PKR-KO en réponse au CyHV-3. Ces observations ouvrent plusieurs pistes intéressantes pour la poursuite des recherches, en particulier la caractérisation de la réponse immunitaire innée chez les mutants de poisson-zèbre dépourvus de ces PRRs importants et l'impact possible de la phosphorylation réduite de l'eIF2α sur la mort cellulaire programmée, s'il y en a une, en réponse à l'infection par le CyHV-3 dans ce modèle.

Cette étude représente le premier rapport sur la génération et l'utilisation de mutants PKR et/ou PKZ-KO de poisson-zèbre, qui constitueront des sujets utiles pour une caractérisation plus poussée et l'étude d'autres virus dans des modèles de poisson-zèbre. Étant donné l'importance de PKR, et potentiellement de PKZ, dans les réponses immunitaires innées et dans de nombreux autres processus cellulaires, ainsi que l'utilisation répandue du poisson-zèbre comme organisme modèle, les mutants KO générés dans cette étude pourront intéresser de nombreux autres chercheurs dans un domaine plus large. Ainsi, le sperme correspondant à ces mutants sera déposé au *European Zebrafish Resource Centre* (EZRC) pour faciliter sa distribution. Il est intéressant de noter que le CyHV-3 peut représenter un modèle idéal pour l'étude de la clairance virale par le système immunitaire inné chez cet hôte important et largement étudié. Cela ouvre de nombreuses voies de futures recherches afin de déterminer quels éléments de la réponse immunitaire sont essentiels pour ce processus. Dans ce contexte, la génération de nouveaux mutants KO, guidée par les données transcriptomiques que nous avons précédemment générées, pourrait conduire au développement de souches de poisson-zèbre plus permissives à ces virus économiquement importants, qui pourraient elles-mêmes être utilisées comme de précieux outils de recherche à l'avenir.

Experimental section

3rd study:

Disruption of PKR and/or PKZ does not impair the clearance of Cyprinid Herpesvirus 3 infection in zebrafish larvae

In Susceptibility and permissivity of zebrafish (*Danio rerio*) larvae to cypriniviruses

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Abstract

In fish cells, viral infections cause the induction of many fish interferon-stimulated genes (ISGs), including the interferon-inducible double−stranded RNA−dependent protein kinase (PKR) and the Z−DNA-dependent protein kinase (PKZ). Our previous *in vitro* and *in vivo* experiments (first chapter) indicated that Cyprinid herpesvirus 3 (CyHV−3) infection was quickly cleared in zebrafish models via programmed cell death. This was supported by the transcriptomic analysis from infected larvae (second chapter), which also supported a potentially important role for the two eIF2α kinases PKR and a related gene encoding PKZ in this process. In this study, the impact of PKR and PKZ on CyHV−3 clearance was investigated using PKR−KO, PKZ−KO, and PKR−PKZ−KO zebrafish mutants developed through CRISPR/Cas9 genome editing. By monitoring the amount of CyHV-3 replication over time using fluorescence microscopy, *in vivo* bioluminescence imaging and relative quantification of qPCR data, we showed that CRISPR/Cas9 knockout of zebrafish PKR and PKZ surprisingly had no impact on CyHV−3 clearance in larvae. Thus, we concluded that PKR and PKZ do not play an essential role in the response against CyHV−3 in this biological model and that the zebrafish immune system exhibits enough redundancy to mount a sufficient response in the absence of these genes, even at this early developmental stage.

Keywords: cyprinid herpesvirus 3; alloherpesvirus; cyprinivirus; zebrafish; PKR; PKZ; CRISPR/Cas9; innate immunity

1. Introduction

Similar to their mammalian counterparts, fish interferons (IFNs) play crucial roles in the antiviral response, inducing the expression of hundreds of IFN−stimulated genes (ISGs) such as ADAR1, ISG15, Mx1, PKR or viperin [1] which act to counteract the replication, assembly, and release of viruses [2]. For instance, the induction of the expression of *viperin* by the IFN contributes to the antiviral response in the zebrafish (*Danio rerio*), a model organism widely used to study the function of genes implicated in the innate immune response [3]. Another important ISG in vertebrates is the double−stranded RNA-dependent protein kinase (PKR), also referred to as the eukaryotic translation initiation factor 2α (eIF2α) kinase 2 (EIF2AK2), which plays a broad role in several anti-viral innate immune responses as outlined in the previous study (second chapter). Similar to mammals, fish PKR consists of a N−terminal dsRNA-binding domain and a C−terminal kinase domain, with a number of N−terminal dsRNA binding motifs (dsRBMs) varying from one to three [4]. Using poly(I:C)−sepharose pull−down assays, the dsRNA binding capacity of the N−terminal dsRBMs has been confirmed for several fish PKRs [5–7]. This is important, as increased levels of cytosolic dsRNA often accumulate during viral infection, and the activation of PKR in response to infection can often be triggered by the binding of dsRNA to dsRBMs. This results in the dimerization and autophosphorylation of PKR [8]. Once activated, PKR contributes to the inhibition of protein translation and restriction of viral replication through the phosphorylation of eIF2α, the activation of apoptosis, as well as the signal transduction of the proinflammatory response and the innate immune signaling pathways [9]. It is also potentially implicated in the regulation of cell growth, proliferation, and differentiation [10,11]. Indeed, the ability of fish PKRs to catalyze the phosphorylation of eIF2α has already been demonstrated [5,6], with carp PKR eIF2α phosphorylation leading to apoptosis [12].Furthermore, overexpression of zebrafish PKR protein in yeast cells was demonstrated to phosphorylate yeast eIF2α. Taken together, these observations indicate the conservation of PKR function across distantly related eukaryotes [4].

In addition to PKR, some teleost species including cyprinids, salmonids, clupeids (herrings) and osteoglossomorphs [13], also encode a paralogue of PKR, known as Z−DNA-dependent protein kinase (PKZ). Initially found in cultured *Carassius auratus* blastulae (CAB) cells [14], zebrafish (*Danio rerio*) [15] and Atlantic salmon (*Salmo salar*) [16], PKZ is a unique eIF2α protein kinase that contains a C−terminal kinase domain and two N−terminal Zalpha (Zα) domains, which are able to bind to Z−DNA/RNA [17]. Phylogenetic analysis revealed that fish PKR kinase domains are more closely related to fish PKZ kinase domains than to mammalian PKRs [4], suggesting that the *pkr* and *pkz* genes are paralogous and are derived from an ancestral kinase gene that was duplicated following divergence from the tetrapod lineage. Given its features, and similarity to PKR, PKZ may also play a similar role in innate immune response to viral infection through the phosphorylation of eIF2α. For example, PKZ from various fish species have been demonstrated to phosphorylate eIF2α when overexpressed, with some inducing apoptosis through this process [4,7,18,19]. Also, like PKR, evidence indicates that it also

acts as a cytosolic nucleic acid sensor via its nucleic acid binding domains, with its subsequent activation initiating an innate immune response [20]. In fish genomes, PKR and PKZ are tandemly arranged in a head−to−tail orientation [4,7], which is thought to be crucial for similar transcriptional activation following immunostimulation. As a consequence, and unsurprisingly, several studies have demonstrated the co−up−regulation of these kinases by viral infections and IFN stimuli [4,7,14–16,18,21–24], including ours as outlined in the previous study (second chapter). Although PKR and PKZ are simultaneously induced upon viral infections, they form homodimers, as opposed to heterodimers, to phosphorylate eIF2α in an independent manner, suggesting that they play cooperative roles in the antiviral response. For instance, it was reported that the overexpression of either Grass carp PKR or PKZ resulted in the inhibition of grass carp reovirus (GCRV) and that the overexpression of both kinases improved antiviral activity [7]. Collectively, these observations indicate that PKZ might act as an antiviral effector with similarities but also complementarities to PKR.

Interestingly, the presence of this PRR in cyprinids may have driven the acquisition and evolution of a Zα containing protein encoded by ORF112 of cyprinid herpesvirus 3 (CyHV−3). In theory, the CyHV-3 ORF112 protein may act to outcompete the binding of PKZ to Z-DNA or RNA during infection, thus supporting a potential importance of PKZ in the detection of viral Z-DNA/RNA in fish cells during infection [25].

We demonstrated in the first chapter of the experimental section that CyHV−3 induced an abortive infection in zebrafish larvae following inoculation by pericardial microinjection, in a process that is preceded by an apoptosis-like death of infected cells. Subsequent characterization of the zebrafish immune response to CyHV−3 by transcriptomic analysis (second chapter) indicated that interferon−stimulated genes, in particular those encoding nucleic acid sensors and potential mediators of programmed cell death, were the most up−regulated in response to infection. These included the genes encoding zebrafish PKR and PKZ, suggesting that they may play an important role in the response to CyHV−3 infection, and its subsequent clearance. Consistent with our observations, others have reported the up−regulation of Gibel carp (*Carassius auratus gibelio*) *pkr* and *pkz* genes in head kidney in response to Cyprinid herpesvirus 2 (CyHV−2) infection [26]. The authors hypothesized that when the head kidney is infected, the p53 signaling pathway is activated through the up−regulation of PKR and PKZ, promoting apoptosis and enabling the host to resist to the viral infection. Collectively these observations provide evidence for the existence of an antiviral pathway involving regulated cell death that is mediated by these protein kinases in fish. Indeed, on this note, it has been demonstrated that the permissivity of fish cells to viral infection can be increased when either or both of these kinases are knocked-down [7].

With this in mind, in this study, we investigated the impact of PKR and PKZ on the clearance of CyHV−3 by zebrafish cells by generating PKR−KO, PKZ−KO, and PKR−PKZ−KO zebrafish mutants

through the CRISPR/Cas9 technology. Results from fluorescence microscopy, *in vivo* bioluminescence imaging and relative quantification of qPCR data revealed that the absence of either or both kinases in mutant zebrafish strains did not impact CyHV−3 clearance in the larvae. These observations indicate that even at this early developmental stage, there is sufficient redundancy within the zebrafish innate immune response to facilitate successful clearance in the absence of these key genes. Thus, PKR and PKZ do not represent essential components of the antiviral response against CyHV−3 in this model. Notably, this is the first report of CRISPR/Cas9 generated PKR−KO and/or PKZ−KO zebrafish mutants.

2. Material and methods

2.1 Zebrafish larvae maintenance

Wild−type (WT, +/+) AB strain adult zebrafish (*Danio rerio*) were obtained by natural spawning and maintained at 27 °C, on a 14/10 h light/dark cycle. They were housed in the GIGA Zebrafish facility in Liège (Belgium) according to animal research guidelines and with the approval of the local ethical commission for animal care and use. Larvae were obtained by pairwise mating of adults in mating cages and maintained in petri dishes with standard embryo medium (E3) and incubated at 25 °C prior to use in experiments.

2.2 Mutant zebrafish experiments

2.2.1. Generation of mutant zebrafish strains using CRISPR/Cas9

The *mut eif2ak2* (*pkr*)^{ulg025}, *mut pkz*^{ulg027}, and *mut eif2ak2* (*pkr*) *L15−1* knockout (KO) zebrafish lines, hereafter referred to as the PKR−KO, PKZ−KO and PKR−PKZ−KO mutant strains, were generated by CRISPR/Cas9 technology as described previously [27–29]. The nls−zCas9−nls mRNA was synthesized by transcription of the plasmid pT3TS−nCas9n (Addgene #46757). First, WT strain AB zebrafish were used to generate the mutant strains PKR−KO and PKZ−KO (Figure 1a). To generate the PKR−KO, PKZ-KO, and the PKR−PKZ−KO mutant strains, CHOPCHOP [30] software was used to design two single−guide RNAs (sgRNA) GAGCACTCACAGTGATGAACCGG and CCACCGTGAACAGGCATCT (PAM motifs are underlined) to target exon 2 of the WT *eif2ak2 (*or *pkr)* gene (NCBI/Entrez/GenBank Gene ID: 100001092) and exon 1 of the WT *pkz* gene (NCBI/Entrez/GenBank Gene ID: 503703), respectively (Figure 1a). sgRNAs were generated by *in vitro* transcription from oligonucleotide templates using the MEGAscript T7 transcription kit (Ambion) as described previously [31]. The DNA templates were prepared by annealing and filling two oligonucleotides containing the T7 promoter sequence and the target sequences as previously described [29]. One−cell stage zebrafish embryos were injected with approximately 1 nL of a solution containing 50 ng sgRNA and 300 ng nls−zCas9−nls mRNA. The efficiency of mutagenesis was checked by genotyping using heteroduplex migration assays after amplification of targeted genomic sequences. Founder embryos (F0 generation) carrying a germline mutation in *eif2ak2* or *pkz* were raised to adulthood and outcrossed with WT fish to generate heterozygous F1 fish. Fish harbouring frameshift

mutations were kept and used to raise F2 homozygous stable knockout lines. Subsequently, PKZ−KO mutant strain zebrafish were then used to generate the double KO PKR−PKZ−KO mutant strain by repeating the process used to generate the PKR−KO mutant strain (Figure 1a). These mutations all resulted in genes producing truncated proteins and were verified by PCR (Figure 1b).

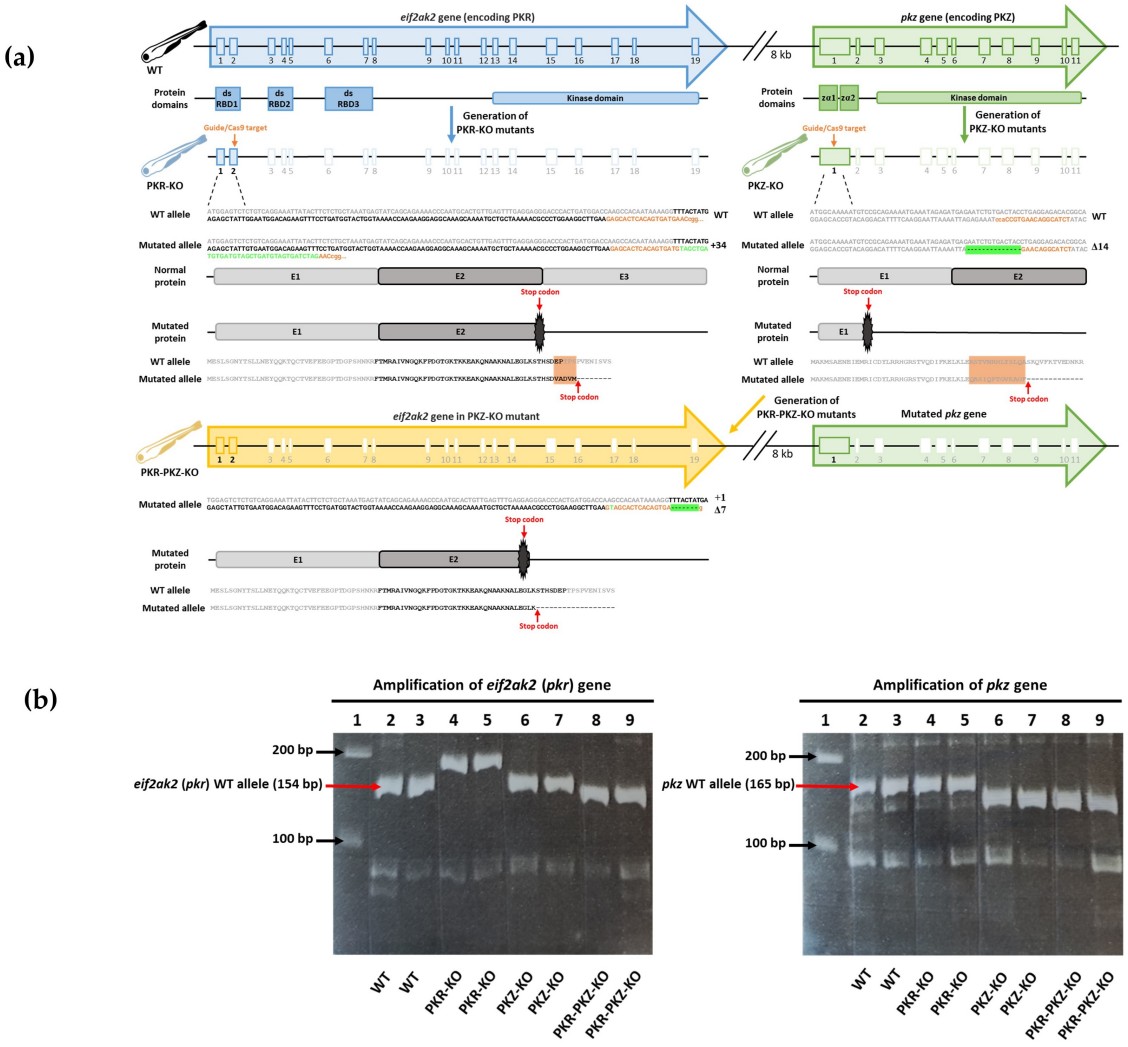

Figure 1. Generation and verification of CRISPR−Cas9 *eif2ak2* (*pkr*) and *pkz* mutations in zebrafish. (**a**) Structure of zebrafish *eif2ak2* (*pkr*) and *pkz* genes and proteins. The protein domains including double stranded RNA−binding domains (dsRB), Z−DNA/RNA binding domains (zα) and kinase domains are aligned to the corresponding exons. The CRISPR/Cas9 gene editing targets were exon 2 in zebrafish *eif2ak2* gene and exon 1 in zebrafish *pkz* gene; sgRNA target sequences are also displayed (orange, PAM lower case). The CRISPR/Cas9−induced changes in the WT *eif2ak2* gene (34−base insertion) to generate PKR−KO, and WT *pkz* gene (14−base deletion) to generate the PKZ−KO mutant strains are displayed. After the generation of the PKZ−KO mutant strain, the WT *eif2ak2* gene in this strain was also mutated, resulting in the PKR−PKZ−KO mutant strain (displayed below). The mutated *eif2ak2* gene in the PKR−PKZ−KO strain exhibits a different mutation (7−base deletion with 1−base insertion) relative to the mutated *eif2ak2* gene in PKR−KO mutant. Inserted and deleted sequences are highlighted in green (deleted sequences are represented by "−"). (**b**) Results from genotyping of homozygous WT, PKR−KO, PKZ−KO, and PKR−PKZ−KO zebrafish groups. This involved PCR amplification of *eif2ak2* (*pkr*) and *pkz* genes, in each mutant group (left and right gel images, respectively, with expected sizes of WT alleles indicated). Each gel consists of the same layout: Lane 1: 1kb Molecular Marker,

Lanes 2–9 each represent a DNA extracted from single whole larva, Lanes 2–3: WT Larvae, Lanes 4–5: PKR−KO mutants, Lanes 6–7 PKZ−KO mutants, Lanes 8–9 PKR−PKZ−KO mutants. Mutant *eif2ak2* (*pkr*) alleles were detected in PKR−KO and PKR−PKZ−KO larvae exhibiting 188−bp and 148−bp amplicons, respectively (left gel). The mutant *pkz* allele was detected in in PKZ−KO and PKR−PKZ−KO larvae, both exhibiting 151−bp amplicons (right gel).

2.2.2. Genotyping of Zebrafish Mutant Lines

The genotyping of WT, PKR−KO, PKZ−KO, and PKR−PKZ−KO zebrafish was performed by polymerase chain reaction (PCR). In order to extract the DNA, two randomly selected zebrafish larvae (4 dpf) were euthanized per mutant line. Each larva was transferred to an Eppendorf tube containing 25 µL 50 mM NaOH, heated at 95 °C for 25 min, and cooled on ice for 10 min. Finally, 2.5 µL 1M Tris−HCl pH8.0 was added, and cellular debris was pelleted by brief centrifugation for 15 sec. DNA concentration was determined by measuring A260 (NanoDrop 2000, Thermo Scientific), and ~2.5 µL of the resulting lysate was used per standard PCR reaction with gene−specific primers (Table S1). PCR reactions consisted of 1× Thermopol buffer (New England Biolabs), 0.025 U/µL Taq Polymerase (New England Biolabs), 300 nM forward and reverse primers, and 60 nM dNTPs (total volume 25 µL). The cycling conditions were as follows: 95 °C for 2 min, 40 cycles of 45 s at 95 °C, 45 s at 60 °C, 20 s at 72 °C, and ending with 72 °C for 10 min.

2.2.3. Inoculation of larvae by microinjection

Borosilicate glass capillaries were loaded with 10 µL of medium containing virus suspensions (1.2 × 10^6 PFU/ml) and then connected to a FemtoJet microinjector (Eppendorf, Framingham, MA, USA) as described elsewhere [32]. After breaking the capillary tip, the pressure was adjusted to obtain droplets with a diameter of ~0.13 mm. Larvae (3 days postfertilization [dpf]) were anesthetized in a bath containing tricaine (0.2 mg/mL). The fish were positioned on a petri dish, and the surface of the dish was dried entirely in order to avoid drifting of the larvae during viral injections. In order to visualize the hearts of the larvae, the petri dish was placed under a binocular magnifier (LEICA MZ6) at 4x magnification and illuminated by an external light source (LEICA CLS 50X). The capillary was then manually inserted into the pericardial cavity and three pulses were performed to inject approximately 3 nL of virus suspension (infected fish) or 3 nL of PBS (mock−infected fish). After microinjection, the larvae were transferred into individual wells in a 24−well plate containing 1 mL E3 medium and incubated at 25 °C.

2.2.4. Epifluorescence microscopy

The progression of infection with a recombinant virus expressing enhanced (EGFP), referred to as the CyHV−3 EGFP strain, was monitored using epifluorescence microscopy. This facilitated longitudinal observation of the same larvae at multiple timepoints. Prior to observation, larvae were anesthetized in a bath of E3 medium containing tricaine (0.2 mg/ml) and methylcellulose (2% w/v) in order to avoid drifting of larvae. Imaging of larvae was performed using a Leica DM2000

epifluorescence microscope at 5x and 10x magnification. After imaging, larvae were immediately transferred back to their individual wells and returned to the incubator. After the final observation timepoint, larvae were euthanized using an overdose of tricaine in E3 media (400 mg/L).

2.2.5. *In vivo* bioluminescent imaging

An *in vivo* imaging (IVIS) system (IVIS Spectrum, PerkinElmer) was used to detect bioluminescence in larvae infected with a Luc2−expressing recombinant virus, referred to as CyHV−3 Luc, thus facilitating the monitoring and quantification of viral levels *in vivo*. At the time of imaging, larvae were anesthetized (as described for epifluorescence microscopy analysis), injected with ~3 nL of D−luciferin (15 mg/mL), and imaged 5 minutes (min) after injection. Images were acquired using the following settings: field of view A, small binning, automatic exposure time with a maximum of 1 min and a subject height of 0.30 cm. Unlike epifluorescence analysis, longitudinal monitoring of individual larvae was not possible due to the harmful effects of repeated D−luciferin injections in the same larvae. Relative bioluminescence intensities were analysed using Living Image software (v4.7.3). Regions of interest (ROIs) were drawn by manually outlining the larval body, and bioluminescence within the ROI was recorded in terms of mean radiance (photons/s/cm²/sr).

2.2.6. Quantification of Viral Genome by TaqMan PCR

Larvae were euthanized using an overdose of tricaine, transferred into RNAlater (Thermo Fisher) and stored at −20 °C. DNA was extracted from whole larvae with a DNeasy Tissue Kit (Qiagen), and approximately 1 ng genomic DNA was used for each TaqMan PCR reaction. TaqMan qPCR reactions consisted of 1× IQ Supermix (Bio−Rad), 200 nM forward and reverse primers, and 400 nM TaqMan probe (total volume of 25 µL). The primers and probes used are provided in Table S1. The PCRs were performed using a CFX96 Touch real−time PCR detection system (Bio−Rad) with detection in the FAM channel. The cycling conditions were as follows: 95 °C for 15 min, 40 cycles of 15 s at 94 °C, and 60 s at 60 °C. Each sample was analysed in triplicate. Viral genome copies were normalized to zebrafish genome copies (internal control) by also amplifying zebrafish genomic DNA as described previously [33]. Viral and zebrafish (internal control) PCRs were performed in separate wells, but always on the same plates. Negative template controls and positive controls were included on each plate. Data were exported to Excel using CFX Manager v3.0 software (Bio−Rad). Relative levels of viral genome copies were calculated using the $2^{-\triangle\triangle CT}$ method as described previously [34].

2.2.7. Ethics statement

The experiments performed in the present study did not require a bioethical permit as they involved the use of larvae before implementation of feeding. However, all experiments were designed and conducted in accord with the 3R rules and other bioethics standards.

2.3 Statistical analysis

Each dataset was first tested for normality using the Shapiro–Wilk test, which was conducted as a stand−alone test or as part of a two−way ANOVA analysis of residuals implemented in GraphPad Prism (v8.0.1). The omnibus tests used were dependent on the outcome of the Shapiro−Wilk tests. For datasets exhibiting normal distribution, One−way ANOVA, Two−way ANOVA, or Two−way repeated measures (RM) ANOVA were used and implemented in GraphPad Prism. For datasets not exhibiting normal distribution, the Durbin test was used (PMCMR package v4.4 [35]), implemented in R (v4.2.0) [36]. The variables of interest relating to each of these tests and their significance are described in the text. Post−hoc multiple comparisons between groups of interest were made using either the Sidak test (two groups) or the Tukey test (more than two groups) implemented in Graphpad Prism (in conjunction with ANOVA tests), for data exhibiting normal distribution. Multiple comparisons were made using Dunn's pairwise test (FSA package v0.9.3 [37]) with Benjamini−Hochberg *p*−value adjustment done using the p.adjust function in R (in conjunction with the Durbin Test), for datasets not exhibiting normal distribution. For the purposes of visual clarity, only significant results from post−hoc multiple comparisons are indicated in each corresponding figure. The results of multiple comparisons tests are represented using the following symbols, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3. Results and Discussion

In vitro and *in vivo* experiments performed in this study indicated that CyHV−3 infection was rapidly cleared in zebrafish models via programmed cell death. This was supported by the transcriptomic analysis from infected larvae, which also supported a potentially important role for the eIF2α kinases PKR and PKZ in this process. Based on this evidence, we tested the impact of these eIF2α kinases on CyHV−3 clearance using CRISPR/Cas9 generated PKR−KO and PKZ−KO zebrafish mutants (Figure 1). Mutant and WT zebrafish larvae were first infected with CyHV−3 EGFP by microinjection as per earlier experiments. As we hypothesized that the onset of infection clearance may take longer to occur in eIF2α kinase KO mutants, we also extended the monitoring period from 4 dpi (in earlier experiments) to 5 days postinfection (dpi). Epifluorescence microscopy suggested that PKR−KO and PKZ−KO mutants cleared viral infection as efficiently as WT larvae (Figure 2a). There was also no difference between the zebrafish strains in terms of the numbers of infected larvae at each timepoint (Two−way RM ANOVA, *p*−value = 0.6440), with all groups exhibiting a dramatic decrease in the number of positive fish at 5 dpi (Figure 2b).

Next, WT, PKR−KO, and PKZ−KO zebrafish strains were infected with CyHV−3 Luc as before, allowing viral replication to be compared between strains (Figure 2c). This revealed no significant difference in viral signal between the three zebrafish strains (Durbin Test, *p*−value = 0.6500). Relative differences in signals between the WT and PKR−KO strains were inconsistent over the monitoring period, with no clear trends to indicate a difference between the two strains. In contrast, virus levels in

the PKZ−KO strain were consistently higher than both WT and PKR−KO strains from 1–4 dpi, with significant differences at 3 dpi. However, viral levels in PKZ−KO larvae were significantly lower than other strains by 5 dpi (Figure 2c), indicating greater clearance, despite higher viral levels from 1–4 dpi.

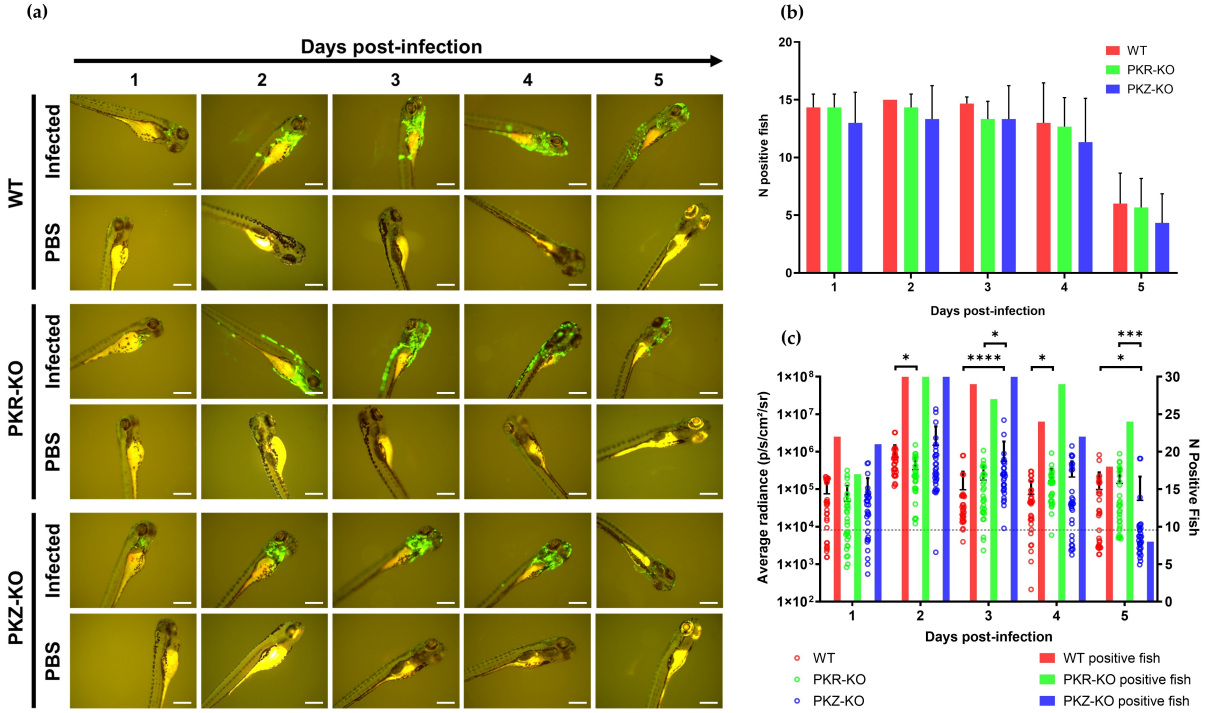

Figure 2. Replication of CyHV−3 in different zebrafish strains. (**a**) Epifluorescence microscopy images representative of larvae inoculated by microinjection with either CyHV−3 EGFP or mock−inoculated with PBS according to time postinfection (longitudinal observation of the same larvae over all timepoints). For all strains infection clearance commenced from 4–5 dpi. Scale bars = 500 µm. (**b**) Numbers of infected larvae among zebrafish strains inoculated with CyHV−3 EGFP (n = 15). Data represents mean ± standard errors from 3 independent experiments (longitudinal observation of the same larvae over all timepoints). (**c**) IVIS analysis measuring Luc2 expression in different zebrafish strains microinjected with CyHV−3 Luc (n = 30). The data points represent the mean radiance per larvae according to time postinfection with mean ± standard error represented for each group at each timepoint. The discontinuous line represents the cut−off for positivity and the mean + 3 × SD of the values obtained for mock−infected larvae. The number of positive larvae at each timepoint is represented by bars. ** $p < 0.01$; * $p < 0.05$.

Cognisant of the possible redundancy in eIF2α kinase function (described earlier), which may have allowed PKZ to compensate for the absence of PKR in the PKR−KO mutant, and *vice versa*, we generated a third mutant, PKR−PKZ−KO, lacking both *pkz* and *eif2ak2* genes (Figure 1). This strain was included in an additional experiment, like the one presented in Figure 2 (Figure 3). However, surprisingly, the viral loads observed in the PKR−PKZ−KO mutants were not significantly different from the WT strain. Taken together, the results from these two experiments indicate that 1) PKR and PKZ are not essential for clearance of CyHV−3 infection in zebrafish larvae, and 2) even at this early developmental stage, the zebrafish immune system exhibits sufficient redundancy to enable clearance of CyHV−3 infection in the absence of PKZ and/or PKR.

If programmed cell death also features heavily in the response to CyHV−3 infection in these mutant zebrafish strains, as earlier observations in the WT strain suggested these processes would need to be mediated via other mechanisms. Notably, in addition to IFN−stimulated PKR/PKZ−mediated programmed cell death [38–40], these processes can be stimulated by other cytokines such as FAS, TNFα, and TRAIL [41–44] (the zebrafish orthologs for these proteins are encoded by the *faslg*, *tnfa*, and *tnfsf10* genes, respectively). Like IFN, these cytokines also operate by binding to their respective cell membrane receptors and downstream interactions between these and various other proteins are required to initiate apoptosis and/or necroptosis. Notably, genes encoding zebrafish orthologs of most of the proteins involved in these processes are also up−regulated in response to infection at 2 dpi, indicating some potential redundancy in terms of the programmed cell death response. However, no expression from the *faslg* and *tnfa* genes was observed in our model. While we did observe expression for *tnfsf10*, it was not up−regulated in response to infection. Therefore, similar to what we have hypothesized regarding IFN expression kinetics, it is possible that with this model, the up−regulation of these three cytokines is also extremely brief, occurring very early after infection with a rapid return to basal levels after. As with IFN, further investigation will be needed to establish the expression kinetics of these cytokines in response to CyHV−3 infection in this host model, and to what extent, if any, they contribute to programmed cell death and clearance of CyHV−3 infection.

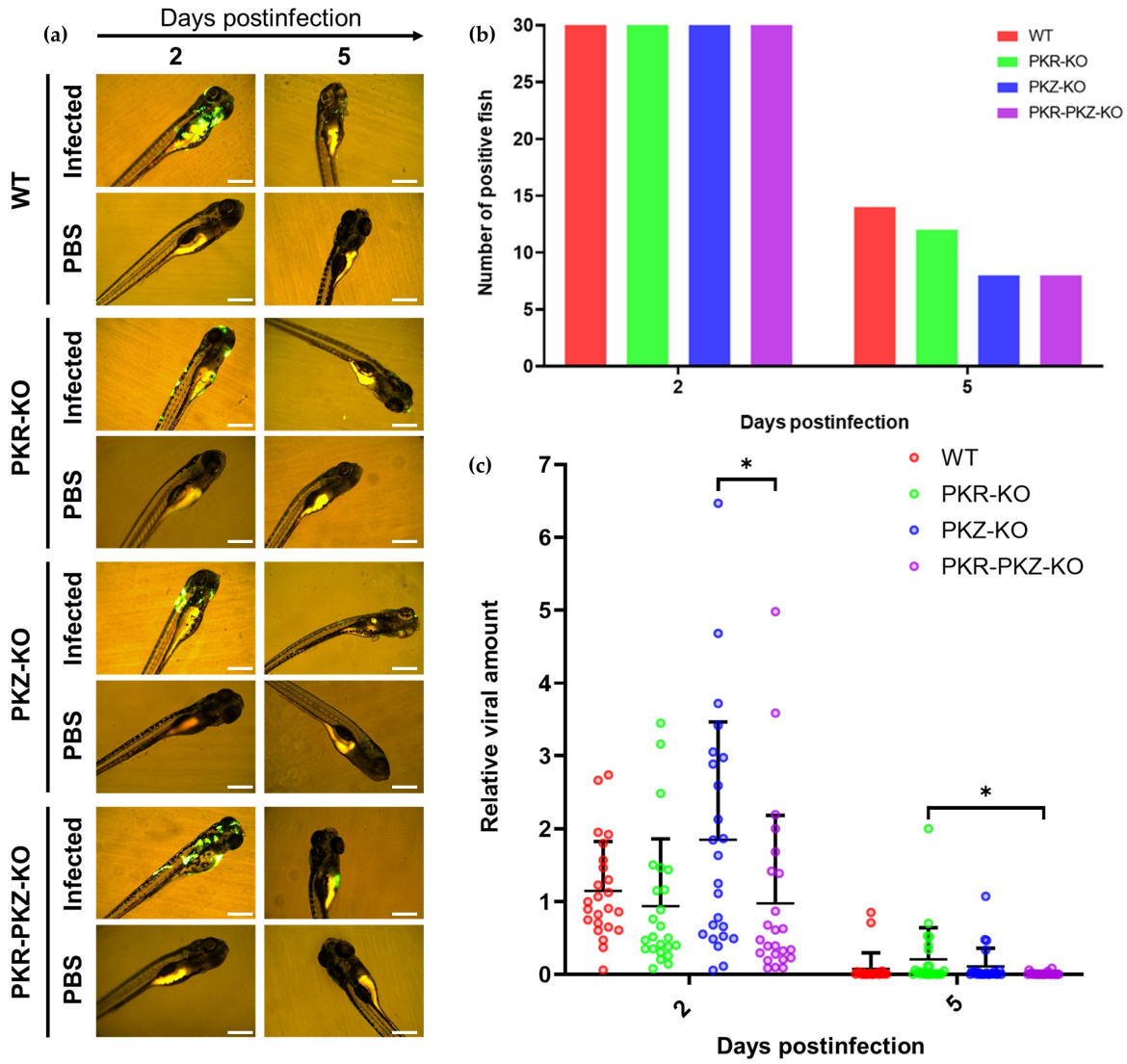

Figure 3. Comparison of CyHV−3 replication and clearance between PKR−PKZ−KO and other zebrafish strains. (**a**) Epifluorescence microscopy images representative of larvae among different zebrafish strains either inoculated with CyHV−3 EGFP or mock−inoculated (PBS) according to time post-infection. Scale bars = 500 µm. (**b**) Numbers of infected individuals among zebrafish larvae strains inoculated with CyHV−3 EGFP (n=30). (**c**) Relative quantification of CyHV-3 load (genome copies) in zebrafish strains (normalized to zebrafish genome copies). Data represent mean ± standard errors of 24 larvae per zebrafish strains per time point. This revealed a significant difference between zebrafish strains in terms of viral load (Durbin test p=0.0168), with post-hoc multiple comparisons (Dunn's test) indicating differences at 2 dpi and 5 dpi. * $p < 0.05$.

In both experiments (Figures 2 and 3), the PKZ−KO mutant exhibited a higher viral load than other strains at the earlier stages of infection. The higher levels of CyHV−3 in the absence of PKZ may indicate the importance of host Zα domain−containing PRRs such as PKZ, in restricting CyHV−3 in the early stages of infection. This is consistent with our recent study where we provide strong evidence that the CyHV−3 ORF112 protein, which also contains a Zα domain, acts as an essential antagonist of RNA PRRs during CyHV−3 infection [45]. However, the absence of PKZ still leads to more dramatic viral clearance at 5 dpi relative to PKZ−competent strains (Figure 2c). We hypothesize that higher viral

replication, from 1–4 dpi, may have ultimately led to an increased innate immune response, priming a more dramatic clearance at 5 dpi. Even if the absence of PKZ does not prevent viral clearance, the higher levels of viral replication in earlier stages, may lead to increased tissue damage via potential inflammatory response, which may ultimately be harmful to the host. Therefore, having the complete repertoire of PRRs necessary for effective restriction of CyHV−3 replication prior to clearance may still be important. Surprisingly, we do not observe higher viral loads at earlier stages of infection in the PKR−PKZ−KO mutant (also lacking PKZ), which instead exhibited a similar phenotype to WT and PKR−KO strains in response to CyHV−3 (Figure 3). These observations open up several interesting avenues for further investigation, in particular the characterization of the innate immune response in zebrafish mutants lacking these important PRRs and the possible impact of reduced eIF2α phosphorylation on programmed cell death, if any, in response to CyHV−3 infection in this model.

4. Conclusions

The aim of this study was to explore the effect of CRISPR/Cas9 knockout of the zebrafish gene encoding PKR and a related gene encoding PKZ on the potential modulation of zebrafish permissivity to CyHV-3 infection. Results revealed that their absence in mutant zebrafish strains does not impact CyHV−3 clearance (Figure 2). This may be due to sufficient levels of redundancy within the zebrafish innate immune response processes, even at this early developmental stage. This is the first report of the generation and use of PKR and/or PKZ KO zebrafish mutants (Figures 1, 2 and 3), and they will represent useful subjects for further characterization and the study of other viruses in zebrafish models. Given the importance of PKR, and potentially PKZ, in the innate immune responses and in many more cellular processes, and the widespread use of zebrafish as a model organism, the KO mutants generated in this study will be of interest to many more researchers in the wider field. Thus, sperm corresponding to these mutants will be deposited in the European Zebrafish Resource Centre (EZRC) for ease of distribution elsewhere. Interestingly, CyHV−3 may represent an ideal model to utilize in the study of viral clearance by the innate immune system in this important and widely studied host. This opens many interesting avenues for future investigation to determine what elements of the immune response are essential for this process. As part of this, the generation of new KO mutants, guided by the transcriptomic data we previously generated, may lead to the development of zebrafish strains that are more permissive to these economically important viruses, which may themselves be utilized as valuable research tools in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v15030768/s1, Table S1 Primers used in this study.

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Discussion - Perspectives

Cypriniviruses induce diseases only within their natural host species, implying constraints linked to host cell susceptibility (defined as the ability to support viral entry) and cell permissivity (defined as the ability to sustain viral replication and the transmission of viable viral progeny to new cells, although the former might occur independently of the latter). Interestingly, *in vitro* studies have shown that cyprinid herpesvirus 2 (CyHV-2) and cyprinid herpesvirus 3 (CyHV-3) are able to infect, albeit with reduced efficiency, cells derived from non-natural hosts (Boutier *et al*., 2015a; Thangaraj *et al*., 2021). Similarly, cell lines derived from a cyprinid fish, the common carp (*Cyprinus carpio*), such as colored carp fin (CCF), common carp fin (CF), and colored carp testis (CCT) cells, were shown to be permissive to anguillid herpesvirus 1 (AngHV-1) (Ueno *et al*., 1992, 1996). Moreover, recent research indicates that CyHV-3 can infect zebrafish (*Danio rerio*) cell lines and induces an abortive infection in adult zebrafish (Rakus *et al*., 2019).

The main objectives of this thesis were to compare AngHV-1, CyHV-2, and CyHV-3 in terms of their ability to infect zebrafish models, both *in vitro* and *in vivo*, as well as to explore the zebrafish model's suitability for investigating the pathogenesis of these cypriniviruses.

In the first chapter, we examined the susceptibility and permissivity of both the ZF4 cell line and zebrafish larvae to AngHV-1, CyHV-2, and CyHV-3 infections. Our results showed that zebrafish larvae did not exhibit susceptibility to these viruses through water immersion, yet infections were achievable through artificial infection models both *in vitro* (using cells derived from zebrafish embryos) and *in vivo* (via microinjection in larvae). However, these infections were short-lived, as rapid viral clearance occurred alongside apoptosis-like death of the infected cells.

The second and third chapters were focused on gaining insights into the antiviral response to cyprinivirus infection in the zebrafish larvae. In order to investigate the potential involvement of programmed cell death and determine the extent of viral gene transcription in CyHV-3-infected zebrafish larvae, the second chapter of this thesis involved the first study of the zebrafish response to CyHV-3 using RNA sequencing (RNAseq) analysis. This transcriptomic analysis showed upregulation of interferon-stimulated genes (ISGs), especially those coding for nucleic acid sensors and mediators of programmed cell death, and of uncharacterized non-coding RNA genes and retrotransposons. Notably, protein kinase R (PKR) and a related protein kinase featuring Z-DNA binding domains (PKZ) were also upregulated in response to CyHV-3 infection and may play a crucial role in both programmed cell death responses and nucleic acid sensing pathways mediated by pattern recognition receptors (PRRs).

In the last chapter, we investigated the impact of these kinases on CyHV-3 clearance by using zebrafish mutants lacking PKR and/or PKZ, generated through CRISPR/Cas9 technology. The results showed that the knockout of PKR and PKZ did not hinder the elimination of CyHV-3 infections in zebrafish larvae. These observations suggest that even in the early developmental stage, the immune

system demonstrates adequate redundancy to mount an effective response in the absence of these genes. This study marks the initial report on the generation and utilization of PKR and/or PKZ knockout zebrafish mutants.

Each of these aspects of the study are discussed in more detail in the following sections.

Zebrafish (ZF4) cells exhibit low susceptibility to all *Cyprinviruses* tested, and moderate permissivity to CyHV-2 and CyHV-3.

Recent *in vitro* studies demonstrated that CyHV-3 was able to replicate in two zebrafish cell lines, a fibroblast cell line derived from amputated caudal fins of an adult zebrafish (SJD.1) and fibroblast cell line derived from 1-day-old zebrafish embryos (ZF4) (Rakus *et al*., 2019). While no CPE was observed in the ZF4 cell line during infection by CyHV-3, the presence of viral transcripts (although at a low level) was detected for CyHV-3 in ZF4 and SJD.1 cells collected at 1 day-post-infection (dpi) and 4 dpi.

At the beginning of this PhD project, the ability of AngHV-1 and CyHV-2 to replicate in zebrafish cells was not reported. Also, the fate of CyHV-3-infected zebrafish cells beyond 4 dpi was unknown. Thus, in the first chapter of this thesis, we first tested the susceptibility and permissivity of the ZF4 cell line to infection with AngHV-1, CyHV-2, and CyHV-3, using recombinant strains expressing green fluorescent proteins (GFP) as reporters and timelapse microscopy to track infected cells from 1 to 11 dpi.

Our results indicate that the ZF4 cell line shows low susceptibility to AngHV-1, CyHV-2 and CyHV-3 in high-MOI conditions, with the lowest number of initial infected cells being observed following inoculation with AngHV-1. This low susceptibility among ZF4 populations may occur for several reasons. Firstly, the entry of herpesviruses relies on several cellular receptors that mediate viral binding and entry (Spear and Longnecker, 2003). In ZF4 cells, which are derived from zebrafish embryos, we hypothesize that the necessary cell surface receptors may be expressed at low levels or not functional during the early developmental stages. This is similar to hypotheses arising from observations in previous studies with carp models of CyHV-3 infection, which indicated that cell surface receptors may be more readily utilized for viral entry at later developmental stages (Ronsmans *et al*., 2014). Secondly, the virus may be able to successfully enter ZF4 cells, however a lack of crucial cellular factors may prevent initiation of virus replication, or replication may only occur in a limited sub-population of cells. The exact reasons why ZF4 cells exhibit low susceptibility to all the cypriniviruses tested have not been evaluated in this project, but this could be very interesting to investigate through single cell sequencing analysis.

In addition to this restricted susceptibility of ZF4 monolayers to cyprinivirus infection, our results also demonstrated that ZF4 cells were not permissive to AngHV-1 and transiently permissive to CyHV-2 and CyHV-3 infection. In this regard, the similarities between CyHV-2 and CyHV-3 as well as their distinctions from AngHV-1 may stem from the closer phylogenetic relationship between CyHV-2 and CyHV-3 compared to either of them with AngHV-1 (Davison *et al*., 2013; Donohoe *et al*., 2021; Van Beurden *et al*., 2010; Waltzek *et al*., 2009). Moreover, considering their natural host species, CyHV-2 and CyHV-3, relative to AngHV-1, may possess inherent adaptations that make them better suited for growth in cells derived from cyprinid host species, such as zebrafish derived ZF-4 cells.

Moreover, while ZF4 cells were more permissive to CyHV-3 relative to CyHV-2, both viruses exhibited evidence of replication and/or transmission between cells, albeit inefficient. First, the transmission of the first generation of viral progeny to the second generation of infected cells yielded low numbers of newly infected cells. Second, the increase in the amount of newly infected cells was linear, not exponential, indicating a poor replication efficiency within infected cells and/or reduced transmission of progeny virus to new cells. Third, we observed isolated infected cells without plaque formation, which highlights the absence of transmission via cell-cell contact in this model and may reveal a high degree of variation in susceptibility to CyHV-2 and CyHV-3 within ZF4 monolayers or very strong or rapid innate responses in adjacent cells. Finally, an absence of syncytia formation was noted among ZF4 cells infected with CyHV-3, which could also play a role in the reduced transmission through cell-cell contact.

Our results also revealed that, following an infection peak observed at 6 dpi, both CyHV-2 and CyHV-3 were gradually cleared from ZF4 cells in a process preceded by what appears to be widespread programmed cell death within infected cells. Halting the replication cycle of CyHV-3, the induction of a programmed cell death response among infected cells before mature progeny is released might explain the absence of sustained productive infection, resulting in the inefficient transmission of viable progeny to new cells. As a result, the propagation of CyHV-3 *in vitro* might be limited enough to enable its elimination through the innate immune response alone.

Regarding this cell death response, timelapse microscopy revealed that death events were mostly morphologically in line with apoptosis, however another distinct type of cell death, somewhat morphologically consistent with necrosis, was also observed. The discrimination between apoptosis, passive necrosis and programmed necrosis (necroptosis) was not possible, based on our morphological observation alone, thus further investigation is needed to definitively identify these types of cell death, notably by using cell staining, flow cytometry, or through the evaluation of the effects of apoptotic inhibitors (Z-VAD-FMK or other poly-caspase inhibitors) and necroptotic inhibitors (necrostatin) on the morphology and kinetics of cell death (Costigan *et al*., 2023).

Overall, our results indicate that the transient permissiveness to CyHV-2 and CyHV-3 exhibited by ZF4 cells is characterized by inefficient viral replication and transmission that fails to overcome the innate immune response among both infected and non-infected cells. Several evidence support this hypothesis: ZF4 cells are able to establish an innate immune response when infected with fish viruses, as demonstrated by studies including the infectious pancreatic necrosis disease virus (IPNV) (Wang *et al*., 2011), nervous necrosis virus (NNV) (Chen *et al*., 2015), snakehead vesiculovirus (SHVV) (Wang *et al*., 2015), or the spring viraemia of carp virus (SVCV) (Espín-Palazón *et al*., 2016; Lu *et al.*, 2023; Zhou *et al*., 2021; Zou *et al*., 2016). Interestingly, Wang *et al.* (2015) demonstrated that initial increases in the amount of SHVV mRNAs and cRNA in ZF4 cells at 3 hours post-infection (hpi) were followed by a dramatic decline in virus levels at 6 hpi. While happening on different timescale, this report parallels our observations in ZF4 cells infected with CyHV-2 and CyHV-3. Furthermore, the abortive infection of SHVV was concurrent with the significant upregulation of the expression of *ifnphi1* (referred to as *IFN* by Wang *et al*., 2015), *mxa* (referred to as *Mx* by Wang *et al*., 2015), *rigi* and *mda5,* revealing an activation of the antiviral response. Regarding cypriniviruses, a very recent study showed that the antiviral genes *ifnphi1* and *irf7* were upregulated in ZF4 cells following CyHV-2 infection, which provides evidence that these cells can exhibit antiviral defense mechanisms, and more broadly, an innate immune response against CyHV-2 (Lu *et al*., 2023). In contrast, although Rakus *et al*. (2019) revealed that CyHV-3 could indeed replicate in ZF4 cells, the induction of an antiviral response was not reported. Indeed, the authors noted that the expression of the two antiviral genes *vig-1* and *mxa*, was not up-regulated in CyHV-3-infected ZF4 cells when analyzed at 1 and 4 dpi. This finding was suggested to be related to the low CyHV-3 transcription levels observed in this cell line, as well as to their relatively modest responsiveness to viral infections. This observation might also be explained by the potential ability of CyHV-3 to inhibit IFN mechanisms, as evidenced by Adamek *et al*. (2012) who reported that a very low type I IFN response was observed in fibroblastic cells derived from the brain of common carp (CCB) exhibiting active CyHV-3 transcription. Interestingly, the induction of the antiviral response in ZF4 cells infected by CyHV-2 and its lack thereof in ZF4 cells inoculated with CyHV-3 might contribute to the differences between the two cypriniviruses in this model and might explain why CyHV-3 exhibits slightly greater fitness *in vitro* relative to CyHV-2.

Although there was an absence of sustained permissivity to CyHV-2 and CyHV-3, leading to abortive infections, we reasoned that these *in vitro* experiments with ZF4 cells offered some evidence that the same recombinant viruses could be employed for further investigation of transient cyprinivirus infection and clearance in an *in vivo* model which may represent a valuable virus-host model, the zebrafish larvae.

Zebrafish larvae are susceptible to CyHV-2 and CyHV-3 but the cyprinivirus infections are rapidly cleared

Zebrafish larvae are increasingly used to study viruses infecting economically important fish species such as SVCV, affecting the common carp (López-Muñoz *et al*., 2010; Varela *et al.*, 2014a). Very recently, Rakus *et al*. (2019) showed that in Tübingen zebrafish larvae infected by CyHV-3 by immersion, no viral RNA and no significant upregulation of *vig-1* and *mxa* expression was detected at 48 hpi. However, the susceptibility of zebrafish larvae to CyHV-3 through another route of infection, microinjection, was not investigated.

Also, when starting this doctoral project, there was no report of the evaluation of the zebrafish larvae as a model to study AngHV-1 and CyHV-2. Thus, as part of the first chapter of this thesis, we also investigated the susceptibility and permissivity of zebrafish larvae to infection with AngHV-1, CyHV-2, and CyHV-3, using two different modes of inoculation, i.e., immersion and microinjection, and exploiting the same recombinant strains previously used in our *in vitro* studies. In addition, we took advantage of the *in vivo* bioluminescent imaging system (IVIS) paired with recombinant strains expressing luciferase to provide insights into cyprinivirus pathogenesis in this *in vivo* model.

Firstly, these experiments revealed that zebrafish larvae are not susceptible to AngHV-1, CyHV-2, and CyHV-3 via immersion. This is consistent with previous findings showing an absence of viral replication in Tübingen zebrafish larvae inoculated with CyHV-3 through bath infection (Rakus *et al*., 2019). This observation may reflect that these viruses are not able to enter zebrafish larvae through natural routes *in vivo*. This is consistent with observations elsewhere indicating that not all viruses that can replicate in zebrafish can infect this model via immersion (Varela *et al*., 2017). In contrast to our observations, the survival rate of zebrafish larvae was demonstrated to be altered following CyHV-2 infections via immersion, reaching less than 50% at 21 hpi (Cai *et al*., 2022). However, this finding might be explained by the viral dose selected by Cai *et al*., but it is not possible to meaningfully compare it to our study, as the viral dose was not expressed in PFU/mL. Furthermore, as the CyHV-2 strain used in this study is not specified, the differences in survival rate might also originate from the use of a different strain than the one selected in our study, i.e., CyHV-2 YC-01. As part of this, the susceptibility to CyHV-2 infections has been suggested to depend on the combination of host and virus strains used. The heterogeneity in virulence among different strains of CyHV-2 and using different goldfish (*Carassius auratus*) strains has already been demonstrated through *in vivo* experiments, with significant differences in mortality reported when using immersion infection methods (He *et al*., 2023; Ito *et al*., 2013). It is also important to note that, strikingly, Cai *et al*. do not describe survival curves for mock-infected larvae. Thus, it is impossible to determine to what extent the survival rate in infected groups differed from the background mortality rate. Given this striking omission, it is difficult to make valid

comparisons between our study and the study from Cai *et al.*, and these results should be interpreted with this caveat in mind.

While we observed that zebrafish larvae exhibit no susceptibility to the cyprinivirus tested when using a natural inoculation route, in contrast we demonstrated that zebrafish larvae are susceptible to CyHV-2 and CyHV-3, but not AngHV-1, via micro-injection, which represents an artificial inoculation route. Thus, zebrafish larvae are not susceptible to AngHV-1 via both modes of inoculation tested in this study. This difference in susceptibility between AngHV-1 and the two cyprinid herpesviruses is in line with our previous observations *in vitro* and indicates that CyHV-2 and CyHV-3 are better adapted to replicating in the zebrafish cells relative to AngHV-1, with the reasons for this outlined earlier (in the first section of this discussion).

Although zebrafish larvae exhibit greater susceptibility to CyHV-2 and CyHV-3, relative to AngHV-1, these infections are quickly eliminated, associated with an absence of mortality, following a peak of infection at 2 dpi. As per observation *in vitro*, we conclude that inoculations with CyHV-2 and CyHV-3 *in vivo* lead to abortive infections. These observations are largely consistent with a previous study involving adult zebrafish infected with CyHV-3 using intraperitoneal injection (Rakus *et al*., 2019), where CyHV-3 mRNA and *vig-1* and *mxa* gene expression were only detected at 1 and 3 dpi (out of the 14-day-long course of the experiment), indicating that the virus was rapidly cleared. Elsewhere, and similar to our results, the amount of CHIKV-infected cells following inoculation of zebrafish larvae was shown to reach a peak by 1-2 dpi, succeeded by the clearance of CHIKV from most organs by 4 dpi (Palha *et al.*, 2013).

Many factors can explain that zebrafish may be resistant to CyHV-2 and CyHV-3 infection. We reasoned that zebrafish may inherently possess strong defenses against viruses closely related to CyHV-2 and CyHV-3, that could have been present in their natural environment throughout their evolutionary history. With regard to this, few viruses are demonstrated to infect zebrafish in natural conditions (Balla *et al*., 2020; Bermúdez *et al*., 2018; Binesh, 2013; Shen and Steiner, 2004), and the natural infection of zebrafish by alloherpesviruses has not been reported. Thus, further investigation is needed to explore whether any currently unidentified member belonging to the family *Alloherpesviridae* can naturally infect zebrafish as a primary host, as it would pave the way for new research opportunities, using a valuable homologous herpesvirus-host model in zebrafish. In addition to evolutionary history or adaptation to existing alloherpesviruses of zebrafish, our experimental conditions may not have been adequate to induce mortality. Indeed, the absence of mortality in our study may also be linked to either the site of inoculation or the viral dose, both being known to influence the severity of viral infections in zebrafish larvae. For instance, Lama *et al*. (2022) demonstrated that depending on the route of infection, the survival rate at 10 dpi could vary between 56 and 94% in zebrafish larvae infected with NNV. Furthermore, mortality of larvae infected with Human herpesvirus 1 (HHV-1, also known as herpesvirus

simplex 1) was revealed to occur in a dose-dependent manner (Ge *et al*., 2015). Finally, as previously discussed, some researchers have noted that strains within each cyprinivirus species clad can show natural heterogeneity regarding replication *in vitro* and/or *in vivo* (Davison *et al*., 2013; Donohoe *et al*., 2021; He *et al*., 2023; Ito *et al*., 2013). Thus, further investigation is needed to establish whether the use of alternative cyprinivirus strains with our zebrafish models would also result in the same outcome.

Similar to our observations *in vitro*, our results reveal that zebrafish larvae show more susceptibility (and possibly more permissivity) to CyHV-3 relative to CyHV-2. As CyHV-3 exhibits greater fitness in the zebrafish models used in this project and as it is considered as the archetype fish alloherpesvirus (Boutier *et al.*, 2015a), we reasoned that this virus represents a more valuable model to use in further investigations of alloherpesvirus infections in zebrafish larvae. Using this CyHV-3 model, light-sheet microscopy revealed that a substantial upsurge in apoptosis-like death among infected cells preceded the elimination of CyHV-3 in zebrafish larvae, which was consistent with observations *in vitro*, discussed earlier. As the identification of the type of cell death observed in response to CyHV-3 was not confirmed via staining, further research is needed to confirm if apoptosis plays a major role in the clearance of CyHV-3 in zebrafish larvae. Elsewhere, and consistent with our observations, apoptosis-like cell death was also observed in CHIKV-infected zebrafish larvae, infected cells presenting features such as membrane blebbing and cellular fragmentation (Palha *et al.*, 2013), indicating that this may be an effective and more general mechanism that is used by zebrafish larvae to clear viral infection, and thus not necessarily unique to our model.

Collectively the experiments performed up to this point in the study indicated that CyHV-3 exhibited higher levels of viral replication and slower clearance in zebrafish larvae relative to CyHV-2. Thus, we reasoned that the CyHV-3 model represented a much more ideal model to use to study viral clearance and determine what elements of the zebrafish innate immune response are essential for this process. Consequently, we chose to work exclusively with the CyHV-3 model to investigate these aspects further in the remaining chapters of this thesis.

Transcriptomic analysis of CyHV-3 infected zebrafish larvae indicate upregulation of ISGs, but not IFN, as well as a potentially important role for two eIF2α kinases in programmed cell death

In the second chapter of this thesis, we studied the zebrafish response to CyHV-3 using RNA sequencing (RNAseq) analysis to determine the extent of viral gene transcription in CyHV-3-infected zebrafish larvae and to investigate the potential involvement of the programmed cell death response. Analysis of our transcriptomic data confirmed the transcription of all 155 known CyHV-3 protein coding genes, indicating that zebrafish cells may indeed be permissive to CyHV-3 *in vivo*. In addition, viral RNA expression kinetics i.e., viral RNA levels reaching a peak at 2 dpi then decreasing considerably at

4 dpi, was consistent with the viral levels observed in the first chapter of this thesis. Furthermore, this transcriptomic analysis led to the following observations:

First, there was an increase in the expression of many ISGs in response to CyHV-3 infection, and this was most pronounced at 2 dpi. Notably, many upregulated genes were responsible for nucleic acid sensing and mediation of programmed cell death. Overall, our observations strongly support the importance of the innate immune response alone in eliminating viral infection and highlight the considerable degree of adaptation cypriniviruses have undergone to ensure successful circulation within their natural hosts. While several ISGs were induced in CyHV-3-infected zebrafish larvae in the present study, we did not observe expression of the two IFN genes, i.e., *ifnphi1* and *ifnphi3* that are active during the stage of development used in this study (Aggad *et al*., 2009). We hypothesize that, RT-qPCR may be more sensitive than RNA-Seq to detect IFN gene transcription under certain circumstances, as *ifnphi1* and *ifnphi3* were successfully detected via this method in WT AB zebrafish larvae infected with SVCV and CHIKV (Aggad *et al*., 2009; Palha *et al*., 2013). As ISG induction was observed in the present study, we suggest that a possible upregulation of IFN genes may have occurred very early after CyHV-3 inoculation, returning quickly to basal levels before our first sampling point. Notably, this induction of ISGs without IFN detection mirrors findings from previous research involving WT zebrafish infected with NNV (Lama *et al.*, 2022). Before this investigation, the response of zebrafish larvae to CyHV-3 challenge regarding type I IFN gene expression was unknown.

Second, uncharacterized non-coding RNA genes and retrotransposons were prominently upregulated in response to CyHV-3 infection, and some were previously not known as contributing to the immune response. It would be interesting to further investigate the importance of these non-coding transcripts during the immune response, especially as one of them was >3000-fold upregulated and the 6th most upregulated gene at 2 dpi. Further research may also be needed to evaluate whether reactivation or upregulation of retrotransposons in response to infection might be advantageous in our model, as their cytoplasmic RNA and/or DNA genome intermediates could potentially act as ligands for PRRs (Chernyavskaya *et al*., 2017).

Finally, our results further indicated a potentially crucial involvement of the eIF2α kinases PKR and PKZ in the programmed cell death response. Among its many roles, PKR activates apoptosis and facilitates signal transduction in both the proinflammatory response and innate immune signaling pathways (García *et al.*, 2006). Notably, carp PKR eIF2α phosphorylation was reported to result in apoptosis (Xu *et al*., 2018). Like PKR, PKZ can phosphorylate eIF2α when overexpressed (Liu *et al*., 2011; Liu *et al*., 2013; Rothenburg *et al*., 2008; Wu *et al*., 2016). Notably, grass carp (*Ctenopharyngodon Idella*) PKZ (CiPkz) was demonstrated to facilitate cell apoptosis through the phosphorylation of eIF2α in CIK cells (Wu *et al*., 2016). Due to their potentially overlapping roles, we reasoned that generating

PKR-KO and PKZ-KO zebrafish mutants would be interesting to explore the role of these eIF2α kinases in clearing CyHV-3 in zebrafish larvae.

The absence of PKR and/or PKZ does not impair the clearance of CyHV-3 infections in zebrafish larvae

Both *in vitro* and *in vivo* experiments performed in the first chapter of this thesis revealed that CyHV-3 infection was rapidly eliminated in ZF4 cells and zebrafish larvae through an apoptosis-like cell death response. The transcriptomic analysis from infected larvae performed in the second chapter further supported this contribution of programmed cell death in the CyHV-3 clearance, as well as a potentially crucial role of PKR and PKZ in this process. Interestingly, a recent study has demonstrated that the *pkr* and *pkz* genes were upregulated in head kidney of Gibel carp (*Carassius auratus gibelio*) in response to CyHV-2 infection (Liu *et al.*, 2018b), the authors suggesting that the up-regulation of eIF2α kinases PKR and PKZ may activate the p53 signaling pathway, which promotes the apoptotic response and improves the host resistance to the viral infection.

In the third chapter of this thesis, we investigated the impact of these eIF2α kinases on the clearance of CyHV-3 by generating PKR-KO, PKZ-KO, and PKR-PKZ-KO zebrafish mutants via CRISPR/Cas9 technology. Our results revealed that the absence of PKR and PKZ in mutant zebrafish strains does not alter the elimination of CyHV-3, indicating that these two eIF2α kinases are not essential for clearance of CyHV-3 infection in zebrafish larvae. One hypothesis that could explain this observation is that the zebrafish innate immune system shows enough levels of redundancy, even at this early life stage, to enable the clearance of CyHV-3 infection when PKR and/or PKZ are knocked-down. This is the first report of the generation and use of PKR and/or PKZ KO zebrafish mutants. Considering the importance of PKR, and possibly PKZ, in innate immune responses and various cellular functions, coupled with the extensive utilization of zebrafish as a model organism, the knockout mutants produced in this study will be valuable subjects for additional characterization and for investigating other viruses in zebrafish models, and are likely to be of interest to researchers across the broader scientific community.

If programmed cell death is also prominently involved in the reaction to CyHV-3 infection in these mutant zebrafish strains, as previous findings in the WT strain indicated, these mechanisms would have to be facilitated through other pathways. Alongside the IFN-stimulated programmed cell death mediated by PKR and PKZ (Su *et al*., 2007; Thapa *et al*., 2013; Zuo *et al*., 2022), this response can be stimulated by other cytokines such as FAS, TNFα, and TRAIL (Berghe *et al*., 2014; Land, 2018; Nailwal and Chan, 2019; Zhou *et al*., 2017) (the zebrafish orthologs for these proteins are encoded by the *faslg*, *tnfa*, and *tnfsf10* genes, respectively). Similar to IFN, these cytokines function by binding to specific cell membrane receptors, and subsequent interactions with various proteins are necessary to trigger apoptosis and/or necroptosis. Importantly, the transcriptomic analysis performed in the second chapter of this

thesis revealed that genes coding for zebrafish orthologs of several proteins involved in these pathways are upregulated in response to infection at 2 dpi, suggesting a degree of redundancy in the programmed cell death response. However, as no expression of *faslg* and *tnfa* was observed in our model and as expression of *tnfsf10* was not upregulated to infection, we hypothesize that, similar to IFN expression kinetics, the upregulation of these cytokines may be brief, taking place very early after infection and going back to basal levels before our first sample point. Thus, establishing the expression kinetics of *faslg*, *tnfa*, and *tnfsf10* genes in response to CyHV-3 in zebrafish larvae as well as investigating the potential role they may play in programmed cell death and CyHV-3 infection, could be interesting avenues for future research.

The use of zebrafish larvae to study cyprinivirus

The zebrafish has been demonstrated to be a relevant and useful model to study viral pathogenicity, vertebrate host immune responses, and viral host-interactions, due to its high fecundity, short generation time, transparent larval stage highly suited to *in vivo* imaging (Levraud *et al*., 2014), and well-developed immune system (Traver *et al*., 2003; Trede *et al*., 2004). Regarding cyprinivirus infection, the zebrafish presents several advantages to investigate the pathogenesis of CyHV-2 and CyHV-3:

Firstly, the evolutionary relatedness between the zebrafish and the common carp, goldfish, crucian carp (*Carassius Carassius*) and Gibel carp suggests that zebrafish may possess similar genetic and physiological factors involved in viral susceptibility and immune response. Notably, our observations in the first chapter of this thesis supports this hypothesis, as zebrafish models, *in vitro* and *in vivo*, were susceptible and transiently permissive to CyHV-2 and CyHV-3, but not to AngHV-1, which infects members of the family *Anguillidae*. Secondly, in contrast to fish species like carp or goldfish, the zebrafish can be easily and rapidly genetically manipulated. The availability of a well-annotated zebrafish reference genome (Howe *et al*., 2013) and large range of recombinant and mutant zebrafish lines (Kettleborough *et al*., 2013) enables investigations into gene function or contribution to various biological processes, including response to cyprinivirus infection.

In this study, these key advantages of the zebrafish model were exploited to study the clearance of CyHV-3 in zebrafish larvae. The generation of new KO mutants using the CRISPR-Cas9 technology, guided by the transcriptomic data performed in the second chapter of this thesis, may lead to the development of zebrafish strains more permissive to these economically important viruses, which may be used as valuable research tools in the future. In addition, our lab has also used this CyHV-3 zebrafish model to compare the transcriptome of carp and zebrafish larvae in response to CyHV-3 infection via microinjection, representing a comparison of the response to infection between the CyHV-3 natural host and our new zebrafish model (unpublished data). The analysis revealed a lack of proteasome complex up-regulation in carp, relative to zebrafish. The proteasome is a protein-destroying complex critical for

cellular functions, including apoptosis (Adams, 2003; Bard *et al*., 2018; Tanaka, 2009) and for the function of the immune response (Saeki and Tanaka, 2008; Strehl *et al*., 2005). This observation may highlight the potential importance of proteasome-mediated responses in controlling CyHV-3. In this way, this approach involving comparative immunology between closely related models could provide valuable insights into what fundamental virus-host interactions are crucial for viral clearance.

When using zebrafish larvae to investigate cyprinivirus pathogenesis, one potential drawback to be considered is the highly artificial nature of the inoculation process. In the first chapter of this thesis, we demonstrated that zebrafish larvae are susceptible to CyHV-2 and CyHV-3, via micro-injection, but not through immersion, which is more natural mode of infection. While the micro-injection allows for precise control over the timing and location of infection, it deviates significantly from the natural route of viral transmission observed in the wild. CyHV-2 and CyHV-3 infections occur primarily via waterborne transmission, with the skin acting as the primary portal of entry of these two cyprinivirus into their respective natural hosts (Costes *et al*., 2009; He *et al*., 2023). Thus, our model does not mimic natural conditions of infection as microinjection bypasses crucial aspects of the host-virus interaction, including the initial entry and dissemination of the virus within the host organism. Despite this and while extrapolation of observations from zebrafish studies to carp or goldfish should be done with caution, this study demonstrates how the experimental advantages of zebrafish provide valuable opportunities to gain insights into the molecular mechanisms underlying CyHV-2 and CyHV-3 infections in cyprinid fish, ultimately contributing to the further understanding of cyprinivirus pathogenesis and interactions with their cyprinid hosts.

To conclude

Today, the zebrafish has emerged as a crucial model organism in virology, notably for the study of virus affecting economically important fish species. Recent studies revealed that CyHV-3 could replicate in zebrafish cells and induce an abortive infection in adult zebrafish. In addition to CyHV-3, the cyprinivirus CyHV-2 and AngHV-1 also affect economically important fish species. However, investigations regarding their pathogenesis remain scarce. In the beginning of this thesis, the ambition was to evaluate the potential use of zebrafish models *in vitro* and *in vivo* to study these three economically important cyprinivirus. Using innovative tools such as IVIS, CRISPR-Cas9, and RNA-Seq, this study unveiled intriguing insights into the antiviral response of zebrafish to cyprinivirus infection and highlighted the importance of the innate immune response in eradicating viral infections. Notably, the second part of this thesis led to the observation of uncharacterized genes previously unreported as contributing to the response to infection or immune stimulation. Overall, this work provides ample subject matter for future projects involving comparisons between CyHV-3-zebrafish and CyHV-3-carp models, the use of PKR and PKR-KO zebrafish mutants, as well as the investigation of the importance of non-coding RNA genes and retrotransposons during the immune response.

As the story of this thesis began with the observation of fluorescent green cells, i.e., zebrafish cells infected with recombinants strains of CyHV-2 and CyHV-3 expressing GFP, I would like to illustrate the sense of awe and curiosity that drew me to this fascinating project with a very adequate quotation of the American writer Francis Scott Fitzgerald: “And as I sat there brooding on the old, unknown world, I thought of Gatsby’s wonder when he first picked out the green light at the end of Daisy’s dock.” The first observation of these fluorescent green cells drew me through a journey of discovery into the promising and expending fields of fish virology and immunology. An integral aspect of this journey was the opportunity to collaborate and learn from remarkable individuals in a top-tier laboratory setting. Ultimately, this thesis served as a catalyst for personal growth, fostering teamwork and collaboration skills, critical thinking and problem-solving aptitudes, and development and adaptability to new techniques. Finally, navigating the challenges and setbacks occurring during this thesis taught me to be resilient and to persevere in the pursuit of knowledge.

“*Discovery should come as an adventure rather than as the result of a logical process of thought. Sharp, prolonged thinking is necessary that we may keep on the chosen road but it does not itself necessarily lead to discovery. The investigator must be ready and on the spot when the light comes from whatever direction*.” Theobald Smith

Conclusion générale

Par son approche mêlant l'exploitation de souches recombinantes de virus exprimant des gènes rapporteurs de la réplication virale, du système d'imagerie par bioluminescence *in vivo* (IVIS), de la microscopie à épifluorescence à long terme (*timelapse*) et de la technologie RNA-Seq, ce travail de thèse a tout d'abord permis de proposer l'utilisation du CyHV-3 pour étudier les infections causées par les alloherpèsvirus chez cet organisme modèle largement étudié qu'est le poisson-zèbre. En effet, les larves de ce poisson présentent une plus grande sensibilité (et peut-être une plus grande permissivité) au CyHV-3 qu'aux CyHV-2 et AngHV-1, compte tenu des niveaux viraux plus élevés et d'une clairance plus lente. Ceci indique que le CyHV-3, considéré comme l'archétype de l'alloherpèsvirus de poisson, associé à la larve de poisson-zèbre, peuvent représenter un modèle virus-hôte précieux à utiliser, notamment dans la poursuite des investigations sur les modalités de la clairance virale stimulée par le système immunitaire. Par ailleurs, ce travail de thèse a contribué à décrire pour la première fois la sensibilité des cellules ZF4 à l'AngHV-1 et au CyHV-2 ainsi que la sensibilité et la permissivité des larves de poisson-zèbre au CyHV-2 et au CyHV-3.

Par ailleurs, nos essais *in vitro* et *in vivo* révèlent que les cellules ZF4 et les larves de poisson-zèbre expriment une faible sensibilité et une permissivité réduite, voire nulle, à l'infection par le CyHV-2 et le CyHV-3, conduisant à une infection avortée des cellules, caractérisée par une clairance des virus précédée par ce qui ressemble morphologiquement à une réponse de mort cellulaire programmée généralisée parmi les populations de cellules infectées. Les raisons exactes de la faible susceptibilité de ces modèles aux *Cyprinivirus* n'ont pas été investiguées lors de ce projet, cependant les recherches concernant ce phénomène pourraient être approfondies en exploitant la technologie de séquençage de l'ARN à cellule unique (« *single cell* »). De même, de futures recherches sont nécessaires afin d'identifier définitivement les types de morts cellulaires observées lors de nos expériences, notamment en utilisant des colorations/marquages cellulaires (« *cell staining* ») en association notamment avec les techniques de microscopie à épifluorescence, de cytométrie en flux ou encore des inhibiteurs d'apoptose et de nécroptose.

Les expérimentations *in vivo* ont révélé que les larves de poisson-zèbre n'étaient sensibles au CyHV-2 et CyHV-3 que par l'inoculation par micro-injection, et non par immersion, cette seconde forme d'exposition étant considérée comme un mode d'infection plus naturel. Or, bien que ces observations soient cohérentes avec une récente étude de Rakus *et al.* (2019), d'autres récents travaux ont démontré qu'une réponse antivirale associée à de la mortalité pouvaient être induits chez des larves de poisson-zèbre infectées avec le CyHV-2 par immersion (Cai *et al.*, 2022; Ouyang *et al.*, 2023). Cette différence de résultats pourrait provenir de la dose virale, de la lignée de poisson-zèbre utilisée ou de la souche virale sélectionnée. Ainsi, l'utilisation de doses de virus plus élevées, de lignées différentes de poisson-

zèbre, ou de souches virales alternatives pourraient aboutir à des résultats différents, ce qui pourrait être intéressant à explorer à l'avenir. Il est cependant important de noter que le taux de survie des poissons-zèbres témoins n'est pas décrit dans les travaux de Cai *et al.* Il n'est donc pas possible de déterminer dans quelle mesure le taux de survie dans les groupes infectés diffère de celui des poissons-zèbres témoins.

L'étude transcriptomique exploitant la technologie RNA-Seq a révélé que de nombreux gènes stimulés par l'interféron (ISGs) étaient régulés positivement en réponse à l'infection par le CyHV-3 chez la larve de poisson-zèbre, malgré une absence de détection de l'expression des gènes codant pour les interférons *ifnphi1* et *ifnphi*3, présents généralement à ce stade de développement du poisson-zèbre. Avant cette étude, la façon dont les larves de poisson-zèbre réagissaient à l'infection par le CyHV-3 en termes d'expression des gènes codant pour les interférons de type I était inconnue. De plus, nous avons noté qu'un grand nombre des gènes les plus significativement régulés positivement en réponse à l'infection par le CyHV-3 chez les larves de poisson-zèbre n'avaient pas été caractérisés auparavant, et certains d'entre eux n'avaient jamais été signalés comme étant impliqués dans la réponse immunitaire. Il s'agit notamment de cinq transcrits non codants (dont l'un est régulé positivement au-delà de 3000 fois et constitue le 6ème gène le plus induit à 2 jours post-infection). Nous proposons de désigner provisoirement ces cinq transcrits sous le nom de "*Zebrafish Non-coding Infection Response Element*" 1-5 (ou ZNIRE 1-5). Cette observation devrait conduire à des recherches plus approfondies sur les rôles de ces gènes au cours de la réponse immunitaire. Nous avons également observé la régulation à la hausse de trois rétrotransposons. Il est possible que cette réactivation/régulation des rétrotransposons en réponse à l'infection soit bénéfique pour l'hôte. Leurs intermédiaires génomiques d'ARN cytoplasmique et/ou d'ADN peuvent potentiellement agir comme des ligands pour les PRR (Chernyavskaya *et al.*, 2017), renforçant ainsi la réponse immunitaire innée à l'infection virale. Ceci constitue une hypothèse intéressante pour une étude plus approfondie avec notre modèle.

Enfin, ce travail de thèse représente le premier rapport sur la génération et l'utilisation de mutants PKR et/ou PKZ-KO chez le poisson-zèbre. Étant donné l'importance de la protéine kinase PKR, et potentiellement de PKZ, dans les réponses immunitaires innées et dans de nombreux autres processus cellulaires, et compte tenu de l'utilisation répandue du poisson-zèbre comme organisme modèle, les mutants KO générés dans cette étude pourront être des modèles d'intérêt pour de nombreux autres chercheurs dans un domaine plus large. Ainsi, les spermatozoïdes correspondant à ces mutants seront déposés au *European Zebrafish Resource Center* (EZRC) afin d'en faciliter la distribution. Bien que l'analyse transcriptomique ait indiqué que ces deux protéines kinases étaient régulées positivement en réponse à l'infection et qu'elles pouvaient contribuer à la mort cellulaire et à la détection d'acides nucléiques par les PRRs, leur absence dans les souches mutantes de poisson-zèbre n'a pas révélé d'impact sur la clairance du CyHV-3. Cela peut être dû à des niveaux suffisants de redondance dans les

processus de réponse immunitaire innée du poisson-zèbre, même à ce stade précoce du développement. Cela ouvre de nombreuses voies intéressantes pour de futures recherches afin de déterminer quels éléments de la réponse immunitaire sont essentiels au phénomène de clairance virale observé chez la larve de poisson-zèbre. Dans ce contexte, la génération de nouveaux mutants KO, guidée par les données transcriptomiques générées dans cette étude, peut conduire au développement de souches de poisson-zèbre plus permissives à ces virus d'importance économique majeure.

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List of publications

a. **Streiff, C.**, He, B., Morvan, L., Zhang, H., Delrez, N., Fourrier, M., Manfroid, I., Suárez, N.M., Betoulle, S., Davison, A.J., Donohoe, O., Vanderplasschen, A. (2023). Susceptibility and Permissivity of Zebrafish (*Danio rerio*) Larvae to Cypriniviruses. *Viruses*, 15, 768. https://doi.org/10.3390/v15030768

b. He, B., Sridhar, A., **Streiff, C.**, Deketelaere, C., Zhang, H., Gao,Y., Hu, Y., Pirotte, S., Delrez, N., Davison, A.J., Donohoe, O., Vanderplasschen, A. (2023). *In Vivo* Imaging Sheds Light on the Susceptibility and Permissivity of *Carassius auratus* to Cyprinid Herpesvirus 2 According to Developmental Stage. *Viruses*, 15, 1746. https://doi.org/10.3390/v15081746

Sensibilité et permissivité des larves de poisson-zèbre (*Danio rerio*) aux cyprinivirus

L'Anguillid herpèsvirus 1 (AngHV-1), le Cyprinid Herpèsvirus (CyHV-2) et le Cyprinid Herpèsvirus 3 (CyHV-3) sont trois cyprinivirus d'importance économique qui provoquent des maladies uniquement chez leurs hôtes naturels. Cependant, les cellules provenant d'hôtes non naturels peuvent être infectées, même de manière inefficace, par des cyprinivirus. Récemment, il a été démontré que le CyHV-3 se répliquait dans des lignées cellulaires de poisson-zèbre (*Danio rerio*) et qu'il induisait une infection avortée après inoculation intrapéritonéale chez le poisson-zèbre adulte. Le poisson-zèbre est un organisme modèle de plus en plus utilisé en virologie. Contrairement aux hôtes naturels des cyprinivirus, ce cyprinidé peut être facilement et rapidement manipulé génétiquement, ce qui facilite les recherches sur la fonction des gènes ou leur contribution à divers processus biologiques. La présente thèse a contribué (i) à évaluer et à comparer l'AngHV-1, le CyHV-2 et le CyHV-3 en termes de capacité à infecter des modèles de poisson-zèbre à la fois *in vitro* et *in vivo* et (ii) à mieux comprendre la réponse antivirale à l'infection par les cyprinivirus chez les larves de poisson-zèbre. Les résultats ont révélé que des infections pouvaient être établies en utilisant des modèles d'infection artificiels *in vitro* (lignées cellulaires de poisson-zèbre) et *in vivo* (micro-injection de larves). Cependant, les infections étaient transitoires, avec une clairance virale rapide associée à une mort des cellules infectées morphologiquement compatible avec l'apoptose. L'analyse transcriptomique des larves infectées par le CyHV-3 a révélé une régulation positive des gènes stimulés par l'interféron, en particulier ceux qui codent pour les médiateurs de la mort cellulaire programmée. Il est à noter que des gènes d'ARN non codants non caractérisés et des rétrotransposons figuraient également parmi les gènes les plus régulés positivement. L'inactivation par CRISPR/Cas9 du gène du poisson-zèbre codant pour la protéine kinase R (PKR) et d'un gène apparenté codant pour une protéine kinase contenant des domaines de liaison à l'ADN Z (PKZ) n'a pas eu d'impact sur la clairance du CyHV-3 chez les larves. En conclusion, ce projet soutient fortement l'importance des interactions entre l'immunité innée et les virus dans l'adaptation des cyprinivirus à leurs hôtes naturels et souligne le potentiel du modèle CyHV-3-poisson-zèbre, par rapport au modèle CyHV-3-carpe, pour l'étude de ces interactions.

Anguillid herpèsvirus 1, Cyprinid herpèsvirus 2, Cyprinid herpèsvirus 3, Alloherpèsvirus, Cyprinivirus, Poisson-zèbre, PKR, PKZ, CRISPR/Cas9, Immunité innée.

Susceptibility and permissivity of zebrafish (*Danio rerio*) larvae to cypriniviruses

The anguillid herpesvirus 1 (AngHV-1), cyprinid herpesvirus 2 (CyHV-2) and cyprinid herpesvirus 3 (CyHV-3) are three economically important cypriniviruses causing diseases solely in their natural host species. However, cells originating from non-natural host species can be infected, even inefficiently, by cypriniviruses. Recently, CyHV-3 was shown to replicate in zebrafish (*Danio rerio*) cell lines and to induce an abortive infection after intraperitoneal inoculation of adult zebrafish. The zebrafish represents an increasingly important model organism in virology. In contrast to natural hosts of cypriniviruses, this cyprinid fish can be easily and rapidly genetically manipulated, thus facilitating investigations into gene function or contribution to various biological processes. The present thesis contributed (i) to evaluate and compare AngHV-1, CyHV-2, and CyHV-3 in terms of their ability to infect zebrafish models both *in vitro* and *in vivo* and (ii) to gain further understanding of the antiviral response to cyprinivirus infection in the zebrafish larvae. Results revealed that infections could be established using artificial infection models *in vitro* (zebrafish cell lines) and *in vivo* (microinjection of larvae). However, infections were transient, with rapid viral clearance associated with apoptosis-like death of infected cells. Transcriptomic analysis of CyHV-3-infected larvae revealed up-regulation of interferon-stimulated genes, in particular those encoding mediators of programmed cell death. It was notable that uncharacterized non-coding RNA genes and retrotransposons were also among those most up-regulated. CRISPR/Cas9 knockout of the zebrafish gene encoding protein kinase R (PKR) and a related gene encoding a protein kinase containing Z-DNA binding domains (PKZ) had no impact on CyHV-3 clearance in larvae. In conclusion, this project strongly supports the importance of innate immunity-virus interactions in the adaptation of cypriniviruses to their natural hosts and highlights the potential of the CyHV-3-zebrafish model, versus the CyHV-3-carp model, for study of these interactions.

Anguillid herpesvirus, Cyprinid herpesvirus 2, Cyprinid herpesvirus 3, Alloherpesvirus, Cyprinivirus, Zebrafish, PKR, PKZ, CRISPR/Cas9, Innate immunity

Discipline : Physiologie et biologie des organismes – Populations et interactions

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