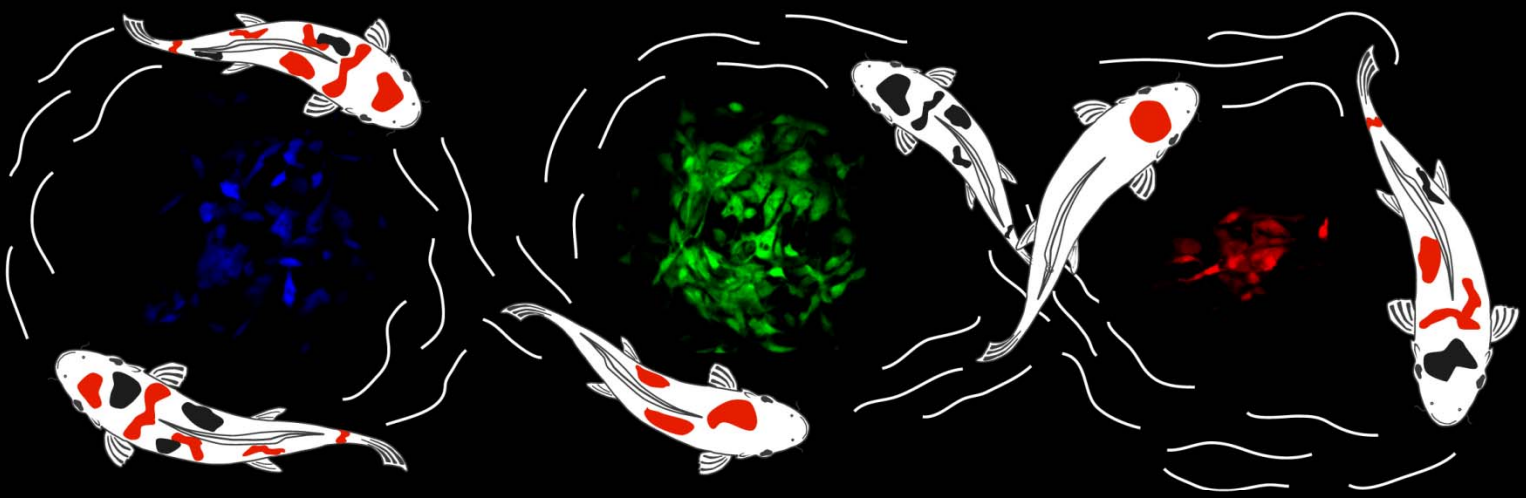
Académie Universitaire Wallonie - Europe
Université de Liège
Faculté de Médecine Vétérinaire
Département des Maladies Infectieuses et Parasitaires
Service d'Immunologie et de Vaccinologie

De la résistance mucosale des larves à la fièvre comportementale des alevins: voyage au sein des défenses immunitaires innées de la carpe commune à l'encontre de l'herpèsvirus cyprin 3

From mucosal resistance of larvae to behavioral fever of fingerlings: a journey in the innate immune defenses of common carp against cyprinid herpesvirus 3



Maygane RONSMANS

Thèse présentée en vue de l'obtention du grade
de Docteur en Sciences Vétérinaires

Année académique 2014-2015

Académie Universitaire Wallonie - Europe
Université de Liège
Faculté de Médecine Vétérinaire
Département des Maladies Infectieuses et Parasitaires
Service d'Immunologie et de Vaccinologie

De la résistance mucosale des larves à la fièvre comportementale des alevins: voyage au sein des défenses immunitaires innées de la carpe commune à l'encontre de l'herpèsvirus cyprin 3

From mucosal resistance of larvae to behavioral fever of fingerlings: a journey in the innate immune defenses of common carp against cyprinid herpesvirus 3

Promoteur : Prof. Alain Vanderplasschen

Maygane RONSMANS

Thèse présentée en vue de l'obtention du grade
de Docteur en Sciences Vétérinaires

Année académique 2014-2015

« Wisdom is to have dreams big enough not to lose sight when we pursue them. »

Oscar Wilde

Remerciements

Liège, le 28 août 2015

Au bout de ces cinq années de recherche au sein du laboratoire d'Immunologie-Vaccinologie, la défense de thèse apporte une certaine reconnaissance du travail réalisé, des efforts et parfois, des sacrifices consentis. Malgré la satisfaction personnelle que l'on ressent, la défense ne représente que le point final d'un parcours dont l'accomplissement a bien plus de valeur que sa finalité. Pendant ce périple que représente la thèse, j'ai rencontré mon lot d'embûches, entraînant de fréquentes remises en question et un moral oscillant tel des montagnes russes… Toutefois, réaliser cette thèse m'a surtout permis de côtoyer et travailler avec des gens de tous horizons, a renforcé mon assurance et la confiance en moi, mais elle m'a aussi apporté de belles amitiés… Il m'est enfin donné l'opportunité de remercier mes collègues, mes amis et ma famille pour toute l'aide, la présence et l'écoute qu'ils m'ont apportés et sans qui cette thèse n'aurait probablement jamais abouti.

Je souhaiterais tout d'abord adresser ma plus profonde reconnaissance à mon promoteur, le Professeur Alain Vanderplasschen. Alors que je me destinais à un autre avenir, il a su éveiller en moi la passion pour la recherche et m'a offert la chance de réaliser une thèse au sein de son laboratoire. A la fin de cette étape importante dans ma courte vie de chercheuse, je me remémore une phrase que tu m'as souvent répétée et qui me semble bien appropriée : « Le bien gagne toujours! ». Merci de m'avoir accordé une telle confiance, de m'avoir soutenue tout au long de ce travail et d'avoir conforté en moi le culte de la perfection.

Je remercie les membres de mon comité d'accompagnement, le Professeur Daniel Desmecht et le Professeur Fabrice Bureau pour leurs conseils et encouragements.

Cette thèse est également le fruit de plusieurs collaborations. Je souhaite ici adresser mes remerciements au Professeur Frédéric Farnir pour sa disponibilité et son aide dans les analyses statistiques, au Dr Michaël Vandecan pour son aide dans l'apprentissage du Femtojet, au Dr Fabien Ectors pour la préparation des pipettes effilées, à Mickaël Sarlet et Joëlle Piret pour leur accueil et la réalisation de nombreuses lames histologiques.

Je tiens à remercier Benjamin Dewals et Guillaume Fournier qui ont été les deux premiers à me guider lors de mes débuts dans la recherche. A mon arrivée, le travail en laboratoire m'était presque inconnu; ce sont leurs conseils avisés et les protocoles explicatifs détaillés qui m'ont aidée à me lancer dans cette aventure! Tout particulièrement, merci à Gui pour ta gentillesse, ta disponibilité, ta patience et ta compréhension. Tu es un collègue exceptionnel et partager mes premières années de recherche avec toi aura été un plaisir et un privilège! Merci aussi à Benja qui, sous ses airs sérieux et réservés, ne m'a jamais laissée tomber lorsqu'on se retrouvait les deux seuls « Travolta » sur la piste de danse!

De par mon sujet de recherche, j'ai eu le plaisir de faire partie de la « fish team », un groupe multiculturel et multilingue mais néanmoins très soudé, où règne entraide et bonne humeur. Il y a d'abord ceux qui sont partis vers d'autres horizons, Ping, thank you for your help during my beginnings and for nice discussions about China; Robert, the best Flemish colleague, teasing each other was so funny; and Anca, with who I shared the desk for a while. Ensuite, il y a ceux qui ont partagé mon aventure pour des périodes plus ou moins longues, Catherine, ma nouvelle voisine de bureau avec qui j'apprécie papoter, Michelle, Léa, Joanna, Natacha et les petits nouveaux, Yuan et Lee. Last but not least, I would like to thank Kris with who I shared the « behavioral fever » story. Working with you was a real pleasure and even in hard times, you kept your smile, good mood and optimism! You were a key member of the team and we will miss you so much when you will go back to Poland... I wish you all the best for your personal and professional life!

Malgré le fait que la "fish team" soit ma team "de cœur", j'ai aussi rencontré de très bons collègues dans les deux autres équipes. Il y a ceux qui ont marqué mon parcours, merci à Sylvie, pour son aide dans la gestion de l'IVIS mais aussi pour son franc parler et son caractère, ne change pas surtout! Je remercie aussi Sarah, de m'avoir fait pousser les portes d'une salle de fitness, et Bérengère, mon acolyte depuis les bancs de l'université. Merci à Laurent pour ses conseils avisés ainsi qu'à toute son équipe, Bénédicte, Céline, Bilal, Mickaël, Caroline, Emeline et Xue. Dans l'équipe « gnou », merci à Françoise et Océane, mes deux collègues et amies avec qui j'ai pu partager de très bons moments au labo mais aussi en dehors, sans compter les nombreuses discussions « bébé » qui ont été très instructives pour moi! Merci aussi à Annette, Marion, Léonor et Steven pour leur sympathie! A côté de l'Immuno, nous partageons aussi le laboratoire avec le groupe de virologie du Professeur Etienne Thiry et je tiens à remercier Axel, Angélique, Elisabeth, Fabiana, Damien et Edmilson. Un tout grand merci à Ana, avec qui j'ai partagé de très bons moments extra-labo, merci d'être telle que tu es, franche et entière!

Un autre membre de la team Viro méritait que je lui consacre quelques lignes de par l'importance qu'il a pris dans ma vie, notre agent double préféré…William alias « Wiwi »! Ce doctorat m'a apporté beaucoup de belles rencontres et tu en fais définitivement partie! Tu es une personne en or et un ami encore meilleur, tu es à l'écoute, comprends, acceptes les qualités mais aussi les défauts de ceux que tu aimes…et tu donnes tellement! Merci d'être là, reste tel que tu es et je te souhaite tout le bonheur possible!

Je tiens également à remercier tous les techniciens, pour leur aide précieuse au jour le jour ainsi que lors des grosses manipes. Merci à Cédric pour son aide lors des nombreuses in vivo « larves » ou « poissons de rivière », les Southern-Blot, les petits soucis informatiques mais aussi, pour les notes d'humour à la ferme… Merci à François et Antoine pour les bons moments passés dans le labo, merci à Juju et Emeline pour leur sympathie et aide indispensable. Merci également aux anciens techniciens Jérémy, Nathalie, Méghann et Christine. J'adresse un merci tout particulier à Christina et Lorène,

pour leur aide, pour les commandes et les soucis administratifs, mais aussi et surtout pour leur écoute et les discussions qui m'ont souvent permis d'oublier les petits soucis du quotidien.

Quand les portes du labo se refermaient, le relai était pris par mes nombreux amis qui ont toujours fait preuve de compréhension face à mon engagement dans cette thèse. Tantôt pour me changer les idées, tantôt pour me soutenir, j'ai pu me reposer sur eux et c'est ce qui m'a permis de tenir lorsque tout ne se passait pas comme prévu. Je tiens à remercier tout particulièrement Amélie, pour ses petits mails journaliers, son soutien et tous les bons moments culinaires, sportifs, cinématographiques…que l'on a partagés! Merci également à Pauline, depuis notre rencontre aux cours préparatoires, tu as pris une place énorme dans ma vie et dans mon cœur. On a partagé de merveilleux moments ensemble, on a aussi pu compter l'une sur l'autre lorsque la vie ne nous a pas épargnées et je souhaite que notre amitié ne finisse jamais! Merci aussi à Yves, Alain, Marjorie, Ludo, Caro, Emilie, Steph, Julien… qui à un moment ou un autre m'ont aidée à mener ce cursus à terme !

Enfin, j'aimerais exprimer toute ma gratitude à ma famille. Depuis toujours, ma famille a été l'élément nécessaire à mon équilibre personnel, représentant mes racines, mes piliers, ceux sur qui je peux toujours compter. Evidemment, mes premiers référents sont mes parents qui m'ont apporté amour, soutien, et aide au quotidien. Maman, Papa, malgré les blocus, stress, insomnies,…que je vous ai fait subir, vous avez toujours été là pour moi et c'est à vous que je dois d'être devenue la femme que je suis… Je vous dédie ce travail! Je remercie aussi leurs moitiés respectives, Riziero et Françoise, qui ont indirectement subi mes frasques, merci pour votre patience et votre écoute. Merci à mes grands-parents maternels, Mamy Gembloux et Papy Jo, et paternels, Mamy Delphine et Parrain Jean-Marie, pour leur soutien et amour indéfectible. Merci à ma marraine Brigitte, mes oncles et tantes, mes cousins et cousines, pour les fêtes de famille et bons moments partagés. Je remercie également ma belle-famille de l'autre côté de la frontière pour sa gentillesse et son accueil. Pour finir, je voudrais remercier mes quatre petits chéris, Louy, Jeanne, Charle et Grégoire, mes petits-frères et sœur, pour les bouffées de bonne humeur qu'ils m'apportent et la fierté qu'ils me procurent!

Pour terminer, je souhaiterais remercier Maxime… J'aurais pu le citer dans la plupart des paragraphes ci-dessus tant il a revêtu de casquettes différentes! D'abord collègue, il est rapidement devenu ami, complice et confident. Depuis près de 3 ans maintenant, il occupe mon quotidien, il est l'homme que j'aime et celui avec qui je veux partager le reste de ma vie, les bons comme les mauvais moments. Sans lui, ce travail n'aurait jamais abouti! Merci pour ton aide et tes réflexions dans le cadre professionnel, ton soutien et ton écoute lors des baisses de moral, mais aussi ton amour et ta tendresse au jour le jour… Merci tout simplement d'exister et d'être toi!

Milles mercis à tous!

Ce travail a été financé par le Fonds National pour la Recherche Scientifique (F.N.R.S.). Je remercie cette institution pour la confiance qu'elle m'a accordée.

List of abbreviations

AciHV-1 :	acipenserid herpesvirus 1
AciHV-2 :	acipenserid herpesvirus 2
ADAR :	double-stranded RNA-specific adenosine deaminase
ADN :	desoxyribonucleic acid
AngHV-1 :	anguillid herpesvirus 1
Ara-C :	cytosine-β-D-arabinofuranoside
Au :	goldfish fin cell
BAC :	bacterial artificial chromosome
BC :	before Christ
BoHV-4 :	bovine herpesvirus 4
bp :	base pair
CaF-2 :	common carp fin cell
CCB :	*Cyprinus carpio* brain
CCF-K104 :	common carp fin cell
CCG :	*Cyprinus carpio* gill
CCO :	channel catfish ovary
cDNA :	complementary DNA
CFC :	carp fin cell
CHSE-214 :	chinook salmon embryo cell
CHX :	cycloheximide
CNS :	central nervous system
CPE :	cytopathic effect
CRD :	cysteine rich domain
CRP :	C-reactive protein
CT :	control
CyHV-1 :	cyprinid herpesvirus 1
CyHV-2 :	cyprinid herpesvirus 2
CyHV-3 :	cyprinid herpesvirus 3
CyHV-4 :	cyprinid herpesvirus 4
Da :	dalton
DAI :	DNA-dependent activator of IFN-regulatory factors
DAMP :	damage-associated molecular pattern
DMEM :	dulbecco's modified essential medium
dpi :	days post-infection
dsDNA :	double-stranded DNA
dsRNA :	double-stranded RNA
E :	early
EBNA :	EBV nuclear antigen
EBV :	epstein-barr virus
EGFP :	enhanced green fluorescent protein
ELISA :	enzyme-linked immunosorbent assay
EM :	electron microscopy
ENT :	equilibrative nucleoside transporter
EPC :	*epithelioma papulosum cyprini*
FAO :	food and agriculture organization
FAT :	fluorescent antibody test
FCS :	fetal calf serum
FHM :	fathead minnow
FL strain :	strain isolated by François Lieffrig
FNRS :	fonds national de la recherche scientifique
GadHV-1:	gadid herpesvirus 1
*gal*K :	galactokinase

gD :	glycoprotein D
HCMV :	human cytomegalovirus
HPA :	hypothalamic-pituitary-adrenal
hpi :	hours post-infection
HSV-1 :	herpes simplex virus 1
HuHV-1 :	human herpesvirus 1
HuHV-5 :	human herpesvirus 5
IcHV-1 :	ictalurid herpesvirus 1
IcHV-2 :	ictalurid herpesvirus 2
ICP :	infected-cell polypeptide
ICTV :	international committee on taxonomy of viruses
IE :	immediate-early
IF :	immunofluorescence
IFN :	interferon
Ig :	immunoglobulin
IHNV :	infectious hematopoietic necrosis virus
IL/Il :	interleukin
IP :	intraperitoneal
ISAV :	infectious salmon anaemia virus
ISH :	*in situ* hybridization
IVIS :	*in vivo* imaging system
kb (p) :	kilobase (pairs)
KCF-1 :	koi carp fin cell
kDa :	kilodalton
KF-1 :	koi carp fin cell
KF-101 :	koi carp fin cell
KFC :	koi carp fin cell
KHV (I, J, U) :	koi herpesvirus (Israel, Japan, USA)
KHVD :	koi herpesvirus disease
KS :	koi carp snout cell
KSHV :	kaposi's sarcoma-associated herpesvirus
L :	late
LacZ :	betagalactosidase gene
LAMP :	loop-mediated isothermal amplification
LUC :	luciferase gene
MCT :	multi-chamber tank
MFC :	common carp fin cell
MHC :	major histocompatibility complex
MM :	molecular mass
MnPO :	median preoptic nucleus
MOI :	multiplicity of infection
MS :	marker size
MSC :	common carp snout cell
NAD(P) :	nicotinamide adenine dinucleotide (phosphate)
NF-κB :	nuclear factor-kappa B
NGF-2 (-3) :	koi carp fin cell
OIE :	World Organization for Animal Health
ORF :	open reading frame
OTU :	ovarian tumor
$p/s/cm^2/sr$:	photon per second per centimeter square per steradian
PAA :	phosphonoacetic acid
PAMP :	pathogen-associated molecular pattern
PBS :	phosphate-buffered saline
PBST :	PBS Tween

PCR :	polymerase chain reaction
pfu :	plaque forming unit
PG :	prostaglandin
pi :	post-infection
PKR :	dsRNA-dependent protein kinase
PKZ :	Z-DNA-dependent protein kinase
pTNFα :	plasmid encoding Tnfα
qPCR	quantitative PCR
RaHV-1 :	ranid herpesvirus 1
RaHV-2 :	ranid herpesvirus 2
RFLP :	restriction fragment length polymorphism
RING :	really interesting new gene
ROI :	region of interest
RT :	room temperature
RT-PCR :	reverse transcription-PCR
SalHV-1 :	salmonid herpesvirus 1
SalHV-2 :	salmonid herpesvirus 2
SalHV-3 :	salmonid herpesvirus 3
SCT :	single chamber tank
SD :	standard deviation
SDS :	sodium dodecyl sulfate
SDS-PAGE :	SDS-polyacrylamide gel electrophoresis
siRNA :	small interfering RNA
SNS :	sympathic nervous system
SPRY :	sprouty
SuHV-1 :	suid herpesvirus 1
SVCV :	spring viremia of carp virus
TK :	thymidine kinase
TNF/Tnf :	tumor necrosis factor
TNFR :	tumor necrosis factor receptor
Tol/FL :	silver carp fin cell
TR :	terminal repeat
TRIM :	tripartite motif
VNTR :	variable number tandem repeat
vs :	versus
WT :	wild-type

Preamble

Common carp is one of the oldest cultivated freshwater fish species and is currently one of the most economically valuable species in aquaculture. It is cultivated for human consumption with a worldwide production of 3.8 million metric tons per year (estimation from the FAO for 2012). While common carp is a cheap source of animal proteins, its colourful ornamental subspecies (koi carp) is grown for personal pleasure and competitive exhibitions. Koi carp represent one of the most expensive markets for individual freshwater fish. In the late 1990s, a highly contagious and fatal disease started to cause severe economic losses in these two carp industries worldwide. The causative agent of the disease, initially called koi herpesvirus (KHV), has been recently renamed cyprinid herpesvirus 3 (CyHV-3) and classified in the *Alloherpesviridae* family of the order *Herpesvirales*.

The economic importance of common and koi carp industries together with the rapid spread of CyHV-3 worldwide, explain why this virus became soon after its isolation a subject of applied research. However, these applied studies rapidly demonstrated that the CyHV-3 – carp model is also an interesting subject for fundamental researches: (i) CyHV-3 is phylogenetically distant from the vast majority of herpesviruses that have been studied so far (the latter belong to the family *Herpesviridae*), thereby providing an original field of research. (ii) The sequence of its genome reveals a fascinating virus with unique properties in the *Herpesvirales*, such as an extremely large genome (295 kb), a high number of sequences which are not homologous to already characterized viral or cellular genes but also sequences which are homologous to cellular genes of the immune system. (iii) It can be studied in laboratories by infection of its natural host (homologous virus-host model) for which substantial information and reagents are available. Altogether, the points listed above demonstrate that the CyHV-3 – carp model is a very good model to study viral-host interactions taking place during the infection of a teleost fish.

Teleost fish possess a complete immune system developing both innate and adaptive immune responses. Compared to higher vertebrates, the innate immune response of teleosts plays a larger role in the immune system for the following reasons. First, the adaptive immune system only matures after few months, so that young fish rely entirely on the innate immune system early in life. Second, the activation of the adaptive immune system is dependent on the temperature of the water. In carp, the adaptive immune response is active only at temperature above 14°C, implying that at lower temperatures fish rely entirely on their innate immune system to prevent and control infections. In this thesis, we addressed two types of innate immune responses developed by carp against CyHV-3 infection: the role of the epidermal mucus as an innate immune barrier against CyHV-3 entry during the early stages of life and the expression of behavioral fever by juvenile fish.

This manuscript is organized as follows. It starts with a broad introduction on CyHV-3 summarizing the knowledge available on this virus. This introduction has been published as a chapter in *Advances in Virus Research*. Following the introduction, the objectives of the thesis are briefly

exposed and are followed by the experimental section. This section consists of two chapters dedicated as mentioned above to innate immune responses developed by carp against CyHV-3. The first has been published in *Veterinary Research*. The second was under submission for publication when this manuscript was printed. In the last section of this manuscript “Discussion and perspectives”, the main results are discussed and some perspectives are presented. While this thesis focused on fundamental aspects of the innate immune mechanisms developed by carp against CyHV-3, it was also an opportunity to contribute to an applied project aiming to develop a safe and efficacious attenuated recombinant vaccine against CyHV-3. This study has been published in *Plos Pathogens* and is provided as an annex at the end of this manuscript.

Introduction

Introduction

Cyprinid herpesvirus 3:
an archetype of fish Alloherpesviruses

Advances in Virus Research (2015), **93**, 161-256

Maxime Boutier, Maygane Ronsmans, Krzysztof Rakus, Joanna Jazowiecka-Rakus, Catherine Vancsok, Léa Morvan, Ma. Michelle D. Peñaranda, David M. Stone, Keith Way, Steven J. van Beurden, Andrew J. Davison and Alain Vanderplasschen

Cyprinid Herpesvirus 3: An Archetype of Fish Alloherpesviruses

Maxime Boutier*, Maygane Ronsmans*, Krzysztof Rakus*, Joanna Jazowiecka-Rakus*, Catherine Vancsok*, Léa Morvan*, Ma. Michelle D. Peñaranda*, David M. Stone†, Keith Way†, Steven J. van Beurden‡, Andrew J. Davison§, Alain Vanderplasschen*,1

*Immunology-Vaccinology (B43b), Department of Infectious and Parasitic Diseases, Fundamental and Applied Research for Animals & Health (FARAH), Faculty of Veterinary Medicine, University of Liège, Liège, Belgium
†The Centre for Environment, Fisheries and Aquaculture Science, Weymouth Laboratory, Weymouth, Dorset, United Kingdom
‡Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands
§MRC-University of Glasgow Centre for Virus Research, Glasgow, United Kingdom
1Corresponding author: e-mail address: a.vdplasschen@ulg.ac.be

Contents

Abstract

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

Advances in Virus Research
ISSN 0065-3527
http://dx.doi.org/10.1016/bs.aivir.2015.03.001

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

1. INTRODUCTION

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

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

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

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

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

2. THE ORDER *HERPESVIRALES*

2.1 Phylogeny

2.1.1 *Phylogeny of the Order* Herpesvirales

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

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

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

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

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

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

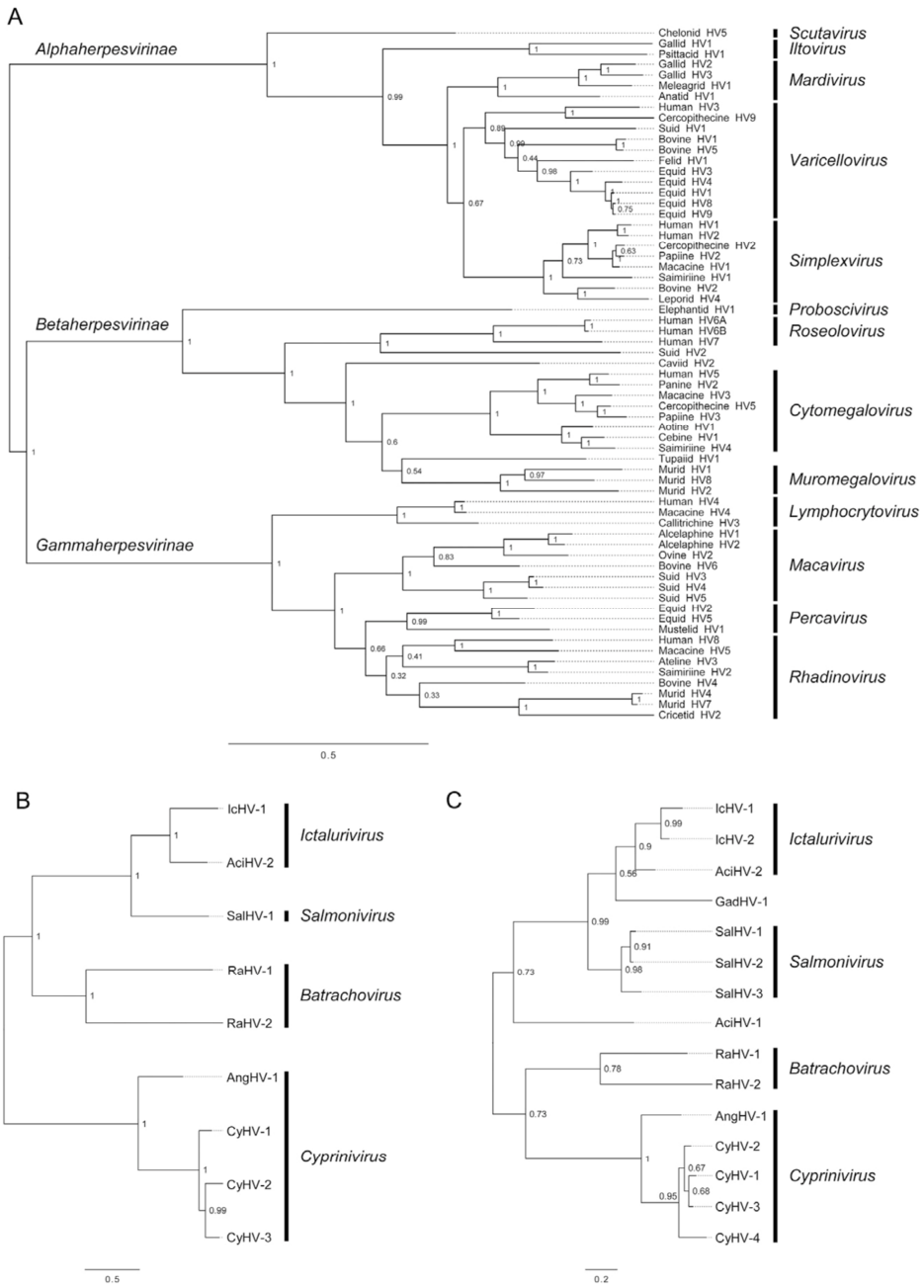


Figure 1 Phylogenetic analysis of the *Herpesviridae* and *Alloherpesviridae* families. Unrooted phylogenetic tree based on (A) the full-length DNA polymerases of members of the family *Herpesviridae*, (B) the full-length DNA polymerases of members of the family *Alloherpesviridae*, and (C) partial DNA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

(*Continued*)

2.1.2 *Phylogeny of the Family* Alloherpesviridae

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

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

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

Table 1 Classification of the Family *Alloherpesviridae*

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

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

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

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

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

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

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

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

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

2.2 Main Biological Properties

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

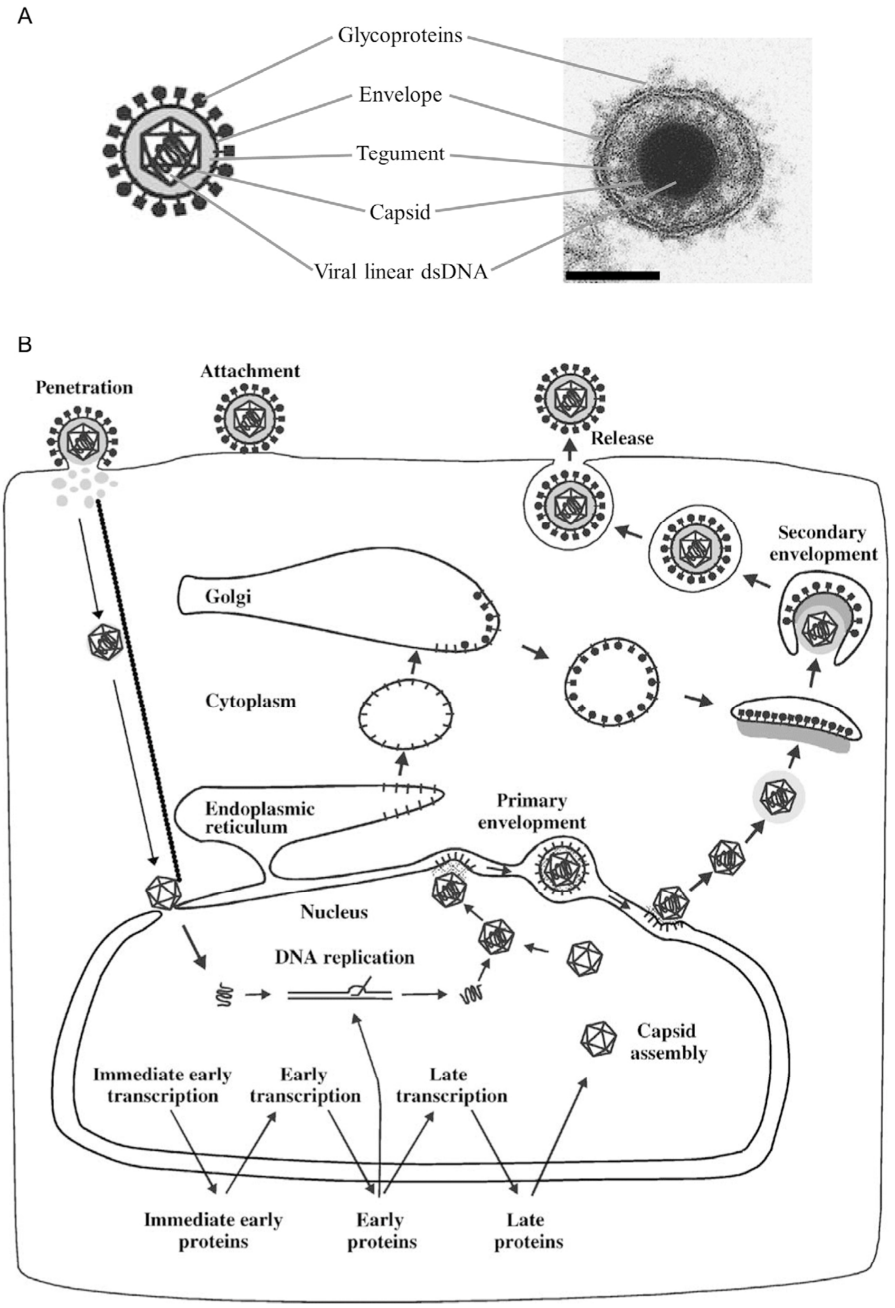


Figure 2 Virion structure and replication cycle of herpesviruses. (A) Schematic representation (left) and electron microscopy examination (right) of CyHV-3 virion. Bar represents 100 nm. (B) Replication cycle of CyHV-3. Diagrammatic representation of the herpesvirus replication cycle, including virus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

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

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

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

2.3 Herpesviruses Infecting Fish

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

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

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

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

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

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

3. CYPRINID HERPESVIRUS 3

3.1 General Description

3.1.1 Morphology and Morphogenesis

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

3.1.2 Genome

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

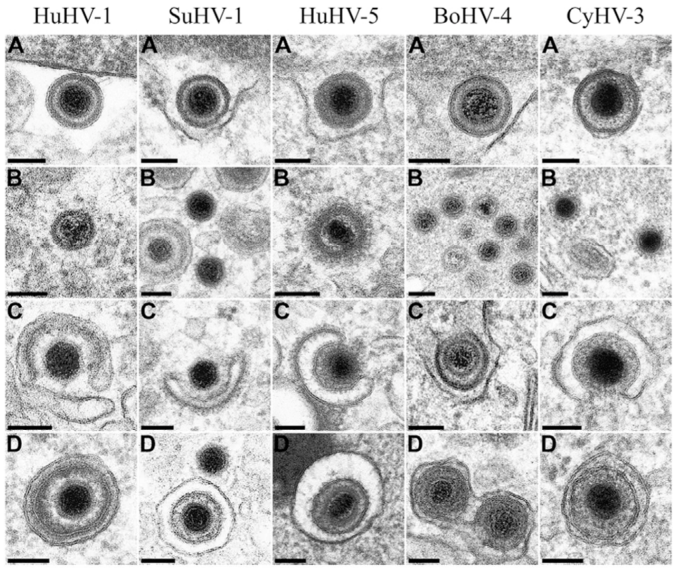


Figure 3 Primary and secondary envelopment of some herpesviruses. (A) Primary-enveloped virions in the perinuclear space. In comparison with Fig. 2, the electron-dense sharply bordered layer of tegument underlying the envelope and the conspicuous absence of envelope glycoprotein spikes are noteworthy.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

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

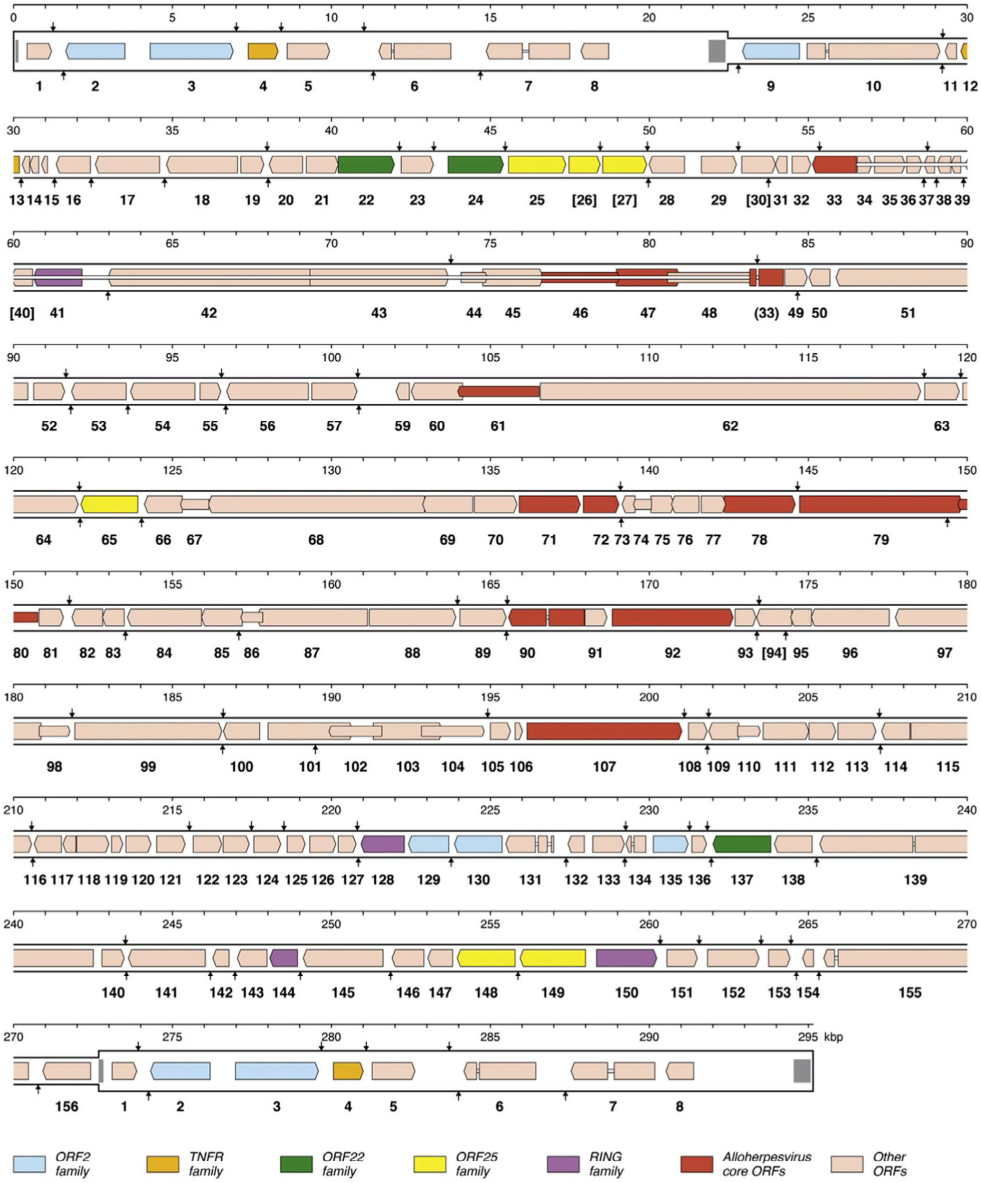


Figure 4 Map of the CyHV-3 genome. The terminal direct repeat (TR) is shown in a thicker format than the rest of the genome. ORFs predicted to encode functional proteins are indicated by arrows (see the key at the foot), with nomenclature lacking the ORF prefix given below. Introns are shown as narrow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

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

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

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

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

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

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

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

Continued

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

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

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

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

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

3.1.3 Genotypes

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

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

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

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

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

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

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

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

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

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

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

3.1.4 Transcriptome

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

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

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

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

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

Table 5 Transcriptomic Classification of CyHV-3 ORFs

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

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

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

Continued

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

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

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

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

Continued

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

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

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

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

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

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

3.1.5 Structural Proteome and Secretome

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

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

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

Table 6 Structural Proteome of CyHV-3

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

Continued

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

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

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

Continued

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

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

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

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

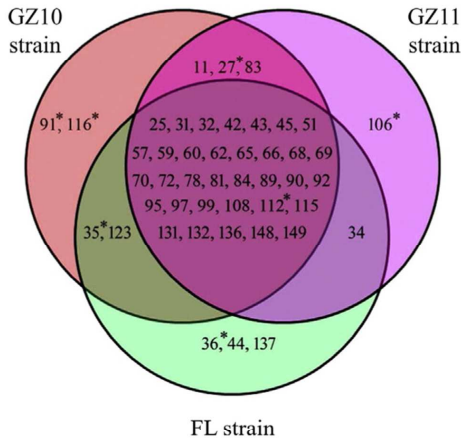


Figure 5 Structural proteome of CyHV-3. Schematic representation of virion-associated proteins from two CyHV-3 Chinese isolates (GZ10 and GZ11) (Yi et al., 2014) and one European isolate (FL) (Michel, Leroy, et al., 2010). Numbers indicate CyHV-3 ORFs. A total of 46 viral proteins were identified from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

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

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

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

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

3.1.6 Viral Replication in Cell Culture

3.1.6.1 Cell Lines Permissive to CyHV-3

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

Table 7 Cell Lines Susceptible to CyHV-3 Infection

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

[a]Only transient CPE.

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

3.1.6.2 Temperature Restriction

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

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

3.2 CyHV-3 Disease

3.2.1 Epidemiology

3.2.1.1 Fish Species Susceptible to CyHV-3 Infection

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

3.2.1.2 Geographical Distribution and Prevalence

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

Table 8 Organisms Tested for CyHV-3 Infection

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

Continued

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.2.1.3 Persistence of CyHV-3 in the Natural Environment

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

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

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

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

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

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

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

3.2.2 Clinical Aspects

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

3.2.2.1 Clinical Signs

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

3.2.2.2 Anatomopathology

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

3.2.2.3 Histopathology

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

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

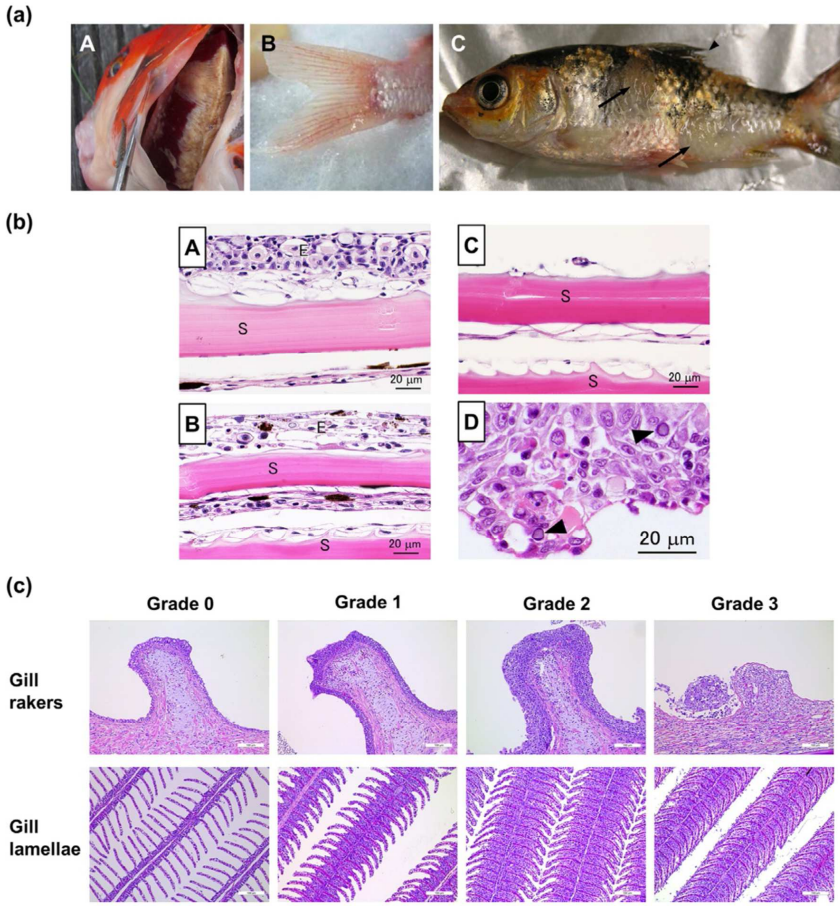


Figure 6 Illustration of anatomopathological and histopathological lesions induced by CyHV-3. (a) Anatomopathological lesions. (A) Severe gill necrosis. (B) Hyperemia at the base of the caudal fin. (C) Extensive necrosis of the skin covering the body (arrows indicate circular herpetic lesion) and fin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

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

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

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

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

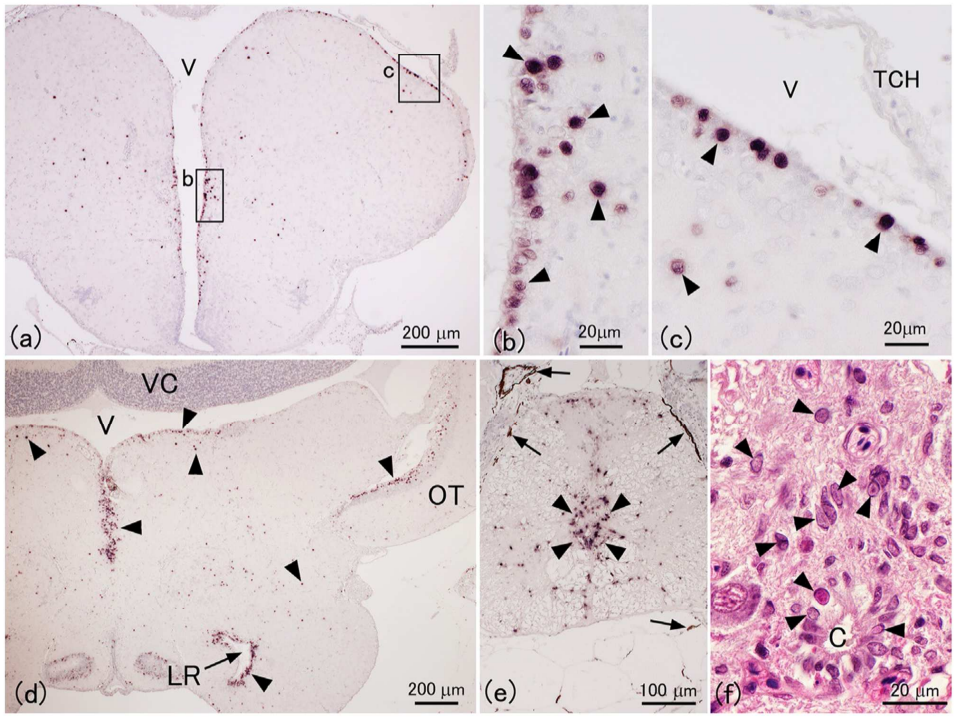


Figure 7 Illustration of histopathological lesions induced in the central nervous system of carp by CyHV-3. (A), (D), and (E) show sections of telencephalon, mesencephalon, and spinal cord hybridized for the viral genome, respectively. Fish were sampled 12 dpi. The hybridization signals (arrowheads)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

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

3.2.3 Pathogenesis

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

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

3.2.3.1 Productive Infection

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

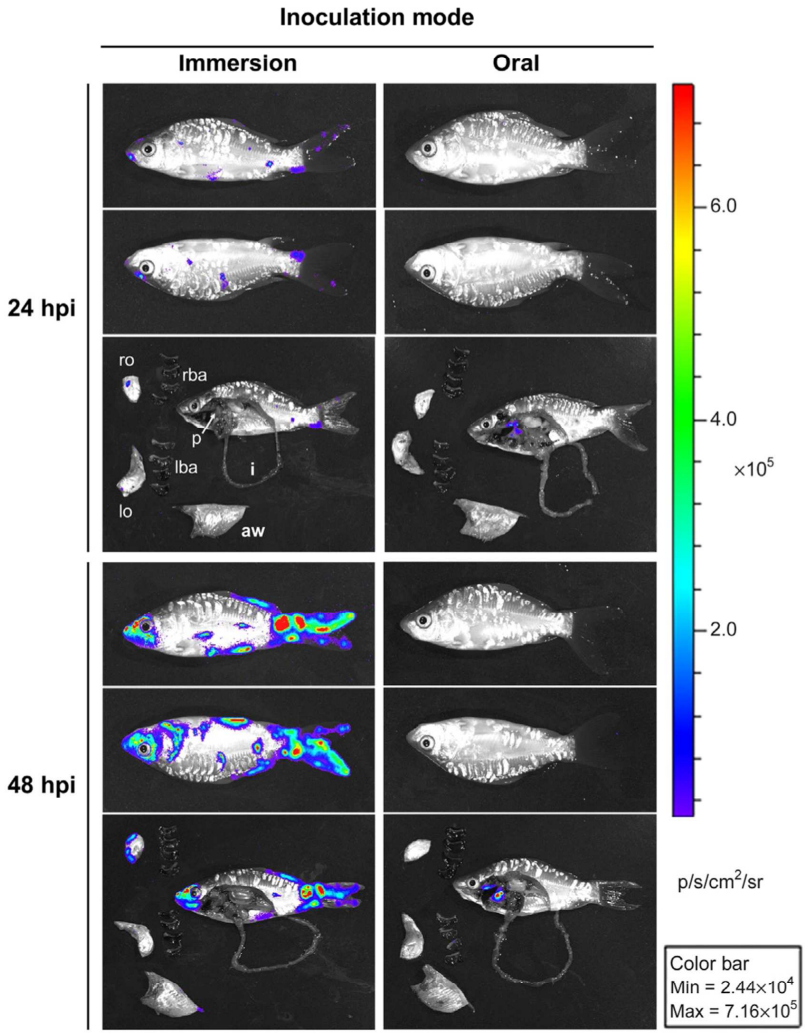


Figure 8 The portals of entry of CyHV-3 in carp analyzed by *in vivo* bioluminescent imaging. Two groups of fish (mean weight 10 g) were infected with a recombinant CyHV-3 strain expressing luciferase as a reporter gene either by bathing them in water containing the virus (immersion, left column) or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

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

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

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

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

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

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

3.2.3.2 Latent Infection

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

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

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

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

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

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

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

3.2.3.3 Effect of Water Temperature

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

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

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

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

3.2.4 Host–Pathogen Interactions

3.2.4.1 Susceptibility of Common Carp According to the Developmental Stage

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

3.2.4.2 Susceptibility of Common Carp According to Host Genetic Background

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

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

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

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

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

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

3.2.4.3 Common Carp Innate Immune Response Against CyHV-3

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

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

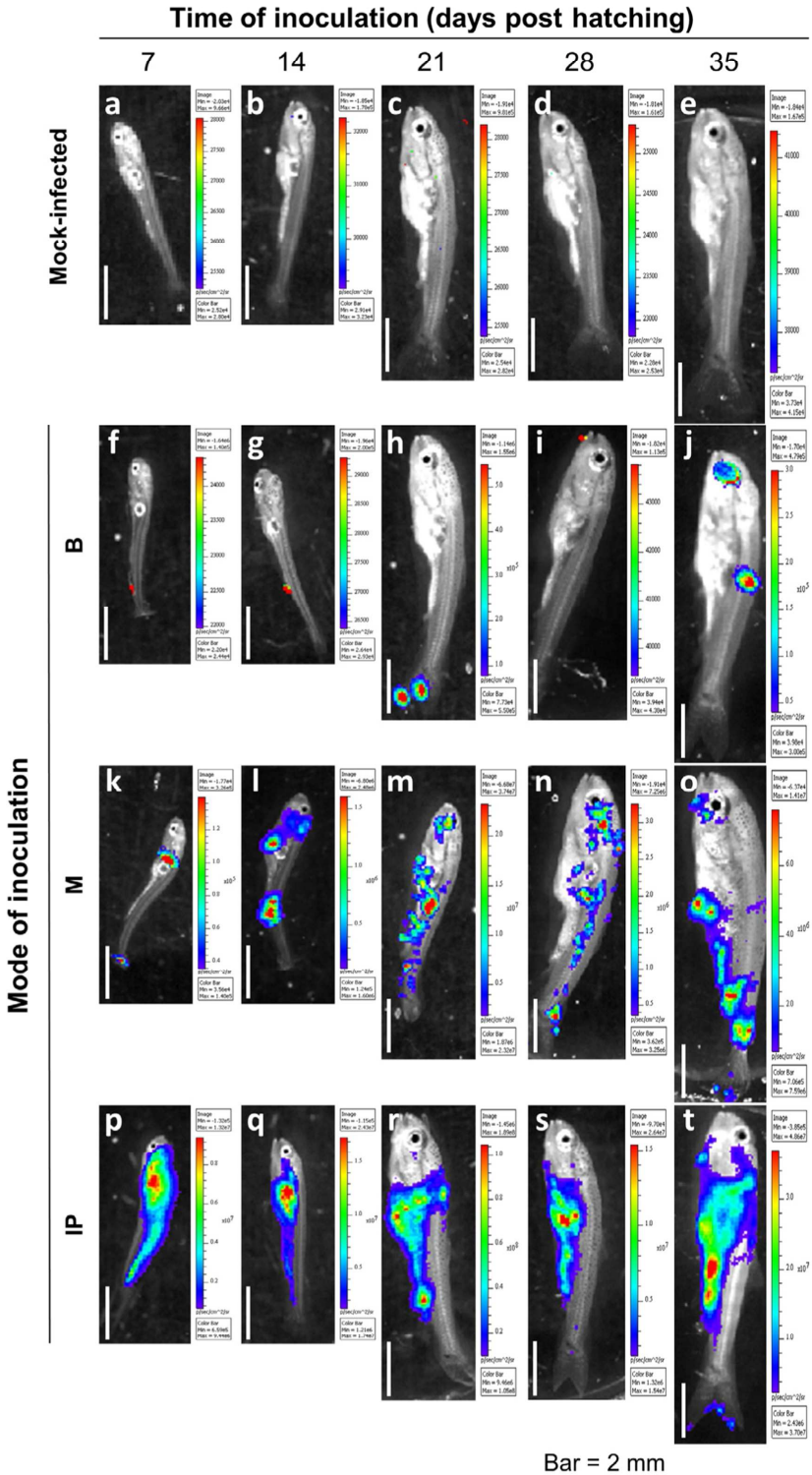


Figure 9 See legend on next page.

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

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

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

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

3.2.4.4 Common Carp Adaptive Immune Response Against CyHV-3

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

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

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

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

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

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

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

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

3.2.4.5 CyHV-3 Genes Involved in Immune Evasion

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

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

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

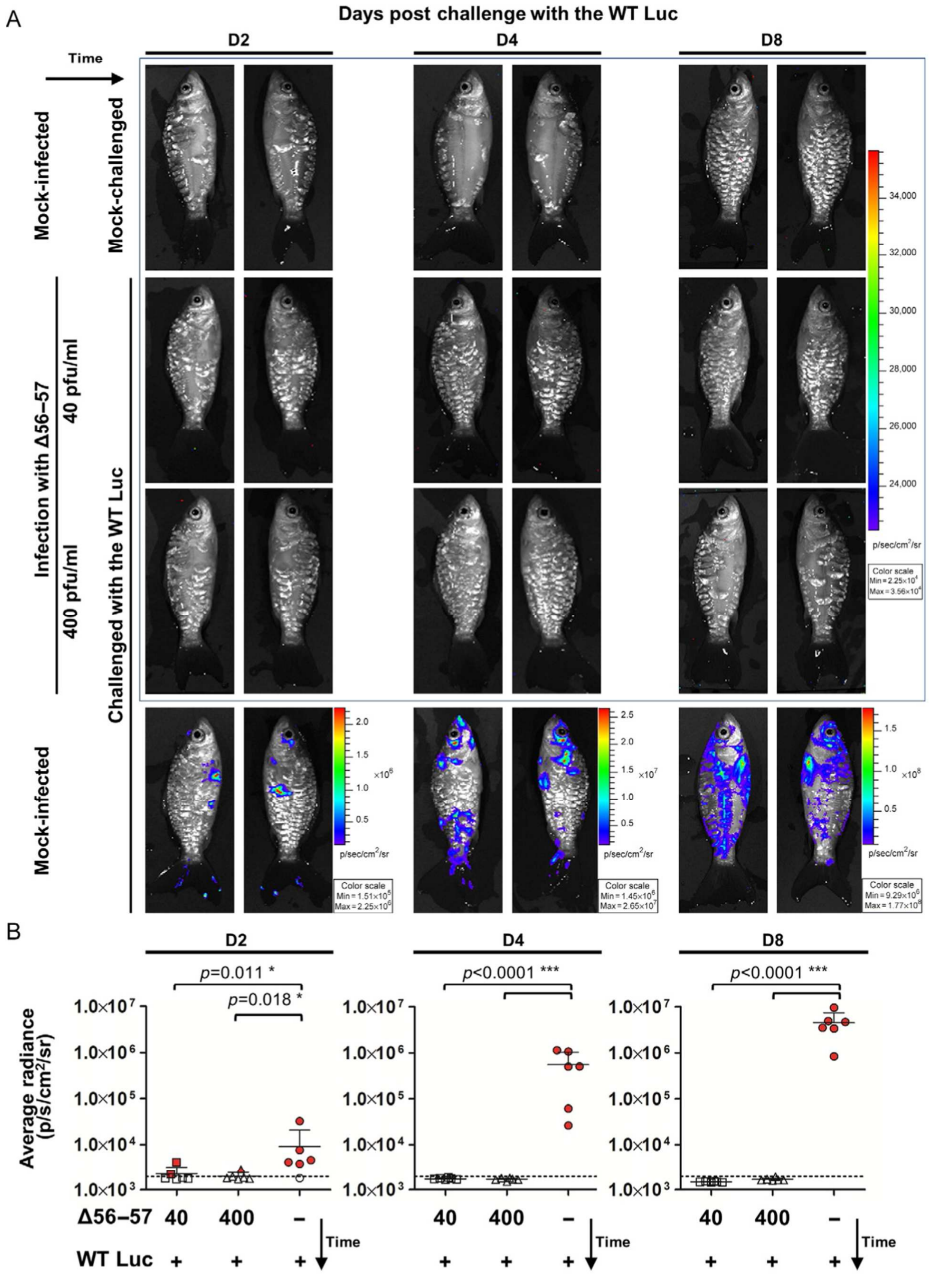


Figure 10 Immune protection conferred by the Δ56–57 attenuated CyHV-3 vaccine revealed by *in vivo* bioluminescent imaging. Common carp (mean ± SD weight 13.82 ± 5.00 g, 9 months old) were infected for 2 h by immersion in water containing 40 or 400 pfu/ml of the Δ56–57 attenuated CyHV-3 strain or mock-infected.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

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

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

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

3.2.5 Diagnosis

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

3.2.5.1 PCR-Based Methods

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

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

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

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

3.2.5.2 Virus Isolation in Cell Culture

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

3.2.5.3 Immunodiagnostic Methods

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

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

3.2.5.4 Other Diagnostic Assays

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

3.2.6 Vaccination

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

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

3.2.6.1 Natural Immunization

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

3.2.6.2 Vaccine Candidates

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

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

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

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

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

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

4. CONCLUSIONS

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

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

ACKNOWLEDGMENTS

This work was supported by Belgian Science Policy Grant R.SSTC.0454-BELVIR IAP7/45 to A.V. and by the Medical Research Council Grant Number MC_UU_12014/3 to A.D.

REFERENCES

Ackermann, M. (2004). Herpesviruses: A brief overview. *Methods in Molecular Biology*, *256*, 199–219. http://dx.doi.org/10.1385/1-59259-753-X:199.

Adamek, M., Rakus, K. L., Brogden, G., Matras, M., Chyb, J., Hirono, I., et al. (2014). Interaction between type I interferon and Cyprinid herpesvirus 3 in two genetic lines of common carp *Cyprinus carpio*. *Diseases of Aquatic Organisms*, *111*(2), 107–118. http://dx.doi.org/10.3354/dao02773.

Adamek, M., Rakus, K. L., Chyb, J., Brogden, G., Huebner, A., Irnazarow, I., et al. (2012). Interferon type I responses to virus infections in carp cells: *In vitro* studies on Cyprinid herpesvirus 3 and Rhabdovirus carpio infections. *Fish & Shellfish Immunology*, *33*(3), 482–493. http://dx.doi.org/10.1016/j.fsi.2012.05.031.

Adamek, M., Steinhagen, D., Irnazarow, I., Hikima, J., Jung, T. S., & Aoki, T. (2014). Biology and host response to Cyprinid herpesvirus 3 infection in common carp. *Developmental and Comparative Immunology*, *43*(2), 151–159. http://dx.doi.org/10.1016/j.dci.2013.08.015.

Adamek, M., Syakuri, H., Harris, S., Rakus, K. L., Brogden, G., Matras, M., et al. (2013). Cyprinid herpesvirus 3 infection disrupts the skin barrier of common carp (*Cyprinus carpio* L.). *Veterinary Microbiology*, *162*(2–4), 456–470. http://dx.doi.org/10.1016/j.vetmic.2012.10.033.

Adkison, M. A., Gilad, O., & Hedrick, R. P. (2005). An enzyme linked immunosorbent assay (ELISA) for detection of antibodies to the koi herpesvirus (KHV) in the serum of koi *Cyprinus carpio*. *Fish Pathology*, *40*, 53–62.

Antychowicz, J., Reichert, M., Matras, M., Bergmann, S. M., & Haenen, O. L. (2005). Epidemiology, pathogenicity and molecular biology of koi herpesvirus isolated in Poland. *Bulletin of the Veterinary Institute in Pulawy*, *49*, 367–373.

Aoki, T., Hirono, I., Kurokawa, K., Fukuda, H., Nahary, R., Eldar, A., et al. (2007). Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide. *Journal of Virology*, *81*(10), 5058–5065. http://dx.doi.org/10.1128/JVI.00146-07.

Aoki, T., Takano, T., Unajak, S., Takagi, M., Kim, Y. R., Park, S. B., et al. (2011). Generation of monoclonal antibodies specific for ORF68 of koi herpesvirus. *Comparative Immunology, Microbiology and Infectious Diseases*, *34*(3), 209–216. http://dx.doi.org/10.1016/j.cimid.2010.11.004.

Ariav, R., Tinman, S., Paperna, I., & Bejerano, I. (1999). In *First report of newly emerging viral disease of Cyprinus carpio species in Israel. Paper presented at the EAFP 9th international conference, Rhodes, Greece.*

Athanasiadis, A. (2012). Zalpha-domains: At the intersection between RNA editing and innate immunity. *Seminars in Cell and Developmental Biology*, *23*(3), 275–280. http://dx.doi.org/10.1016/j.semcdb.2011.11.001.

Avarre, J. C., Madeira, J. P., Santika, A., Zainun, Z., Baud, M., Cabon, J., et al. (2011). Investigation of Cyprinid herpesvirus-3 genetic diversity by a multi-locus variable

number of tandem repeats analysis. *Journal of Virological Methods*, *173*(2), 320–327. http://dx.doi.org/10.1016/j.jviromet.2011.03.002.

Avarre, J. C., Santika, A., Bentenni, A., Zainun, Z., Madeira, J. P., Maskur, M., et al. (2012). Spatio-temporal analysis of cyprinid herpesvirus 3 genetic diversity at a local scale. *Journal of Fish Diseases*, *35*(10), 767–774. http://dx.doi.org/10.1111/j.1365-2761.2012.01404.x.

Balon, E. K. (1995). Origin and domestication of the wild carp, *Cyprinus carpio*: From Roman gourmets to the swimming flowers. *Aquaculture*, *129*, 3–48.

Baumer, A., Fabian, M., Wilkens, M. R., Steinhagen, D., & Runge, M. (2013). Epidemiology of cyprinid herpesvirus-3 infection in latently infected carp from aquaculture. *Diseases of Aquatic Organisms*, *105*(2), 101–108. http://dx.doi.org/10.3354/dao02604.

Bercovier, H., Fishman, Y., Nahary, R., Sinai, S., Zlotkin, A., Eyngor, M., et al. (2005). Cloning of the koi herpesvirus (KHV) gene encoding thymidine kinase and its use for a highly sensitive PCR based diagnosis. *BMC Microbiology*, *5*, 13. http://dx.doi.org/10.1186/1471-2180-5-13.

Bergmann, S. M., & Kempter, J. (2011). Detection of koi herpesvirus (KHV) after re-activation in persistently infected common carp (*Cyprinus carpio* L.) using non-lethal sampling methods. *Bulletin of the European Association of Fish Pathologists*, *31*(3), 92–100.

Bergmann, S. M., Kempter, J., Sadowski, J., & Fichtner, D. (2006). First detection, confirmation and isolation of koi herpesvirus (KHV) in cultured common carp (*Cyprinus carpio* L.) in Poland. *Bulletin of the European Association of Fish Pathologists*, *26*(2), 97–104.

Bergmann, S. M., Lutze, P., Schütze, H., Fischer, U., Dauber, M., Fichtner, D., et al. (2010). Goldfish (*Carassius auratus auratus*) is a susceptible species for koi herpesvirus (KHV) but not for KHV disease (KHVD). *Bulletin of the European Association of Fish Pathologists*, *30*(2), 74–84.

Bergmann, S. M., Riechardt, M., Fichtner, D., Lee, P., & Kempter, J. (2010). Investigation on the diagnostic sensitivity of molecular tools used for detection of koi herpesvirus. *Journal of Virological Methods*, *163*(2), 229–233. http://dx.doi.org/10.1016/j.jviromet.2009.09.025.

Bergmann, S. M., Sadowski, J., Kielpinski, M., Bartlomiejczyk, M., Fichtner, D., Riebe, R., et al. (2010). Susceptibility of koi x crucian carp and koi x goldfish hybrids to koi herpesvirus (KHV) and the development of KHV disease (KHVD). *Journal of Fish Diseases*, *33*(3), 267–272. http://dx.doi.org/10.1111/j.1365-2761.2009.01127.x.

Bergmann, S. M., Schütze, H., Fischer, U., Fichtner, D., Riechardt, M., Meyer, K., et al. (2009). Detection KHV genome in apparently health fish. *Bulletin of the European Association of Fish Pathologists*, *29*(5), 145–152.

Bigarré, L., Baud, M., Cabon, J., Antychowicz, J., Bergmann, S. M., Engelsma, M., et al. (2009). Differentiation between Cyprinid herpesvirus type-3 lineages using duplex PCR. *Journal of Virological Methods*, *158*(1–2), 51–57. http://dx.doi.org/10.1016/j.jviromet.2009.01.023.

Bly, J. E., & Clem, W. (1992). Temperature and teleost immune functions. *Fish & Shellfish Immunology*, *2*, 159–171.

Bondad-Reantaso, M. G., Subasinghe, R. P., Arthur, J. R., Ogawa, K., Chinabut, S., Adlard, R., et al. (2005). Disease and health management in Asian aquaculture. *Veterinary Parasitology*, *132*(3–4), 249–272. http://dx.doi.org/10.1016/j.vetpar.2005.07.005.

Booy, F. P., Trus, B. L., Davison, A. J., & Steven, A. C. (1996). The capsid architecture of channel catfish virus, an evolutionarily distant herpesvirus, is largely conserved in the absence of discernible sequence homology with herpes simplex virus. *Virology*, *215*(2), 134–141. http://dx.doi.org/10.1006/viro.1996.0016.

Boutier, M., Ronsmans, M., Ouyang, P., Fournier, G., Reschner, A., Rakus, K., et al. (2015). Rational development of an attenuated recombinant cyprinid herpesvirus 3

vaccine using prokaryotic mutagenesis and in vivo bioluminescent imaging. *PLoS Pathogens*, *11*(2), e1004690. http://dx.doi.org/10.1371/journal.ppat.1004690.

Bretzinger, A., Fischer-Scherl, T., Oumouma, R., Hoffmann, R., & Truyen, U. (1999). Mass mortalities in koi, *Cyprinus carpio*, associated with gill and skin disease. *Bulletin of the European Association of Fish Pathologists*, *19*, 182–185.

Brudeseth, B. E., Wiulsrod, R., Fredriksen, B. N., Lindmo, K., Lokling, K. E., Bordevik, M., et al. (2013). Status and future perspectives of vaccines for industrialised fin-fish farming. *Fish & Shellfish Immunology*, *35*(6), 1759–1768. http://dx.doi.org/10.1016/j.fsi.2013.05.029.

Chen, J., Chee, D., Wang, Y., Lim, G. Y., Chong, S. M., Lin, Y. N., et al. (2014). Identification of a novel cyprinid herpesvirus 3 genotype detected in koi from the East Asian and South-East Asian Regions. *Journal of Fish Diseases*. http://onlinelibrary.wiley.com/doi/10.1111/jfd.12305/abstract.

Chistiakov, D. A., & Voronova, N. V. (2009). Genetic evolution and diversity of common carp *Cyprinus carpio* L. *Central European Journal of Biology*, *4*(3), 304–312. http://dx.doi.org/10.2478/s11535-009-0024-2.

Cho, M. Y., Won, K. M., Kim, J. W., Jee, B. Y., Park, M. A., & Hong, S. (2014). Detection of koi herpesvirus (KHV) in healthy cyprinid seed stock. *Diseases of Aquatic Organisms*, *112*(1), 29–36. http://dx.doi.org/10.3354/dao02784.

Costes, B., Fournier, G., Michel, B., Delforge, C., Raj, V. S., Dewals, B., et al. (2008). Cloning of the koi herpesvirus genome as an infectious bacterial artificial chromosome demonstrates that disruption of the thymidine kinase locus induces partial attenuation in *Cyprinus carpio koi*. *Journal of Virology*, *82*(10), 4955–4964. http://dx.doi.org/10.1128/JVI.00211-08.

Costes, B., Raj, V. S., Michel, B., Fournier, G., Thirion, M., Gillet, L., et al. (2009). The major portal of entry of koi herpesvirus in *Cyprinus carpio* is the skin. *Journal of Virology*, *83*(7), 2819–2830. http://dx.doi.org/10.1128/JVI.02305-08.

Davidovich, M., Dishon, A., Ilouze, M., & Kotler, M. (2007). Susceptibility of cyprinid cultured cells to cyprinid herpesvirus 3. *Archives of Virology*, *152*(8), 1541–1546. http://dx.doi.org/10.1007/s00705-007-0975-4.

Davison, A. J. (1992). Channel catfish virus: A new type of herpesvirus. *Virology*, *186*(1), 9–14.

Davison, A. J. (1998). The genome of salmonid herpesvirus 1. *Journal of Virology*, *72*(3), 1974–1982.

Davison, A. J., Cunningham, C., Sauerbier, W., & McKinnell, R. G. (2006). Genome sequences of two frog herpesviruses. *Journal of General Virology*, *87*(Pt 12), 3509–3514. http://dx.doi.org/10.1099/vir.0.82291-0.

Davison, A. J., & Davison, M. D. (1995). Identification of structural proteins of channel catfish virus by mass spectrometry. *Virology*, *206*(2), 1035–1043. http://dx.doi.org/10.1006/viro.1995.1026.

Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., McGeoch, D. J., Minson, A. C., et al. (2009). The order Herpesvirales. *Archives of Virology*, *154*(1), 171–177. http://dx.doi.org/10.1007/s00705-008-0278-4.

Davison, A. J., Kurobe, T., Gatherer, D., Cunningham, C., Korf, I., Fukuda, H., et al. (2013). Comparative genomics of carp herpesviruses. *Journal of Virology*, *87*(5), 2908–2922. http://dx.doi.org/10.1128/JVI.03206-12.

Davison, A. J., Trus, B. L., Cheng, N., Steven, A. C., Watson, M. S., Cunningham, C., et al. (2005). A novel class of herpesvirus with bivalve hosts. *Journal of General Virology*, *86*(Pt 1), 41–53. http://dx.doi.org/10.1099/vir.0.80382-0.

Deback, C., Boutolleau, D., Depienne, C., Luyt, C. E., Bonnafous, P., Gautheret-Dejean, A., et al. (2009). Utilization of microsatellite polymorphism for differentiating herpes simplex virus type 1 strains. *Journal of Clinical Microbiology*, *47*(3), 533–540. http://dx.doi.org/10.1128/JCM.01565-08.

Dishon, A., Davidovich, M., Ilouze, M., & Kotler, M. (2007). Persistence of cyprinid herpesvirus 3 in infected cultured carp cells. *Journal of Virology*, *81*(9), 4828–4836. http://dx.doi.org/10.1128/JVI.02188-06.

Dishon, A., Perelberg, A., Bishara-Shieban, J., Ilouze, M., Davidovich, M., Werker, S., et al. (2005). Detection of carp interstitial nephritis and gill necrosis virus in fish droppings. *Applied and Environmental Microbiology*, *71*(11), 7285–7291. http://dx.doi.org/10.1128/AEM.71.11.7285-7291.2005.

Dixon, P. F., Joiner, C. L., Way, K., Reese, R. A., Jeney, G., & Jeney, Z. (2009). Comparison of the resistance of selected families of common carp, *Cyprinus carpio* L., to koi herpesvirus: Preliminary study. *Journal of Fish Diseases*, *32*(12), 1035–1039. http://dx.doi.org/10.1111/j.1365-2761.2009.01081.x.

Dong, C., Li, X., Weng, S., Xie, S., & He, J. (2013). Emergence of fatal European genotype CyHV-3/KHV in mainland China. *Veterinary Microbiology*, *162*(1), 239–244. http://dx.doi.org/10.1016/j.vetmic.2012.10.024.

Dong, C., Weng, S., Li, W., Li, X., Yi, Y., Liang, Q., et al. (2011). Characterization of a new cell line from caudal fin of koi, *Cyprinus carpio koi*, and first isolation of cyprinid herpesvirus 3 in China. *Virus Research*, *161*(2), 140–149. http://dx.doi.org/10.1016/j.virusres.2011.07.016.

Doszpoly, A., Benko, M., Bovo, G., Lapatra, S. E., & Harrach, B. (2011). Comparative analysis of a conserved gene block from the genome of the members of the genus ictalurivirus. *Intervirology*, *54*(5), 282–289. http://dx.doi.org/10.1159/000319430.

Doszpoly, A., Kovacs, E. R., Bovo, G., LaPatra, S. E., Harrach, B., & Benko, M. (2008). Molecular confirmation of a new herpesvirus from catfish (*Ameiurus melas*) by testing the performance of a novel PCR method, designed to target the DNA polymerase gene of alloherpesviruses. *Archives of Virology*, *153*(11), 2123–2127. http://dx.doi.org/10.1007/s00705-008-0230-7.

Doszpoly, A., Papp, M., Deakne, P. P., Glavits, R., Ursu, K., & Dan, A. (2015). Molecular detection of a putatively novel cyprinid herpesvirus in sichel (*Pelecus cultratus*) during a mass mortality event in Hungary. *Archives of Virology*, *160*(5), 1279–1283. http://dx.doi.org/10.1007/s00705-015-2348-8.

Doszpoly, A., Somogyi, V., LaPatra, S. E., & Benko, M. (2011). Partial genome characterization of acipenserid herpesvirus 2: Taxonomical proposal for the demarcation of three subfamilies in Alloherpesviridae. *Archives of Virology*, *156*(12), 2291–2296. http://dx.doi.org/10.1007/s00705-011-1108-7.

Eide, K. E., Miller-Morgan, T., Heidel, J. R., Bildfell, R. J., & Jin, L. (2011). Results of total DNA measurement in koi tissue by Koi Herpes Virus real-time PCR. *Journal of Virological Methods*, *172*(1–2), 81–84. http://dx.doi.org/10.1016/j.jviromet.2010.12.012.

Eide, K. E., Miller-Morgan, T., Heidel, J. R., Kent, M. L., Bildfell, R. J., Lapatra, S., et al. (2011). Investigation of koi herpesvirus latency in koi. *Journal of Virology*, *85*(10), 4954–4962. http://dx.doi.org/10.1128/JVI.01384-10.

El-Din, M. M. M. (2011). Histopathological studies in experimentally infected koi carp (*Cyprinus carpio koi*) with koi herpesvirus in Japan. *World Journal of Fish and Marine Science*, *3*, 252–259.

Ellis, A. E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. *Developmental and Comparative Immunology*, *25*(8–9), 827–839. http://dx.doi.org/10.1016/s0145-305x(01)00038-6.

El-Matbouli, M., Saleh, M., & Soliman, H. (2007). Detection of cyprinid herpesvirus type 3 in goldfish cohabiting with CyHV-3-infected koi carp (*Cyprinus carpio koi*). *Veterinary Record*, *161*(23), 792–793.

El-Matbouli, M., & Soliman, H. (2011). Transmission of cyprinid herpesvirus-3 (CyHV-3) from goldfish to naive common carp by cohabitation. *Research in Veterinary Science*, *90*(3), 536–539. http://dx.doi.org/10.1016/j.rvsc.2010.07.008.

Engelsma, M. Y., Way, K., Dodge, M. J., Voorbergen-Laarman, M., Panzarin, V., Abbadi, M., et al. (2013). Detection of novel strains of cyprinid herpesvirus closely related to koi herpesvirus. *Diseases of Aquatic Organisms*, *107*(2), 113–120. http://dx.doi.org/10.3354/dao02666.

Fabian, M., Baumer, A., & Steinhagen, D. (2013). Do wild fish species contribute to the transmission of koi herpesvirus to carp in hatchery ponds? *Journal of Fish Diseases*, *36*(5), 505–514. http://dx.doi.org/10.1111/jfd.12016.

FAO. (2012). Global Aquaculture Production 1950–2012. Retrieved 15/02/2015, from http://www.fao.org/fishery/statistics/global-aquaculture-production/query/fr.

Fawcett, D. W. (1956). Electron microscope observations on intracellular virus-like particles associated with the cells of the Lucke renal adenocarcinoma. *The Journal of Biophysical and Biochemical Cytology*, *2*(6), 725–741.

Fournier, G., Boutier, M., Stalin Raj, V., Mast, J., Parmentier, E., Vanderwalle, P., et al. (2012). Feeding *Cyprinus carpio* with infectious materials mediates cyprinid herpesvirus 3 entry through infection of pharyngeal periodontal mucosa. *Veterinary Research*, *43*, 6. http://dx.doi.org/10.1186/1297-9716-43-6.

Fuchs, W., Fichtner, D., Bergmann, S. M., & Mettenleiter, T. C. (2011). Generation and characterization of koi herpesvirus recombinants lacking viral enzymes of nucleotide metabolism. *Archives of Virology*, *156*(6), 1059–1063. http://dx.doi.org/10.1007/s00705-011-0953-8.

Fuchs, W., Granzow, H., Dauber, M., Fichtner, D., & Mettenleiter, T. C. (2014). Identification of structural proteins of koi herpesvirus. *Archives of Virology*, *159*(12), 3257–3268. http://dx.doi.org/10.1007/s00705-014-2190-4.

Fujioka, H., Yamasaki, K., Furusawa, K., Tamura, K., Oguro, K., Kurihara, S., et al. (2015). Prevalence and characteristics of Cyprinid herpesvirus 3 (CyHV-3) infection in common carp (*Cyprinus carpio* L.) inhabiting three rivers in Kochi Prefecture, Japan. *Veterinary Microbiology*, *175*(2–4), 362–368. http://dx.doi.org/10.1016/j.vetmic.2014.12.002.

Garver, K. A., Al-Hussinee, L., Hawley, L. M., Schroeder, T., Edes, S., LePage, V., et al. (2010). Mass mortality associated with koi herpesvirus in wild common carp in Canada. *Journal of Wildlife Diseases*, *46*(4), 1242–1251. http://dx.doi.org/10.7589/0090-3558-46.4.1242.

Gilad, O., Yun, S., Adkison, M. A., Way, K., Willits, N. H., Bercovier, H., et al. (2003). Molecular comparison of isolates of an emerging fish pathogen, koi herpesvirus, and the effect of water temperature on mortality of experimentally infected koi. *Journal of General Virology*, *84*(Pt 10), 2661–2667.

Gilad, O., Yun, S., Andree, K. B., Adkison, M. A., Zlotkin, A., Bercovier, H., et al. (2002). Initial characteristics of koi herpesvirus and development of a polymerase chain reaction assay to detect the virus in koi, *Cyprinus carpio koi*. *Diseases of Aquatic Organisms*, *48*(2), 101–108. http://dx.doi.org/10.3354/dao048101.

Gilad, O., Yun, S., Zagmutt-Vergara, F. J., Leutenegger, C. M., Bercovier, H., & Hedrick, R. P. (2004). Concentrations of a Koi herpesvirus (KHV) in tissues of experimentally infected *Cyprinus carpio koi* as assessed by real-time TaqMan PCR. *Diseases of Aquatic Organisms*, *60*(3), 179–187. http://dx.doi.org/10.3354/dao060179.

Gomez, D. K., Joh, S. J., Jang, H., Shin, S. P., Choresca, C. H., Han, J. E., et al. (2011). Detection of koi herpesvirus (KHV) from koi (*Cyprinus carpio koi*) broodstock in South Korea. *Aquaculture*, *311*(1–4), 42–47. http://dx.doi.org/10.1016/j.aquaculture.2010.11.021.

Gomez, D., Sunyer, J. O., & Salinas, I. (2013). The mucosal immune system of fish: The evolution of tolerating commensals while fighting pathogens. *Fish & Shellfish Immunology*, *35*(6), 1729–1739. http://dx.doi.org/10.1016/j.fsi.2013.09.032.

Goodwin, A. E., Sadler, J., Merry, G. E., & Marecaux, E. N. (2009). Herpesviral haematopoietic necrosis virus (CyHV-2) infection: Case studies from commercial

goldfish farms. *Journal of Fish Diseases*, *32*(3), 271–278. http://dx.doi.org/10.1111/j.1365-2761.2008.00988.x.

Gotesman, M., Kattlun, J., Bergmann, S. M., & El-Matbouli, M. (2013). CyHV-3: The third cyprinid herpesvirus. *Diseases of Aquatic Organisms*, *105*(2), 163–174. http://dx.doi.org/10.3354/dao02614.

Gotesman, M., Soliman, H., Besch, R., & El-Matbouli, M. (2014). *In vitro* inhibition of cyprinid herpesvirus-3 replication by RNAi. *Journal of Virological Methods*, *206*, 63–66. http://dx.doi.org/10.1016/j.jviromet.2014.05.022.

Gravell, M., Granoff, A., & Darlington, R. W. (1968). Viruses and renal carcinoma of *Rana pipiens*. VII. Propagation of a herpes-type frog virus. *Virology*, *36*(3), 467–475. http://dx.doi.org/10.1016/0042-6822(68)90172-4.

Gray, W. L., Mullis, L., LaPatra, S. E., Groff, J. M., & Goodwin, A. (2002). Detection of koi herpesvirus DNA in tissues of infected fish. *Journal of Fish Diseases*, *25*, 171–178.

Grimmett, S. G., Warg, J. V., Getchell, R. G., Johnson, D. J., & Bowser, P. R. (2006). An unusual koi herpesvirus associated with a mortality event of common carp *Cyprinus carpio* in New York State, USA. *Journal of Wildlife Diseases*, *42*(3), 658–662. http://dx.doi.org/10.7589/0090-3558-42.3.658.

Gunimaladevi, I., Kono, T., Venugopal, M. N., & Sakai, M. (2004). Detection of koi herpesvirus in common carp, *Cyprinus carpio* L., by loop-mediated isothermal amplification. *Journal of Fish Diseases*, *27*(10), 583–589. http://dx.doi.org/10.1111/j.1365-2761.2004.00578.x.

Haenen, O. L. M., Dijkstra, S. G., Van Tulden, P. W., Davidse, A., Van Nieuwstadt, A. P., Wagenaar, F., et al. (2002). Herpesvirus anguillae (HVA) isolations from disease outbreaks in cultured European eel, *Anguilla anguilla* in the Netherlands since 1996. *Bulletin of the European Association of Fish Pathologists*, *22*(4), 247–257.

Haenen, O. L. M., Way, K., Bergmann, S. M., & Ariel, E. (2004). The emergence of koi herpesvirus and its significance to European aquaculture. *Bulletin of the European Association of Fish Pathologists*, *24*(6), 293–307.

Han, J. E., Kim, J. H., Renault, T., Jr., Choresca, C., Shin, S. P., Jun, J. W., et al. (2013). Identifying the viral genes encoding envelope glycoproteins for differentiation of cyprinid herpesvirus 3 isolates. *Viruses*, *5*(2), 568–576. http://dx.doi.org/10.3390/v5020568.

Hansen, J. D., Landis, E. D., & Phillips, R. B. (2005). Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(19), 6919–6924. http://dx.doi.org/10.1073/pnas.0500027102.

Hanson, L., Dishon, A., & Kotler, M. (2011). Herpesviruses that infect fish. *Viruses*, *3*(11), 2160–2191. http://dx.doi.org/10.3390/v3112160.

Hara, H., Aikawa, H., Usui, K., & Nakanishi, T. (2006). Outbreaks of koi herpesvirus disease in rivers of Kanagawa prefecture. *Fish Pathology*, *41*(2), 81–83. http://dx.doi.org/10.3147/jsfp.41.81.

Haramoto, E., Kitajima, M., Katayama, H., Ito, T., & Ohgaki, S. (2009). Development of virus concentration methods for detection of koi herpesvirus in water. *Journal of Fish Diseases*, *32*(3), 297–300. http://dx.doi.org/10.1111/j.1365-2761.2008.00977.x.

Haramoto, E., Kitajima, M., Katayama, H., & Ohgaki, S. (2007). Detection of koi herpesvirus DNA in river water in Japan. *Journal of Fish Diseases*, *30*(1), 59–61. http://dx.doi.org/10.1111/j.1365-2761.2007.00778.x.

Harmache, A., LeBerre, M., Droineau, S., Giovannini, M., & Bremont, M. (2006). Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for Novirhabdovirus. *Journal of Virology*, *80*(7), 3655–3659. http://dx.doi.org/10.1128/JVI.80.7.3655-3659.2006.

Hedrick, R. P., Gilad, O., Yun, S., Spangenberg, J., Marty, R., Nordhausen, M., et al. (2000). A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. *Journal of Aquatic Animal Health*, *12*, 44–55.

Hedrick, R. P., Waltzek, T. B., & McDowell, T. S. (2006). Susceptibility of koi carp, common carp, goldfish, and goldfish × common carp hybrids to cyprinid herpesvirus-2 and herpesvirus-3. *Journal of Aquatic Animal Health*, *18*(1), 26–34. http://dx.doi.org/10.1577/h05-028.1.

Honjo, M. N., Minamoto, T., & Kawabata, Z. (2012). Reservoirs of Cyprinid herpesvirus 3 (CyHV-3) DNA in sediments of natural lakes and ponds. *Veterinary Microbiology*, *155*(2–4), 183–190. http://dx.doi.org/10.1016/j.vetmic.2011.09.005.

Honjo, M. N., Minamoto, T., Matsui, K., Uchii, K., Yamanaka, H., Suzuki, A. A., et al. (2010). Quantification of cyprinid herpesvirus 3 in environmental water by using an external standard virus. *Applied and Environmental Microbiology*, *76*(1), 161–168. http://dx.doi.org/10.1128/AEM.02011-09.

Horst, D., Ressing, M. E., & Wiertz, E. J. (2011). Exploiting human herpesvirus immune evasion for therapeutic gain: Potential and pitfalls. *Immunology and Cell Biology*, *89*((3), 359–366. http://dx.doi.org/10.1038/icb.2010.129.

Hutoran, M., Ronen, A., Perelberg, A., Ilouze, M., Dishon, A., Bejerano, I., et al. (2005). Description of an as yet unclassified DNA virus from diseased *Cyprinus carpio* species. *Journal of Virology*, *79*(4), 1983–1991. http://dx.doi.org/10.1128/JVI.79.4.1983-1991.2005.

Ilouze, M., Davidovich, M., Diamant, A., Kotler, M., & Dishon, A. (2010). The outbreak of carp disease caused by CyHV-3 as a model for new emerging viral diseases in aquaculture: A review. *Ecological Research*, *26*(5), 885–892. http://dx.doi.org/10.1007/s11284-010-0694-2.

Ilouze, M., Dishon, A., Kahan, T., & Kotler, M. (2006). Cyprinid herpes virus-3 (CyHV-3) bears genes of genetically distant large DNA viruses. *FEBS Letters*, *580*(18), 4473–4478. http://dx.doi.org/10.1016/j.febslet.2006.07.013.

Ilouze, M., Dishon, A., & Kotler, M. (2006). Characterization of a novel virus causing a lethal disease in carp and koi. *Microbiology and Molecular Biology Reviews*, *70*(1), 147–156. http://dx.doi.org/10.1128/MMBR.70.1.147-156.2006.

Ilouze, M., Dishon, A., & Kotler, M. (2012a). Coordinated and sequential transcription of the cyprinid herpesvirus-3 annotated genes. *Virus Research*, *169*(1), 98–106. http://dx.doi.org/10.1016/j.virusres.2012.07.015.

Ilouze, M., Dishon, A., & Kotler, M. (2012b). Down-regulation of the cyprinid herpesvirus-3 annotated genes in cultured cells maintained at restrictive high temperature. *Virus Research*, *169*(1), 289–295. http://dx.doi.org/10.1016/j.virusres.2012.07.013.

Imajoh, M., Fujioka, H., Furusawa, K., Tamura, K., Yamasaki, K., Kurihara, S., et al. (2014). Establishment of a new cell line susceptible to Cyprinid herpesvirus 3 (CyHV-3) and possible latency of CyHV-3 by temperature shift in the cells. *Journal of Fish Diseases*. http://dx.doi.org/10.1111/jfd.12252.

Ishioka, T., Yoshizumi, M., Izumi, S., Suzuki, K., Suzuki, H., Kozawa, K., et al. (2005). Detection and sequence analysis of DNA polymerase and major envelope protein genes in koi herpesviruses derived from *Cyprinus carpio* in Gunma prefecture, Japan. *Veterinary Microbiology*, *110*(1–2), 27–33. http://dx.doi.org/10.1016/j.vetmic.2005.07.002.

Ito, T., Kurita, J., & Yuasa, K. (2014). Differences in the susceptibility of Japanese indigenous and domesticated Eurasian common carp (*Cyprinus carpio*), identified by mitochondrial DNA typing, to cyprinid herpesvirus 3 (CyHV-3). *Veterinary Microbiology*, *171*(1–2), 31–40. http://dx.doi.org/10.1016/j.vetmic.2014.03.002.

Ito, T., & Maeno, Y. (2014). Effects of experimentally induced infections of goldfish *Carassius auratus* with cyprinid herpesvirus 2 (CyHV-2) at various water temperatures. *Diseases of Aquatic Organisms*, *110*(3), 193–200. http://dx.doi.org/10.3354/dao02759.

Ito, T., Sano, M., Kurita, J., Yuasa, K., & Iida, T. (2007). Carp larvae are not susceptible to Koi Herpesvirus. *Fish Pathology*, *42*(2), 107–109.

Kattlun, J., Menanteau-Ledouble, S., & El-Matbouli, M. (2014). Non-structural protein pORF 12 of cyprinid herpesvirus 3 is recognized by the immune system of the common carp *Cyprinus carpio*. *Diseases of Aquatic Organisms*, *111*(3), 269–273. http://dx.doi.org/10.3354/dao02793.

Kelley, G. O., Waltzek, T. B., McDowell, T. S., Yun, S. C., LaPatra, S. E., & Hedrick, R. P. (2005). Genetic relationships among Herpes-like viruses isolated from sturgeon. *Journal of Aquatic Animal Health*, *17*(4), 297–303. http://dx.doi.org/10.1577/h05-002.1.

Kempter, J., & Bergmann, S. M. (2007). Detection of koi herpesvirus (KHV) genome in wild and farmed fish from Northern Poland. *Aquaculture*, *272*(Suppl. 1), S275.

Kempter, J., Kielpinski, M., Panicz, R., Sadowski, J., Myslowski, B., & Bergmann, S. M. (2012). Horizontal transmission of koi herpes virus (KHV) from potential vector species to common carp. *Bulletin of the European Association of Fish Pathologists*, *32*(6), 212–219.

Kempter, J., Sadowski, J., Schütze, H., Fischer, U., Dauber, M., Fichtner, D., et al. (2009). Koi herpes virus: Do acipenserid restitution programs pose a threat to carp farms in the disease-free zones? *Acta Ichthyologica Et Piscatoria*, *39*(2), 119–126. http://dx.doi.org/10.3750/aip2009.39.2.06.

Kielpinski, M., Kempter, J., Panicz, R., Sadowski, J., Schütze, H., Ohlemeyer, S., et al. (2010). Detection of KHV in freshwater mussels and crustaceans from ponds with KHV history in common carp (*Cyprinus carpio*). *Israeli Journal of Aquaculture-Bamidgeh*, *62*(1), 28–37.

Kim, H. J., & Kwon, S. R. (2013). Evidence for two koi herpesvirus (KHV) genotypes in South Korea. *Diseases of Aquatic Organisms*, *104*(3), 197–202. http://dx.doi.org/10.3354/dao02590.

Kongchum, P., Sandel, E., Lutzky, S., Hallerman, E. M., Hulata, G., David, L., et al. (2011). Association between IL-10a single nucleotide polymorphisms and resistance to cyprinid herpesvirus-3 infection in common carp (*Cyprinus carpio*). *Aquaculture*, *315*(3–4), 417–421. http://dx.doi.org/10.1016/j.aquaculture.2011.02.035.

Kucuktas, H., & Brady, Y. J. (1999). Molecular biology of channel catfish virus. *Aquaculture*, *172*(1–2), 147–161. http://dx.doi.org/10.1016/s0044-8486(98)00442-6.

Kurita, J., Yuasa, K., Ito, T., Sano, M., Hedrick, R. P., Engelsma, M. Y., et al. (2009). Molecular epidemiology of koi herpesvirus. *Fish Pathology*, *44*(2), 59–66.

Kurobe, T., Kelley, G. O., Waltzek, T. B., & Hedrick, R. P. (2008). Revised phylogenetic relationships among herpesviruses isolated from sturgeons. *Journal of Aquatic Animal Health*, *20*(2), 96–102. http://dx.doi.org/10.1577/H07-028.1.

Lee, N. S., Jung, S. H., Park, J. W., & Do, J. W. (2012). *In situ* hybridization detection of koi herpesvirus in paraffin-embedded tissues of common carp *Cyprinus carpio* collected in 1998 in Korea. *Fish Pathology*, *47*(3), 100–103. http://dx.doi.org/10.3147/jsfp.47.100.

Li, W., Lee, X., Weng, S., He, J., & Dong, C. (2015). Whole-genome sequence of a novel Chinese cyprinid herpesvirus 3 isolate reveals the existence of a distinct European genotype in East Asia. *Veterinary Microbiology*, *175*(2–4), 185–194. http://dx.doi.org/10.1016/j.vetmic.2014.11.022.

Lin, S. L., Cheng, Y. H., Wen, C. M., & Chen, S. N. (2013). Characterization of a novel cell line from the caudal fin of koi carp *Cyprinus carpio*. *Journal of Fish Biology*, *82*(6), 1888–1903. http://dx.doi.org/10.1111/jfb.12116.

Lio-Po, G. D. (2011). Recent developments in the study and surveillance of koi herpesvirus (KHV) in Asia. In M. G. Bondad-Reantaso, J. B. Jones, F. Corsin, & T. Aoki (Eds.), *Proceedings of the seventh symposium on diseases in Asian Aquaculture* (385 p.). Selangor, Malaysia: Fish Health Section, Asian Fisheries Society.

Liu, Z., Ke, H., Ma, Y., Hao, L., Feng, G., Ma, J., et al. (2014). Oral passive immunization of carp *Cyprinus carpio* with anti-CyHV-3 chicken egg yolk immunoglobulin (IgY). *Fish Pathology*, *49*(3), 113–120.

Loret, S., Guay, G., & Lippe, R. (2008). Comprehensive characterization of extracellular herpes simplex virus type 1 virions. *Journal of Virology*, *82*(17), 8605–8618. http://dx.doi.org/10.1128/JVI.00904-08.

Mabuchi, K., Senou, H., Suzuki, T., & Nishida, M. (2005). Discovery of an ancient lineage of *Cyprinus carpio* from Lake Biwa, central Japan, based on mtDNA sequence data, with reference to possible multiple origins of koi. *Journal of Fish Biology*, *66*(6), 1516–1528. http://dx.doi.org/10.1111/j.0022-1112.2005.00676.x.

Marcos-Lopez, M., Waltzek, T. B., Hedrick, R. P., Baxa, D. V., Garber, A. F., Liston, R., et al. (2012). Characterization of a novel alloherpesvirus from Atlantic cod (*Gadus morhua*). *Journal of Veterinary Diagnostic Investigation*, *24*(1), 65–73. http://dx.doi.org/10.1177/1040638711416629.

McCleary, S., Ruane, N. M., Cheslett, D., Hickey, C., Rodger, H. D., Geoghegan, F., et al. (2011). Detection of koi herpesvirus (KHV) in koi carp (*Cyprinus carpio* L.) imported into Ireland. *Bulletin of the European Association of Fish Pathologists*, *31*(3), 124–128.

McColl, K. A., Cooke, B. D., & Sunarto, A. (2014). Viral biocontrol of invasive vertebrates: Lessons from the past applied to cyprinid herpesvirus-3 and carp (*Cyprinus carpio*) control in Australia. *Biological Control*, *72*, 109–117. http://dx.doi.org/10.1016/j.biocontrol.2014.02.014.

McDermott, C., & Palmeiro, B. (2013). Selected emerging infectious diseases of ornamental fish. *Veterinary Clinics of North America: Exotic Animal Practice*, *16*(2), 261–282. http://dx.doi.org/10.1016/j.cvex.2013.01.006.

McGeoch, D. J., Dolan, A., & Ralph, A. C. (2000). Toward a comprehensive phylogeny for mammalian and avian herpesviruses. *Journal of Virology*, *74*(22), 10401–10406.

McGeoch, D. J., & Gatherer, D. (2005). Integrating reptilian herpesviruses into the family *Herpesviridae*. *Journal of Virology*, *79*(2), 725–731.

McGeoch, D. J., Rixon, F. J., & Davison, A. J. (2006). Topics in herpesvirus genomics and evolution. *Virus Research*, *117*(1), 90–104. http://dx.doi.org/10.1016/j.virusres.2006.01.002.

McKinnell, R. G., & Tarin, D. (1984). Temperature-dependent metastasis of the Lucke renal carcinoma and its significance for studies on mechanisms of metastasis. *Cancer and Metastasis Reviews*, *3*(4), 373–386.

Meeusen, E. N., Walker, J., Peters, A., Pastoret, P. P., & Jungersen, G. (2007). Current status of veterinary vaccines. *Clinical Microbiology Reviews*, *20*(3), 489–510. http://dx.doi.org/10.1128/CMR.00005-07.

Mettenleiter, T. C. (2004). Budding events in herpesvirus morphogenesis. *Virus Research*, *106*(2), 167–180. http://dx.doi.org/10.1016/j.virusres.2004.08.013.

Mettenleiter, T. C., Klupp, B. G., & Granzow, H. (2009). Herpesvirus assembly: An update. *Virus Research*, *143*(2), 222–234. http://dx.doi.org/10.1016/j.virusres.2009.03.018.

Michel, B., Fournier, G., Lieffrig, F., Costes, B., & Vanderplasschen, A. (2010). Cyprinid herpesvirus 3. *Emerging Infectious Diseases*, *16*(12), 1835–1843. http://dx.doi.org/10.3201/eid1612.100593.

Michel, B., Leroy, B., Stalin Raj, V., Lieffrig, F., Mast, J., Wattiez, R., et al. (2010). The genome of cyprinid herpesvirus 3 encodes 40 proteins incorporated in mature virions. *Journal of General Virology*, *91*(Pt 2), 452–462. http://dx.doi.org/10.1099/vir.0.015198-0.

Midtlyng, P. J., Storset, A., Michel, C., Slierendrecht, W. J., & Okamoto, N. (2002). Breeding for disease resistance in fish. *Bulletin of the European Association of Fish Pathologists*, *22*(2), 166–172.

Miest, J. J., Adamek, M., Pionnier, N., Harris, S., Matras, M., Rakus, K. L., et al. (2015). Differential effects of alloherpesvirus CyHV-3 and rhabdovirus SVCV on apoptosis in fish cells. *Veterinary Microbiology*, *176*(1–2), 19–31. http://dx.doi.org/10.1016/j.vetmic.2014.12.012.

Minamoto, T., Honjo, M. N., & Kawabata, Z. (2009). Seasonal distribution of cyprinid herpesvirus 3 in Lake Biwa, Japan. *Applied and Environmental Microbiology*, *75*(21), 6900–6904. http://dx.doi.org/10.1128/AEM.01411-09.

Minamoto, T., Honjo, M. N., Uchii, K., Yamanaka, H., Suzuki, A. A., Kohmatsu, Y., et al. (2009). Detection of cyprinid herpesvirus 3 DNA in river water during and after an outbreak. *Veterinary Microbiology*, *135*(3–4), 261–266. http://dx.doi.org/10.1016/j.vetmic.2008.09.081.

Minamoto, T., Honjo, M. N., Yamanaka, H., Tanaka, N., Itayama, T., & Kawabata, Z. (2011). Detection of cyprinid herpesvirus-3 DNA in lake plankton. *Research in Veterinary Science*, *90*(3), 530–532. http://dx.doi.org/10.1016/j.rvsc.2010.07.006.

Minamoto, T., Honjo, M. N., Yamanaka, H., Uchii, K., & Kawabata, Z. (2012). Nationwide Cyprinid herpesvirus 3 contamination in natural rivers of Japan. *Research in Veterinary Science*, *93*(1), 508–514. http://dx.doi.org/10.1016/j.rvsc.2011.06.004.

Miwa, S., Ito, T., & Sano, M. (2007). Morphogenesis of koi herpesvirus observed by electron microscopy. *Journal of Fish Diseases*, *30*(12), 715–722. http://dx.doi.org/10.1111/j.1365-2761.2007.00850.x.

Miwa, S., Kiryu, I., Yuasa, K., Ito, T., & Kaneko, T. (2014). Pathogenesis of acute and chronic diseases caused by cyprinid herpesvirus-3. *Journal of Fish Diseases*. http://dx.doi.org/10.1111/jfd.12282.

Miyazaki, T., Kuzuya, Y., Yasumoto, S., Yasuda, M., & Kobayashi, T. (2008). Histopathological and ultrastructural features of Koi herpesvirus (KHV)-infected carp *Cyprinus carpio*, and the morphology and morphogenesis of KHV. *Diseases of Aquatic Organisms*, *80*(1), 1–11. http://dx.doi.org/10.3354/dao01929.

Miyazaki, T., Yasumoto, S., Kuzuya, Y., & Yoshimura, T. (2005). In *A primary study on oral vaccination with liposomes entrapping koi herpesvirus (KHV) antigens against KHV infection in carp. Paper presented at the diseases in Asian aquaculture, Colombo, Sri Lanka.*

Monaghan, S. J., Thompson, K. D., Adams, A., & Bergmann, S. M. (2015). Sensitivity of seven PCRs for early detection of koi herpesvirus in experimentally infected carp, *Cyprinus carpio* L., by lethal and non-lethal sampling methods. *Journal of Fish Diseases*, *38*(3), 303–319. http://dx.doi.org/10.1111/jfd.12235.

Monaghan, S. J., Thompson, K. D., Adams, A., Kempter, J., & Bergmann, S. M. (2015). Examination of the early infection stages of koi herpesvirus (KHV) in experimentally infected carp Cyprinus carpio L. using in situ hybridization. *Journal of Fish Diseases*, *38*(5), 477–489. http://dx.doi.org/10.1111/jfd.12260.

Musa, N., Leong, N. K., & Sunarto, A. (2005). *Koi herpesvirus (KHV)—An emerging pathogen in koi. Paper presented at the Colloquium on Viruses of Veterinary and Public Health Importance.* Malaysia: Bangi.

Neukirch, M., Böttcher, K., & Bunnajrakul, S. (1999). Isolation of a virus from koi with altered gills. *Bulletin of the European Association of Fish Pathologists*, *19*, 221–224.

Neukirch, M., & Kunz, U. (2001). Isolation and preliminary characterization of several viruses from koi (*Cyprinus carpio*) suffering gill necrosis and mortality. *Bulletin of the European Association of Fish Pathologists*, *21*(4), 125–135.

O'Connor, M. R., Farver, T. B., Malm, K. V., Yun, S. C., Marty, G. D., Salonius, K., et al. (2014). Protective immunity of a modified-live cyprinid herpesvirus 3 vaccine in koi (*Cyprinus carpio koi*) 13 months after vaccination. *American Journal of Veterinary Research*, *75*(10), 905–911. http://dx.doi.org/10.2460/ajvr.75.10.905.

Ødegård, J., Olesena, I., Dixonb, P., Jeneyc, Z., Nielsena, H.-M., Wayb, K., et al. (2010). Genetic analysis of common carp (*Cyprinus carpio*) strains II: Resistance to koi herpesvirus and Aeromonas hydrophila and their relationship with pond survival. *Aquaculture*, *304*(1–4), 7–13.

OIE. (2012). Chapter 2.3.6. Koi herpesvirus disease. In *Manual of Diagnostic Tests for Aquatic Animals* (pp. 328–344).

Ouyang, P., Rakus, K., Boutier, M., Reschner, A., Leroy, B., Ronsmans, M., et al. (2013). The IL-10 homologue encoded by cyprinid herpesvirus 3 is essential neither for viral replication *in vitro* nor for virulence *in vivo*. *Veterinary Research*, *44*(1), 53.

Ouyang, P., Rakus, K., van Beurden, S. J., Westphal, A. H., Davison, A. J., Gatherer, D., et al. (2014). IL-10 encoded by viruses: A remarkable example of independent acquisition of a cellular gene by viruses and its subsequent evolution in the viral genome. *Journal of General Virology*, *95*(Pt 2), 245–262. http://dx.doi.org/10.1099/vir.0.058966-0.

Pellett, P. E., Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., Lacoste, V., et al. (2011a). Alloherpesviridae. In A. M. Q. King, E. Lefkowitz, M. J. Adams, & E. B. Carstens (Eds.), *Virus taxonomy: Ninth report of the International Committee on Taxonomy of Viruses* (pp. 108–110). London, United Kingdom: Elsevier Academic Press.

Pellett, P. E., Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., Lacoste, V., et al. (2011b). Herpesvirales. In A. M. Q. King, E. Lefkowitz, M. J. Adams, & E. B. Carstens (Eds.), *Virus taxonomy: Ninth report of the International Committee on Taxonomy of Viruses* (pp. 99–107). London, United Kingdom: Elsevier Academic Press.

Pellett, P. E., Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., Lacoste, V., et al. (2011c). Herpesviridae. In A. M. Q. King, E. Lefkowitz, M. J. Adams, & E. B. Carstens (Eds.), *Virus taxonomy: Ninth report of the International Committee on Taxonomy of Viruses* (pp. 111–122). London, United Kingdom: Elsevier Academic Press.

Pellett, P. E., Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., Lacoste, V., et al. (2011d). Malacoherpesviridae. In A. M. Q. King, E. Lefkowitz, M. J. Adams, & E. B. Carstens (Eds.), *Virus taxonomy: Ninth report of the International Committee on Taxonomy of Viruses* (p. 123). London, United Kingdom: Elsevier Academic Press.

Perelberg, A., Ilouze, M., Kotler, M., & Steinitz, M. (2008). Antibody response and resistance of *Cyprinus carpio* immunized with cyprinid herpes virus 3 (CyHV-3). *Vaccine*, *26*(29–30), 3750–3756. http://dx.doi.org/10.1016/j.vaccine.2008.04.057.

Perelberg, A., Ronen, A., Hutoran, M., Smith, Y., & Kotler, M. (2005). Protection of cultured *Cyprinus carpio* against a lethal viral disease by an attenuated virus vaccine. *Vaccine*, *23*(26), 3396–3403. http://dx.doi.org/10.1016/j.vaccine.2005.01.096.

Perelberg, A., Smirnov, M., Hutoran, M., Diamant, A., Bejerano, Y., & Kotler, M. (2003). Epidemiological description of a new viral disease afflicting cultured *Cyprinus carpio* in Israel. *Israeli Journal of Aquaculture-Bamidgeh*, *55*(1), 5–12.

Piackova, V., Flajshans, M., Pokorova, D., Reschova, S., Gela, D., Cizek, A., et al. (2013). Sensitivity of common carp, *Cyprinus carpio* L., strains and crossbreeds reared in the Czech Republic to infection by cyprinid herpesvirus 3 (CyHV-3; KHV). *Journal of Fish Diseases*, *36*(1), 75–80. http://dx.doi.org/10.1111/jfd.12007.

Piazzon, M. C., Savelkoul, H. F., Pietretti, D., Wiegertjes, G. F., & Forlenza, M. (2015). Carp Il10 has anti-inflammatory activities on phagocytes, promotes proliferation of memory T cells, and regulates B cell differentiation and antibody secretion. *Journal of Immunology*, *194*(1), 187–199. http://dx.doi.org/10.4049/jimmunol.1402093.

Pikarsky, E., Ronen, A., Abramowitz, J., Levavi-Sivan, B., Hutoran, M., Shapira, Y., et al. (2004). Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. *Journal of Virology*, *78*(17), 9544–9551. http://dx.doi.org/10.1128/JVI.78.17.9544-9551.2004.

Pikulkaew, S., Meeyam, T., & Banlunara, W. (2009). The outbreak of koi herpesvirus (KHV) in koi (*Cyprinus carpio koi*) from Chiang Mai province, Thailand. *Thai Journal of Veterinary Medicine*, *39*(1), 53–58.

Pionnier, N., Adamek, M., Miest, J. J., Harris, S. J., Matras, M., Rakus, K. L., et al. (2014). C-reactive protein and complement as acute phase reactants in common carp *Cyprinus carpio* during CyHV-3 infection. *Diseases of Aquatic Organisms*, *109*(3), 187–199. http://dx.doi.org/10.3354/dao02727.

Plant, K. P., & Lapatra, S. E. (2011). Advances in fish vaccine delivery. *Developmental and Comparative Immunology*, *35*(12), 1256–1262. http://dx.doi.org/10.1016/j.dci.2011.03.007.

Pokorova, D., Reschova, S., Hulova, J., Vicenova, M., Vesely, T., & Piackova, V. (2010). Detection of cyprinid herpesvirus-3 in field samples of common and koi carp by various single-round and nested PCR methods. *Journal of the World Aquaculture Society*, *41*(5), 773–779. http://dx.doi.org/10.1111/j.1749-7345.2010.00419.x.

Pokorova, D., Vesely, T., Piackova, V., Reschova, S., & Hulova, J. (2005). Current knowledge on koi herpesvirus (KHV): A review. *Veterinarni Medicina*, *5*(4), 139–147.

Pretto, T., Manfrin, A., Ceolin, C., Dalla Pozza, M., Zelco, S., Quartesan, R., et al. (2013). First isolation of koi herpes virus (KHV) in Italy from imported koi (*Cyprinus carpio koi*). *Bulletin of the European Association of Fish Pathologists*, *33*(4), 126–133.

Radosavljevic, V., Jeremic, S., Cirkovic, M., Lako, B., Milicevic, V., Potkonjak, A., et al. (2012). Common fish species in polyculture with carp as cyprinid herpes virus 3 carriers. *Acta Veterinaria*, *62*(5–6), 675–681. http://dx.doi.org/10.2298/avb1206675r.

Raj, V. S., Fournier, G., Rakus, K., Ronsmans, M., Ouyang, P., Michel, B., et al. (2011). Skin mucus of *Cyprinus carpio* inhibits cyprinid herpesvirus 3 binding to epidermal cells. *Veterinary Research*, *42*, 92. http://dx.doi.org/10.1186/1297-9716-42-92.

Rakus, K. L., Irnazarow, I., Adamek, M., Palmeira, L., Kawana, Y., Hirono, I., et al. (2012). Gene expression analysis of common carp (*Cyprinus carpio* L.) lines during Cyprinid herpesvirus 3 infection yields insights into differential immune responses. *Developmental and Comparative Immunology*, *37*(1), 65–76. http://dx.doi.org/10.1016/j.dci.2011.12.006.

Rakus, K. L., Ouyang, P., Boutier, M., Ronsmans, M., Reschner, A., Vancsok, C., et al. (2013). Cyprinid herpesvirus 3: An interesting virus for applied and fundamental research. *Veterinary Research*, *44*, 85. http://dx.doi.org/10.1186/1297-9716-44-85.

Rakus, K. L., Wiegertjes, G. F., Adamek, M., Siwicki, A. K., Lepa, A., & Irnazarow, I. (2009). Resistance of common carp (*Cyprinus carpio* L.) to cyprinid herpesvirus-3 is influenced by major histocompatibility (MH) class II *B* gene polymorphism. *Fish & Shellfish Immunology*, *26*(5), 737–743. http://dx.doi.org/10.1016/j.fsi.2009.03.001.

Rakus, K. L., Wiegertjes, G. F., Jurecka, P., Walker, P. D., Pilarczyk, A., & Irnazarow, I. (2009). Major histocompatibility (MH) class II *B* gene polymorphism influences disease resistance of common carp (*Cyprinus carpio* L.). *Aquaculture*, *288*(1–2), 44–50. http://dx.doi.org/10.1016/j.aquaculture.2008.11.016.

Reed, A. N., Izume, S., Dolan, B. P., LaPatra, S., Kent, M., Dong, J., et al. (2014). Identification of B cells as a major site for cyprinid herpesvirus 3 latency. *Journal of Virology*, *88*(16), 9297–9309. http://dx.doi.org/10.1128/JVI.00990-14.

Rixon, F. J., & Schmid, M. F. (2014). Structural similarities in DNA packaging and delivery apparatuses in Herpesvirus and dsDNA bacteriophages. *Current Opinion in Virology*, *5*, 105–110. http://dx.doi.org/10.1016/j.coviro.2014.02.003.

Rombout, J. H., Yang, G., & Kiron, V. (2014). Adaptive immune responses at mucosal surfaces of teleost fish. *Fish & Shellfish Immunology*, *40*(2), 634–643. http://dx.doi.org/10.1016/j.fsi.2014.08.020.

Ronen, A., Perelberg, A., Abramowitz, J., Hutoran, M., Tinman, S., Bejerano, I., et al. (2003). Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*. *Vaccine*, *21*(32), 4677–4684.

Ronsmans, M., Boutier, M., Rakus, K., Farnir, F., Desmecht, D., Ectors, F., et al. (2014). Sensitivity and permissivity of *Cyprinus carpio* to cyprinid herpesvirus 3 during the early stages of its development: Importance of the epidermal mucus as an innate immune barrier. *Veterinary Research*, *45*(1), 100. http://dx.doi.org/10.1186/s13567-014-0100-0.

Rosenkranz, D., Klupp, B. G., Teifke, J. P., Granzow, H., Fichtner, D., Mettenleiter, T. C., et al. (2008). Identification of envelope protein pORF81 of koi herpesvirus. *Journal of General Virology*, *89*(Pt 4), 896–900. http://dx.doi.org/10.1099/vir.0.83565-0.

Rothenburg, S., Deigendesch, N., Dittmar, K., Koch-Nolte, F., Haag, F., Lowenhaupt, K., et al. (2005). A PKR-like eukaryotic initiation factor 2alpha kinase from zebrafish contains Z-DNA binding domains instead of dsRNA binding domains. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(5), 1602–1607. http://dx.doi.org/10.1073/pnas.0408714102.

Rueckert, C., & Guzman, C. A. (2012). Vaccines: From empirical development to rational design. *PLoS Pathogens*, *8*(11), e1003001. http://dx.doi.org/10.1371/journal.ppat.1003001.

Ryo, S., Wijdeven, R. H., Tyagi, A., Hermsen, T., Kono, T., Karunasagar, I., et al. (2010). Common carp have two subclasses of bonyfish specific antibody IgZ showing differential expression in response to infection. *Developmental and Comparative Immunology*, *34*(11), 1183–1190. http://dx.doi.org/10.1016/j.dci.2010.06.012.

Sadler, J., Marecaux, E., & Goodwin, A. E. (2008). Detection of koi herpes virus (CyHV-3) in goldfish, *Carassius auratus* (L.), exposed to infected koi. *Journal of Fish Diseases*, *31*(1), 71–72. http://dx.doi.org/10.1111/j.1365-2761.2007.00830.x.

Sano, T., Fukuda, H., & Furukawa, M. (1985). Herpesvirus cyprini: Biological and oncogenic properties. *Fish Pathology*, *20*(2/3), 381–388. http://dx.doi.org/10.3147/jsfp.20.381.

Sano, M., Fukuda, H., & Sano, T. (1990). Isolation and characterization of a new herpesvirus from eel. In P. O. Perkins & T. C. Cheng (Eds.), *Pathology in marine sciences* (pp. 15–31). San Diego, CA: Academic Press.

Sano, M., Ito, T., Kurita, J., Yanai, T., Watanabe, N., Satoshi, M., et al. (2004). First detection of koi herpes virus in cultured common carp *Cyprinus carpio* in Japan. *Fish Pathology*, *39*, 165–168.

Schubert, G. H. (1966). The infective agent in carp pox. *Bulletin de l'Office International des Epizooties*, *65*(7), 1011–1022.

Shapira, Y., Magen, Y., Zak, T., Kotler, M., Hulata, G., & Levavi-Sivan, B. (2005). Differential resistance to koi herpes virus (KHV)/carp interstitial nephritis and gill necrosis virus (CNGV) among common carp (*Cyprinus carpio* L.) strains and crossbreds. *Aquaculture*, *245*(1–4), 1–11.

Shephard, K. L. (1994). Functions for fish mucus. *Reviews in Fish Biology and Fisheries*, *4*(4), 401–429. http://dx.doi.org/10.1007/bf00042888.

Shimizu, T., Yoshida, N., Kasai, H., & Yoshimizu, M. (2006). Survival of koi herpesvirus (KHV) in environmental water. *Fish Pathology*, *41*(4), 153–157.

Sievers, F., & Higgins, D. G. (2014). Clustal omega. *Current Protocols in Bioinformatics*, *48*, 3.13.11–13.13.16. http://dx.doi.org/10.1002/0471250953.bi0313s48.

Siwicki, A., Kazuń, K., Kazuń, B., & Majewicz-Zbikowska, E. (2012). Impact of cyprinid herpesvirus-3, which causes interstitial nephritis and gill necrosis, on the activity of carp (*Cyprinus carpio* L.) macrophages and lymphocytes. *Archives of Polish Fisheries*, *20*(2), 123–128. http://dx.doi.org/10.2478/v10086-012-0014-2.

Soliman, H., & El-Matbouli, M. (2010). Loop mediated isothermal amplification combined with nucleic acid lateral flow strip for diagnosis of cyprinid herpes virus-3. *Molecular and Cellular Probes*, *24*(1), 38–43. http://dx.doi.org/10.1016/j.mcp.2009.09.002.

Sommerset, I., Krossoy, B., Biering, E., & Frost, P. (2005). Vaccines for fish in aquaculture. *Expert Review of Vaccines*, *4*(1), 89–101. http://dx.doi.org/10.1586/14760584.4.1.89.

St-Hilaire, S., Beevers, N., Joiner, C., Hedrick, R. P., & Way, K. (2009). Antibody response of two populations of common carp, *Cyprinus carpio* L., exposed to koi herpesvirus. *Journal of Fish Diseases*, *32*(4), 311–320. http://dx.doi.org/10.1111/j.1365-2761.2008.00993.x.

St-Hilaire, S., Beevers, N., Way, K., Le Deuff, R. M., Martin, P., & Joiner, C. (2005). Reactivation of koi herpesvirus infections in common carp *Cyprinus carpio*. *Diseases of Aquatic Organisms*, *67*(1–2), 15–23. http://dx.doi.org/10.3354/dao067015.

Stingley, R. L., & Gray, W. L. (2000). Transcriptional regulation of the channel catfish virus genome direct repeat region. *Journal of General Virology*, *81*(Pt 8), 2005–2010.

Sunarto, A., Liongue, C., McColl, K. A., Adams, M. M., Bulach, D., Crane, M. S., et al. (2012). Koi herpesvirus encodes and expresses a functional interleukin-10. *Journal of Virology*, *86*(21), 11512–11520. http://dx.doi.org/10.1128/JVI.00957-12.

Sunarto, A., McColl, K. A., Crane, M. S., Schat, K. A., Slobedman, B., Barnes, A. C., et al. (2014). Characteristics of cyprinid herpesvirus 3 in different phases of infection: Implications for disease transmission and control. *Virus Research*, *188*, 45–53. http://dx.doi.org/10.1016/j.virusres.2014.03.024.

Sunarto, A., McColl, K. A., Crane, M. S., Sumiati, T., Hyatt, A. D., Barnes, A. C., et al. (2011). Isolation and characterization of koi herpesvirus (KHV) from Indonesia: Identification of a new genetic lineage. *Journal of Fish Diseases*, *34*(2), 87–101. http://dx.doi.org/10.1111/j.1365-2761.2010.01216.x.

Sunarto, A., Rukyani, A., & Itami, T. (2005). Indonesian experience on the outbreak of koi herpesvirus in koi and carp (*Cyprinus carpio*). *Bulletin of Fisheries Research Agency*, (Suppl. 2), 15–21.

Syakuri, H., Adamek, M., Brogden, G., Rakus, K. L., Matras, M., Irnazarow, I., et al. (2013). Intestinal barrier of carp (*Cyprinus carpio* L.) during a cyprinid herpesvirus 3-infection: Molecular identification and regulation of the mRNA expression of claudin encoding genes. *Fish & Shellfish Immunology*, *34*(1), 305–314. http://dx.doi.org/10.1016/j.fsi.2012.11.010.

Takahara, T., Honjo, M. N., Uchii, K., Minamoto, T., Doi, H., Ito, T., et al. (2014). Effects of daily temperature fluctuation on the survival of carp infected with Cyprinid herpesvirus 3. *Aquaculture*, *433*, 208–213. http://dx.doi.org/10.1016/j.aquaculture.2014.06.001.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*(12), 2725–2729. http://dx.doi.org/10.1093/molbev/mst197.

Taylor, N. G., Dixon, P. F., Jeffery, K. R., Peeler, E. J., Denham, K. L., & Way, K. (2010). Koi herpesvirus: Distribution and prospects for control in England and Wales. *Journal of Fish Diseases*, *33*(3), 221–230. http://dx.doi.org/10.1111/j.1365-2761.2009.01111.x.

Taylor, N. G., Norman, R. A., Way, K., & Peeler, E. J. (2011). Modelling the koi herpesvirus (KHV) epidemic highlights the importance of active surveillance within a national control policy. *Journal of Applied Ecology*, *48*(2), 348–355.

Taylor, N. G., Way, K., Jeffery, K. R., & Peeler, E. J. (2010). The role of live fish movements in spreading koi herpesvirus throughout England and Wales. *Journal of Fish Diseases*, *33*(12), 1005–1007.

Terhune, J. S., Grizzle, J. M., Hayden, K., McClenahan, S. D., Lamprecht, S. D., & White, M. G. (2004). First report of koi herpesvirus in wild common carp in the Western Hemisphere. *Fish Health Newsletter*, *32*(3), 8–9.

Tischer, B. K., & Kaufer, B. B. (2012). Viral bacterial artificial chromosomes: Generation, mutagenesis, and removal of mini-F sequences. *Journal of Biomedicine and Biotechnology*, *2012*, 472537. http://dx.doi.org/10.1155/2012/472537.

Tome, A. R., Kus, K., Correia, S., Paulo, L. M., Zacarias, S., de Rosa, M., et al. (2013). Crystal structure of a poxvirus-like zalpha domain from cyprinid herpesvirus 3. *Journal of Virology*, *87*(7), 3998–4004. http://dx.doi.org/10.1128/JVI.03116-12.

Tu, C., Lu, Y. P., Hsieh, C. Y., Huang, S. M., Chang, S. K., & Chen, M. M. (2014). Production of monoclonal antibody against ORF72 of koi herpesvirus isolated in Taiwan. *Folia Microbiologica*, *59*(2), 159–165. http://dx.doi.org/10.1007/s12223-013-0261-7.

Tu, C., Weng, M. C., Shiau, J. R., & Lin, S. Y. (2004). Detection of koi herpesvirus in koi *Cyprinus carpio* in Taiwan. *Fish Pathology*, *39*(2), 109–110.

Uchii, K., Matsui, K., Iida, T., & Kawabata, Z. (2009). Distribution of the introduced cyprinid herpesvirus 3 in a wild population of common carp, *Cyprinus carpio* L. *Journal of Fish Diseases*, *32*(10), 857–864. http://dx.doi.org/10.1111/j.1365-2761.2009.01064.x.

Uchii, K., Minamoto, T., Honjo, M. N., & Kawabata, Z. (2014). Seasonal reactivation enables Cyprinid herpesvirus 3 to persist in a wild host population. *FEMS Microbiology Ecology*, *87*(2), 536–542. http://dx.doi.org/10.1111/1574-6941.12242.

Uchii, K., Okuda, N., Minamoto, T., & Kawabata, Z. (2013). An emerging infectious pathogen endangers an ancient lineage of common carp by acting synergistically with conspecific exotic strains. *Animal Conservation*, *16*(3), 324–330. http://dx.doi.org/10.1111/j.1469-1795.2012.00604.x.

Uchii, K., Telschow, A., Minamoto, T., Yamanaka, H., Honjo, M. N., Matsui, K., et al. (2011). Transmission dynamics of an emerging infectious disease in wildlife through host reproductive cycles. *ISME Journal*, *5*(2), 244–251. http://dx.doi.org/10.1038/ismej.2010.123.

van Beurden, S. J., Bossers, A., Voorbergen-Laarman, M. H., Haenen, O. L., Peters, S., Abma-Henkens, M. H., et al. (2010). Complete genome sequence and taxonomic position of anguillid herpesvirus 1. *Journal of General Virology*, *91*(Pt 4), 880–887. http://dx.doi.org/10.1099/vir.0.016261-0.

van Beurden, S. J., & Engelsma, M. Y. (2012). Herpesviruses of fish, amphibians and invertebrates. In G. D. Magel & S. Tyring (Eds.), *Herpesviridae—A look into this unique family of viruses* (pp. 217–242). Rijeka: InTech.

van Beurden, S. J., Engelsma, M. Y., Roozenburg, I., Voorbergen-Laarman, M. A., van Tulden, P. W., Kerkhoff, S., et al. (2012). Viral diseases of wild and farmed European eel Anguilla anguilla with particular reference to the Netherlands. *Diseases of Aquatic Organisms*, *101*(1), 69–86. http://dx.doi.org/10.3354/dao02501.

van Beurden, S. J., Forlenza, M., Westphal, A. H., Wiegertjes, G. F., Haenen, O. L., & Engelsma, M. Y. (2011). The alloherpesviral counterparts of interleukin 10 in European eel and common carp. *Fish & Shellfish Immunology*, *31*(6), 1211–1217. http://dx.doi.org/10.1016/j.fsi.2011.08.004.

van Beurden, S. J., Gatherer, D., Kerr, K., Galbraith, J., Herzyk, P., Peeters, B. P., et al. (2012). Anguillid herpesvirus 1 transcriptome. *Journal of Virology*, *86*(18), 10150–10161. http://dx.doi.org/10.1128/JVI.01271-12.

van Beurden, S. J., Leroy, B., Wattiez, R., Haenen, O. L., Boeren, S., Vervoort, J. J., et al. (2011). Identification and localization of the structural proteins of anguillid herpesvirus 1. *Veterinary Research*, *42*(1), 105. http://dx.doi.org/10.1186/1297-9716-42-105.

van Beurden, S. J., Peeters, B. P., Rottier, P. J., Davison, A. J., & Engelsma, M. Y. (2013). Genome-wide gene expression analysis of anguillid herpesvirus 1. *BMC Genomics*, *14*, 83. http://dx.doi.org/10.1186/1471-2164-14-83.

van der Marel, M., Adamek, M., Gonzalez, S. F., Frost, P., Rombout, J. H., Wiegertjes, G. F., et al. (2012). Molecular cloning and expression of two beta-defensin and two mucin genes in common carp (*Cyprinus carpio* L.) and their up-regulation after beta-glucan feeding. *Fish & Shellfish Immunology*, *32*(3), 494–501. http://dx.doi.org/10.1016/j.fsi.2011.12.008.

Vrancken, R., Boutier, M., Ronsmans, M., Reschner, A., Leclipteux, T., Lieffrig, F., et al. (2013). Laboratory validation of a lateral flow device for the detection of CyHV-3 antigens in gill swabs. *Journal of Virological Methods*, *193*(2), 679–682. http://dx.doi.org/10.1016/j.jviromet.2013.07.034.

Walster, C. I. (1999). Clinical observations of severe mortalities in koi carp, *Cyprinus carpio*, with gill disease. *Fish Veterinary Journal*, *3*, 54–58.

Waltzek, T. B., Kelley, G. O., Alfaro, M. E., Kurobe, T., Davison, A. J., & Hedrick, R. P. (2009). Phylogenetic relationships in the family *Alloherpesviridae*. *Diseases of Aquatic Organisms*, *84*(3), 179–194. http://dx.doi.org/10.3354/dao02023.

Waltzek, T. B., Kelley, G. O., Stone, D. M., Way, K., Hanson, L., Fukuda, H., et al. (2005). Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family *Herpesviridae*. *Journal of General Virology*, *86*(Pt 6), 1659–1667. http://dx.doi.org/10.1099/vir.0.80982-0.

Wang, Y., Zeng, W., Li, Y., Liang, H., Liu, C., Pan, H., et al. (2015). Development and characterization of a cell line from the snout of koi (*Cyprinus carpio* L.) for detection of koi herpesvirus. *Aquaculture*, *435*, 310–317. http://dx.doi.org/10.1016/j.aquaculture.2014.10.006.

Weber, E. P., 3rd, Malm, K. V., Yun, S. C., Campbell, L. A., Kass, P. H., Marty, G. D., et al. (2014). Efficacy and safety of a modified-live cyprinid herpesvirus 3 vaccine in koi (*Cyprinus carpio koi*) for prevention of koi herpesvirus disease. *American Journal of Veterinary Research*, *75*(10), 899–904. http://dx.doi.org/10.2460/ajvr.75.10.899.

Whittington, R. J., Crockford, M., Jordan, D., & Jones, B. (2008). Herpesvirus that caused epizootic mortality in 1995 and 1998 in pilchard, *Sardinops sagax neopilchardus* (Steindachner), in Australia is now endemic. *Journal of Fish Diseases*, *31*(2), 97–105. http://dx.doi.org/10.1111/j.1365-2761.2007.00869.x.

Winton, J., Batts, W., deKinkelin, P., LeBerre, M., Bremont, M., & Fijan, N. (2010). Current lineages of the *epithelioma papulosum cyprini* (EPC) cell line are contaminated with fathead minnow, *Pimephales promelas*, cells. *Journal of Fish Diseases*, *33*(8), 701–704. http://dx.doi.org/10.1111/j.1365-2761.2010.01165.x.

Wolf, K. (1988). Channel catfish virus disease. In K. Wolf (Ed.), *Fish viruses and fish viral diseases* (pp. 21–42). Ithaca, New York: Cornell University Press.

Wolf, K., & Darlington, R. W. (1971). Channel catfish virus: A new herpesvirus of ictalurid fish. *Journal of Virology*, *8*(4), 525–533.

Xu, J. R., Bently, J., Beck, L., Reed, A., Miller-Morgan, T., Heidel, J. R., et al. (2013). Analysis of koi herpesvirus latency in wild common carp and ornamental koi in Oregon, USA. *Journal of Virological Methods*, *187*(2), 372–379. http://dx.doi.org/10.1016/j.jviromet.2012.11.015.

Xu, Z., Parra, D., Gomez, D., Salinas, I., Zhang, Y. A., von Gersdorff Jorgensen, L., et al. (2013). Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(32), 13097–13102. http://dx.doi.org/10.1073/pnas.1304319110.

Yasumoto, S., Kuzuya, Y., Yasuda, M., Yoshimura, T., & Miyazaki, T. (2006). Oral immunization of common carp with a liposome vaccine fusing koi herpesvirus antigen. *Fish Pathology*, *41*(4), 141–145.

Yi, Y., Zhang, H., Lee, X., Weng, S., He, J., & Dong, C. (2014). Extracellular virion proteins of two Chinese CyHV-3/KHV isolates, and identification of two novel envelope proteins. *Virus Research*, *191*, 108–116. http://dx.doi.org/10.1016/j.virusres.2014.07.034.

Yoshida, N., Sasaki, R. K., Kasai, H., & Yoshimizu, M. (2013). Inactivation of koi-herpesvirus in water using bacteria isolated from carp intestines and carp habitats. *Journal of Fish Diseases*, *36*(12), 997–1005. http://dx.doi.org/10.1111/j.1365-2761.2012.01449.x.

Yoshino, M., Watari, H., Kojima, T., & Ikedo, M. (2006). Sensitive and rapid detection of koi herpesvirus by LAMP method. *Fish Pathology*, *41*(1), 19–27. http://dx.doi.org/10.3147/jsfp.41.19.

Yoshino, M., Watari, H., Kojima, T., Ikedo, M., & Kurita, J. (2009). Rapid, sensitive and simple detection method for koi herpesvirus using loop-mediated isothermal amplification. *Microbiology and Immunology*, *53*(7), 375–383. http://dx.doi.org/10.1111/j.1348-0421.2009.00145.x.

Yuasa, K., Ito, T., & Sano, M. (2008). Effect of water temperature on mortality and virus shedding in carp experimentally infected with koi herpesvirus. *Fish Pathology*, *43*(2), 83–85. http://dx.doi.org/10.3147/jsfp.43.83.

Yuasa, K., Kurita, J., Kawana, M., Kiryu, I., Oseko, N., & Sano, M. (2012). Development of mRNA-specific RT-PCR for the detection of koi herpesvirus (KHV) replication stage. *Diseases of Aquatic Organisms*, *100*(1), 11–18. http://dx.doi.org/10.3354/dao02499.

Yuasa, K., & Sano, M. (2009). Koi herpesvirus: Status of outbreaks, diagnosis, surveillance, and research. *Israeli Journal of Aquaculture-Bamidgeh*, *61*(3), 169–179.

Yuasa, K., Sano, M., Kurita, J., Ito, T., & Iida, T. (2005). Improvement of a PCR method with the Sph I-5 primer set for the detection of koi herpesvirus (KHV). *Fish Pathology*, *40*(1), 37–39.

Yuasa, K., Sano, M., & Oseko, N. (2012). Effective procedures for culture isolation of koi herpesvirus (KHV). *Fish Pathology*, *47*(3), 97–99. http://dx.doi.org/10.3147/jsfp.47.97.

Yuasa, K., Sano, M., & Oseko, N. (2013). Goldfish is not a susceptible host of koi herpesvirus (KHV) disease. *Fish Pathology*, *48*(2), 52–55. http://dx.doi.org/10.3147/jsfp.48.52.

Zak, T., Perelberg, A., Magen, I., Milstein, A., & Joseph, D. (2007). Heterosis in the growth rate of hungarian-israeli common carp crossbreeds and evaluation of their sensitivity to koi herpes virus (KHV) disease. *Israeli Journal of Aquaculture-Bamidgeh*, *59*(2), 63–72.

Zhang, Y. A., Salinas, I., Li, J., Parra, D., Bjork, S., Xu, Z., et al. (2010). IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nature Immunology*, *11*(9), 827–835. http://dx.doi.org/10.1038/ni.1913.

Zhou, J., Wang, H., Li, X. W., Zhu, X., Lu, W., & Zhang, D. (2014). Construction of KHV-CJ ORF25 DNA vaccine and immune challenge test. *Journal of Fish Diseases*, *37*(4), 319–325. http://dx.doi.org/10.1111/jfd.12105.

Zhou, J., Wang, H., Zhu, X., Li, X., Lv, W., & Zhang, D. (2013). The primary culture of mirror carp snout and caudal fin tissues and the isolation of koi herpesvirus. *In Vitro Cellular and Developmental Biology: Animal*, *49*(9), 734–742. http://dx.doi.org/10.1007/s11626-013-9661-x.

Zhou, J., Xue, J., Wang, Q., Zhu, X., Li, X., Lv, W., et al. (2014). Vaccination of plasmid DNA encoding ORF81 gene of CJ strains of KHV provides protection to immunized carp. *Vitro Cellular and Developmental Biology: Animal*, *50*(6), 489–495. http://dx.doi.org/10.1007/s11626-014-9737-2.

Objectives

The objectives of this thesis were to investigate the role of two unrelated innate immune mechanisms of carp in anti-CyHV-3 immunity. The first objective was to determine the role of epidermal mucus as an innate immune barrier against CyHV-3 entry during the early developmental stages of carp. The rational of this question relied on different independent observations: (i) The early developmental stages of carp were reported to be resistant to CyHV-3 infection (Ito *et al.*, 2007). (ii) The host laboratory demonstrated using *in vivo* bioluminescence imaging that the epidermis covering the fins and the body of carp is the major portal of entry for CyHV-3 (Costes *et al.*, 2009). And (iii) The epidermal mucus of adult carp was proved to act as an innate immune barrier reducing the binding of CyHV-3 to epidermal cells (Raj *et al.*, 2011). Based on these observations, we postulated that the early developmental stages of carp could be resistant to CyHV-3 infection due to stronger inhibition of viral binding to epidermal cells by epidermal mucus. To test this hypothesis, we investigated the sensitivity and the permissivity of carp to CyHV-3 during the early stages of its development.

The second objective of this thesis consisted to investigate whether carp express behavioral fever when infected by CyHV-3; and if so, what is the effect of this innate immune reaction on the development of CyHV-3 disease. When infected by pathogens, both endotherms and ectotherms can express a salutary reaction by increasing their body temperature. While in endotherms this reaction is called fever and depends on intrinsic thermogenesis, ectotherms like teleosts can only upregulate their body temperature by moving to warmer places, hence the term behavioral fever. When studying the pathogenesis of CyHV-3 (Boutier *et al.*, 2015), we observed that carp infected at 24°C (the thermal preference of healthy carp) tended to concentrate around the tank heater when it was running. This observation led us to postulate that infected subjects could express behavioral fever in natural environments where temperature gradients exist.

Experimental section

Experimental section

1st chapter:

Sensitivity and permissivity of *Cyprinus carpio* to cyprinid herpesvirus 3 during the early stages of its development: importance of the epidermal mucus as an innate immune barrier

Veterinary Research (2014), **45**:100

Maygane Ronsmans, Maxime Boutier, Krzysztof Rakus, Frédéric Farnir, Daniel Desmecht, Fabien Ectors, Michaël Vandecan, François Lieffrig, Charles Mélard and Alain Vanderplasschen

Preamble

Common carp and its subspecies koi undergo important morphological, histological and physiological modifications during their development which encompasses successive stages. From hatching to 3-5 days post-hatching, fish are called embryos. When the yolk sac is entirely absorbed (by 3 to 5 days post-hatching) carp are named larvae. This stage lasts until a metamorphosis around 2 to 3 weeks post-hatching, when fish become juveniles. Finally, around 35 days post-hatching, carp are called fingerlings that appear just like adults.

The susceptibility of carp to CyHV-3 infection has been tested by immersion of different developmental stages in infectious water (Ito *et al.*, 2007; Michel *et al.*, 2010; Perelberg *et al.*, 2003). These studies suggested that carp are susceptible to the infection from the juvenile stage onwards with a maximum of susceptibility at the fingerlings stage. Ito *et al* postulated that the two earlier developmental stages (embryo and larvae) are resistant to CyHV-3 infection (Ito *et al.*, 2007). Interestingly, this resistance could be due to a lack of expression of a component essential for completion of the viral infection (e.g., a cell surface binding receptor) by the early developmental stages and/or the expression of an innate immune mechanism capable of preventing the infection that is not expressed or at a lower level by later developmental stages.

Two recent studies of the host laboratory led us to investigate these two non-exclusive hypotheses at the level of the portal of entry for CyHV-3. Using *in vivo* bioluminescence imaging and a CyHV-3 recombinant strain expressing luciferase as a constitutive reporter gene (revealing replicative and non-replicative infections), Costes *et al.* demonstrated that the epidermis covering the fins and the body of carp is the major portal of entry for CyHV-3 (Costes *et al.*, 2009). A possible hypothesis to explain the resistance of the early developmental stages could be that their epidermal cells are not sensitive to CyHV-3 infection. A second study relying on the same tools by Raj *et al.* proved that the epidermal mucus of adult carp acts as an innate immune barrier reducing the binding of CyHV-3 to epidermal cells (Raj *et al.*, 2011). This study led us to hypothesize that early developmental stages of carp could be resistant to CyHV-3 due to a more efficient anti-viral activity of their epidermal mucus.

In the present study, we tested the two hypotheses described above using *in vivo* bioluminescence imaging and a CyHV-3 recombinant strain expressing luciferase. The different developmental stages of carp were infected through different routes (natural and artificial) of infection to clarify the determinism of the resistance observed for the two early stages.

Ronsmans *et al. Veterinary Research* 2014, **45**:100
http://www.veterinaryresearch.org/content/45/1/100

VETERINARY RESEARCH

RESEARCH Open Access

Sensitivity and permissivity of *Cyprinus carpio* to cyprinid herpesvirus 3 during the early stages of its development: importance of the epidermal mucus as an innate immune barrier

Maygane Ronsmans[1], Maxime Boutier[1], Krzysztof Rakus[1], Frédéric Farnir[2], Daniel Desmecht[3], Fabien Ectors[4], Michaël Vandecan[5], François Lieffrig[6], Charles Mélard[5] and Alain Vanderplasschen[1*]

Abstract

Cyprinid herpesvirus 3 (CyHV-3) causes a lethal disease in common and koi carp (*Cyprinus carpio*). The present study investigated the ability of CyHV-3 to infect common carp during the early stages of its development (from embryos to fingerlings) after inoculation by immersion in water containing the virus. Fish were inoculated at different times after hatching with a pathogenic recombinant CyHV-3 strain expressing luciferase. The sensitivity and permissivity of carp to CyHV-3 were investigated using in vivo bioluminescence imaging. The susceptibility of carp to CyHV-3 disease was investigated by measuring the survival rate. Carp were sensitive and permissive to CyHV-3 infection and susceptible to CyHV-3 disease at all stages of development, but the sensitivity of the two early developmental stages (embryo and larval stages) was limited compared to later stages. The lower sensitivity observed for the early developmental stages was due to stronger inhibition of viral entry into the host by epidermal mucus. In addition, independent of the developmental stage at which inoculation was performed, the localization of light emission suggested that the skin is the portal of CyHV-3 entry. Taken together, the results of the present study demonstrate that carp are sensitive and permissive to CyHV-3 at all stages of development and confirm that the skin is the major portal of entry after inoculation by immersion in infectious water. The results also stress the role of epidermal mucus as an innate immune barrier against pathogens even and especially at the early stages of development.

Introduction

The common carp (*Cyprinus carpio*) is one of the oldest cultivated fish species. In China, the cultivation of carp dates back to at least the 5th century BC and in Europe, carp farming began during the Roman Empire [1]. Common carp is currently one of the most economically valuable species in aquaculture, as it is one of the main cultivated fish for human consumption with worldwide production of 3.4 million tons per year [2]. Common carp is also produced and stocked in fishing areas for angling purposes. Its colorful ornamental varieties (koi carp), grown for personal pleasure and competitive exhibitions, represent one of the most expensive markets for individual freshwater fish, with some prize-winners being sold for US $10 000–1 000 000 [3].

Carp development comprises successive stages associated with specific morphological and physiological characteristics. The incubation period of fertilized eggs varies between 48 and 72 h depending on water parameters [4,5]. From hatching to 3–5 days post-hatching, fish are called embryos. The digestive tract is not fully developed so the fish rely entirely on the yolk sac as a source of nutrients [6,7]. Because the gills are not yet formed, gas exchange occurs through the blood vessels of the yolk sac and the caudal fin. By 3 to 5 days post-hatching the yolk sac is entirely absorbed and most organs formed and functional. From this moment, the fish are referred to as larvae. In contrast to embryos, larvae rely on exogenous feeding and their respiration is mediated by the gills.

* Correspondence: a.vdplasschen@ulg.ac.be
[1]Immunology-Vaccinology, Department of Infectious and Parasitic Diseases (B43b), Fundamental and Applied Research for Animals & Health, Faculty of Veterinary Medicine, University of Liège, 4000 Liège, Belgium
Full list of author information is available at the end of the article

Around 2–3 weeks post-hatching, larvae acquire an adult shape and are no longer transparent. When the metamorphosis is complete, the fish are referred to as juveniles. The fish then develop paired fins and maturation of the organs is finalized. Around 35 days post-hatching, the fish are called fingerlings. Fingerlings are fully developed, entirely covered with scales (with the exception of fish that have no scales), and appear just like adults [6,7]. Despite their adult appearance, the immune system of fingerlings is not yet fully mature and functional.

Teleosts develop both innate and adaptive immune responses [8]. During the early stages of life described above, the lymphomyeloid organs of teleosts are not yet fully mature and the fish are unable to develop an effective adaptive immune response. In carp, adaptive immune competency occurs roughly 2 months after hatching [4,9]. Early in life, fish immunity relies on passively transferred maternal factors and on innate immune mechanisms which are activated just after egg fertilization and fully functional at hatching [10,11]. Among the components of the innate immune system, the mucus covering the external and internal surfaces of the fish is thought to play a key role in the inhibition of pathogen entry into the host [8,11].

Koi herpesvirus (KHV), also known as cyprinid herpesvirus 3 (CyHV-3) (genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales*), is the etiological agent of an emerging and lethal disease in common and koi carp. Since its emergence in the late 1990s, this highly contagious and dreadful disease has caused severe economic losses in both the common and koi carp culture industries worldwide. Publication of the CyHV-3 sequence [12] and the cloning of its genome as an infectious bacterial artificial chromosome (BAC) [13] allowed the production of CyHV-3 recombinant strains. Recently, we took advantage of these advances to construct a luciferase (LUC)-expressing recombinant strain via intergenic insertion of a LUC expression cassette. Using this recombinant strain and in vivo imaging system (IVIS), we showed that the skin covering the fins and body of carp fingerlings, but not the gills, is the major portal of entry after inoculation by immersion in water containing the virus [14]. Using similar approaches, epidermal mucus has been shown to act as an innate immune barrier, drastically reducing CyHV-3 binding to epidermal cells [15].

The epidermis of adult teleost fish is a living stratified squamous epithelium divided into three layers. The surface and basal layers are single-cell layers composed of keratinocytes and undifferentiated basal cells, respectively. The pluristratified intermediate layer is composed of unicellular glands (mucous cells and club cells), ionocytes and undifferentiated cells. All three layers contain cells capable of mitotic division. The scales are dermal structures covered by the epidermis [16]. Compared to adults, the epidermis of embryos has a simplified structure consisting of surface and basal layers (single-cell layers) interrupted by unicellular glands and ionocytes.

The susceptibility of carp to CyHV-3 disease has been tested at different developmental stages after inoculation by immersion in water containing the virus. Though juveniles, fingerlings, and adults are susceptible to CyHV-3 disease [17-19], larvae have been reported to be resistant to infection based on PCR analysis of experimentally inoculated subjects [19]. These results suggest that the early stages of carp development are resistant to CyHV-3. Interestingly, this resistance could be due to a lack of expression of a component essential for completion of the viral infection (e.g., a cell surface binding receptor) by the early developmental stages and/or the expression of an innate immune mechanism capable of preventing the infection that is not expressed by later development stages.

In the present study, we investigated the ability of CyHV-3 to infect common carp during the early stages of its development (from embryos to fingerlings) after inoculation by immersion in water containing the virus. Fish were inoculated at different times post-hatching with a pathogenic recombinant CyHV-3 strain expressing LUC as a reporter gene. The sensitivity (ability to support viral entry into host cells) and permissivity (ability to support viral replication) of carp to CyHV-3 infection were investigated using IVIS. The susceptibility of carp to CyHV-3 disease was investigated by measuring the survival rate over a period of one month after inoculation. The results of the present study demonstrate that carp are sensitive and permissive to CyHV-3 infection and susceptible to CyHV-3 disease at all developmental stages. However, the fish express reduced sensitivity during the two earlier stages of development (embryo and larval stages) due to efficient inhibition of viral entry into the host by epidermal mucus. This study further supports the importance of the skin as the major portal of entry of CyHV-3 after inoculation by immersion in infectious water and the role of the epidermal mucus as an innate immune barrier against pathogens even and especially during the early developmental stages.

Materials and methods

Cells and virus

Common carp brain (CCB) cells [20] were cultured in minimum essential medium (Sigma) containing 4.5 g/L of glucose (D-glucose monohydrate; Merck) and 10% fetal calf serum. The cells were cultured at 25 °C in a humid atmosphere containing 5% CO_2. CCB cells were used to produce and titrate CyHV-3. The FL BAC revertant ORF136 LUC strain of CyHV-3, hereafter referred to as the FL LUC strain, was described previously [14]. This

recombinant strain derived from the FL BAC clone of CyHV-3 encodes a wild type ORF55 locus (in which the BAC cassette was initially cloned) and a firefly (*Photinus pyralis*) luciferase expression cassette under control of the human cytomegalovirus immediate early promoter. The LUC cassette was inserted in the intergenic region between open reading frames ORF136 and ORF137.

Carp reproduction

Common carp (*Cyprinus carpio*) were produced by artificial reproduction. Breeders free of CyHV-3 based on PCR analysis of peripheral mononuclear blood cells and serological analysis that generated offspring sensitive to CyHV-3 disease were hosted in a recirculating aquaculture system at 23 °C (CEFRA, University of Liège, Belgium). Mature breeders were selected based on morphological characteristics. Ovulation and spawning were induced by two intraperitoneal (IP) injections of Ovaprim (Syndel) performed at an 8 h interval. The first injection was a dose of 0.05 mL/kg of body weight. The dose of the second injection differed between males and females: 0.25 and 0.5 mL/kg of body weight, respectively. Eggs were stripped 12 h after the second hormonal injection and fertilized with stripped sperm. All manipulations of the breeders were performed under anesthetic tranquilization with benzocaine (50 mg/L of water). Fertilized eggs were incubated in Zoug bottles until hatching. Embryos (mean body weight 1.2 mg) were transferred to a 50 L tank (2500 larvae per tank). The water temperature was regulated at 24 °C and the O_2 concentration maintained above 6 ppm. Larvae were fed *ad libitum* with artificial larval food Gemma micro (Skretting). The food was distributed manually 6 times per day during the first 45 days post-hatching. Juveniles reached a mean body weight of 500 mg within their first month of life.

CyHV-3 inoculation of fish

For viral inoculation mimicking natural infection, fish were kept for 2 h in water containing the FL LUC strain of CyHV-3 (400 plaque-forming units (pfu)/mL). At the end of the incubation period, the fish were returned to 50 L tanks where they were kept in floating breeding nests (SERA). To avoid removal of the epidermal mucus, fish were caught using a container rather than a fish net. In some experiments, the skin mucus was partially removed before inoculation. Carp were caught with a fish net, placed on a plastic sheet, and the upper side of the body was gently rubbed with a floqswab ultrathin (Copan flock technologies, Brescia, Italy) to remove the epidermal mucus. Carp were also inoculated by IP injection as follows. Carp were anesthetized by immersion in water containing benzocaine (25 mg/L) and distributed individually to the wells of a macroscopic slide (12 cavity microscopic slide, VWR international) containing a viscous solution (NaCl 292 mg/L, KCl 12.6 mg/L, $CaCl_2.2H_2O$ 48.3 mg/L, $MgSO_4.7H_2O$ 81.6 mg/L, methylene blue 1 mg/L, carboxyl methyl cellulose high viscosity 20 g/L). Culture medium containing 3.6×10^6 pfu/mL of the FL LUC strain was injected intraperitoneally (corresponding to an approximate dose of 40 pfu per fish) using a microinjector (Femtojet, Eppendorf, VWR international) and tapered beveled pipettes (15–20 μm). Injection was performed using the following parameters, leading to the administration of an approximate volume of 0.01 μL: automatic mode, injection pressure of 200 hPa, compensation pressure of 0 hPa, and injection time of 0.2 - 0.8 s according to fish size. After injection, the fish were returned to 50 L tanks. This animal study was accredited by the local ethics committee of the University of Liège.

In vivo bioluminescence imaging

Imaging of firefly LUC expression was performed using IVIS (IVIS spectrum, Xenogen, Caliper LifeSciences) as described previously [14,15,21]. D-luciferin (Xenogen, Caliper LifeSciences) (15 mg/mL) in phosphate-buffered saline (PBS) was microinjected into the peritoneal cavity as described above. Efficient injection of D-luciferin into fish was controlled by examination of the fish using full field epifluorescent microscopy (Eclipse TE2000-S, Nikon). D-luciferin emits a green fluorescence when excited with a blue light (488 nm). After an incubation period of 10 min, the carp were analyzed by IVIS. Images were collected using the following settings: field of view A, binning on small, automatic exposure time with a maximum of 1 min, and a subject height of 0.35 cm. The relative intensities of transmitted light from bioluminescence were represented as a pseudocolor image ranging from violet (least intense) to red (most intense). For quantitative comparisons, regions of interest were manually drawn by surrounding the fish outline, and the Living Image software 3.2 (Caliper Life Sciences) was used to calculate the corrected average radiance ($p/s/cm^2/sr$) by subtracting the average radiance of the background for each image.

Histological analysis

Euthanized fish were fixed by immersion in Carnoy solution (ethanol:acetic acid:chloroform 6:1:3, v/v/v) for 24 h at 4 °C. After dehydration with ethanol, samples were embedded in paraffin blocks [15]. Five micron sections were stained with a combined Periodic Acid-Schiff, Alcian Blue and hematoxylin-eosin staining prior to microscopic analysis using a Leica DM 2000 LED microscope equipped with a DFC 450C camera (Leica, Heerbrugg, Switzerland).

Statistical analysis

Significant differences in the log of the average radiance emitted by IVIS-positive fish, according to time of

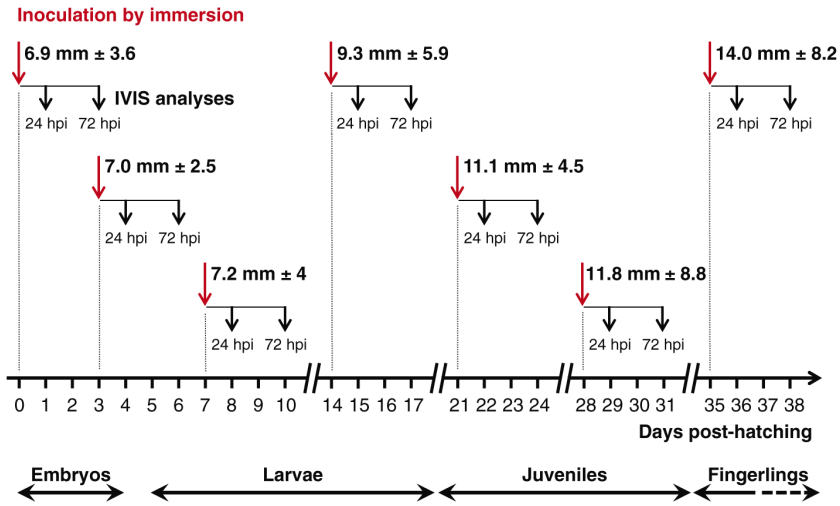


Figure 1 Timeline of the experiments performed to investigate the sensitivity and permissivity of carp to CyHV-3 during the early stages of development. At different times post-hatching (indicated by red arrows), carp were inoculated by immersion in water containing the FL LUC strain. At 24 and 72 hpi, 30 carp were analyzed individually by IVIS. The two-headed arrows below the time scale represent the period of time during which the indicated developmental stage represented at least 80% of the fish population. The size of fish (mean ± SD, based on 15 measurements) at the time of inoculation (indicated by vertical red arrows) is indicated.

inoculation (days post-hatching) and time post-inoculation or according to time of inoculation and mode of inoculation, were assessed using two-way ANOVA with interactions. Significant differences in the number of IVIS-positive fish according to time of inoculation (days post-hatching), time post-inoculation and mode of inoculation were assessed using logistical analysis and the chi-squared test. Significant differences in survival rates according to time of inoculation (days post-hatching) were assessed using a contingency table and chi-squared test.

Results

The goal of the present study was to investigate the ability of CyHV-3 to infect *Cyprinus carpio* during the early stages of its development. The data presented in this manuscript are representative of duplicate independent experiments. Importantly, though the different experiments were initially performed successively, the data set presented in this manuscript represents the parallel repetition of all experiments with the same batch of fish.

Sensitivity and permissivity of *Cyprinus carpio* to CyHV-3 during the early stages of development

Figure 1 presents the design of the experiment performed to address the sensitivity and permissivity of the early developmental stages of common carp to CyHV-3 after inoculation by immersion in infectious water. Carp were inoculated at different times post-hatching that are representative of the different developmental stages: days 0 and 3 for the embryo stage, days 7 and 14 for the larval stage, days 21 and 28 for the juvenile stage, and day 35 for the fingerling stage. At 24 and 72 h post-inoculation (hpi), fish were injected intraperitoneally with D-luciferin. The small size of the earlier stages of development made the injection of D-luciferin a critical step. Taking advantage of the autofluorescence of D-luciferin, the success of the injection was controlled by full field epifluorescent examination of each fish prior to IVIS analysis (Figure 2). In contrast to fish injected with PBS,

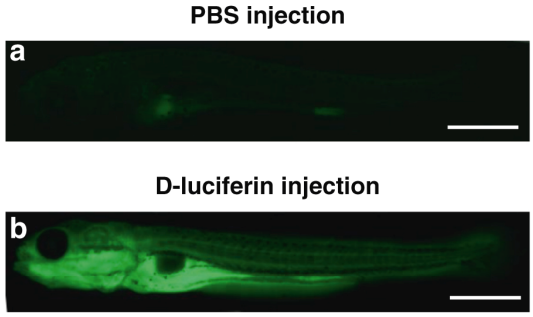


Figure 2 Quality control of D-luciferin microinjection by epifluorescent microscopy. Prior to IVIS analysis, D-luciferin was microinjected into the peritoneal cavity of carp. The success of the injection was controlled by epifluorescent microscopy of the subjects with D-luciferin autofluorescence. The upper and lower panels represent fish injected with PBS **(panel a)** and D-luciferin **(panel b)**, respectively. Both subjects illustrating the larval stage (15 days post-hatching) were examined under full field 488 nm excitation. Scale bars = 1 mm.

Ronsmans *et al. Veterinary Research* 2014, **45**:100
http://www.veterinaryresearch.org/content/45/1/100

fish successfully injected with D-luciferin expressed green fluorescence in the peritoneal cavity and vascularized tissues. Twenty-four and 72 h after each inoculation time point (0, 3, 7, 14, 21, 28, and 35 days post-hatching), 30 successfully injected fish were analyzed using the IVIS (Figures 3 and 4). The threshold of positivity was determined based on the analysis of mock-inoculated fish (mean plus 3 standard deviations (SD), $p < 0.00135$; Figure 3). Fish with an average radiance higher than the threshold were classified as positive. Examination of IVIS images revealed that the vast majority exhibited at least one focal source of light. Examination of IVIS images from both the mock-inoculated and inoculated groups that had an average radiance lower than

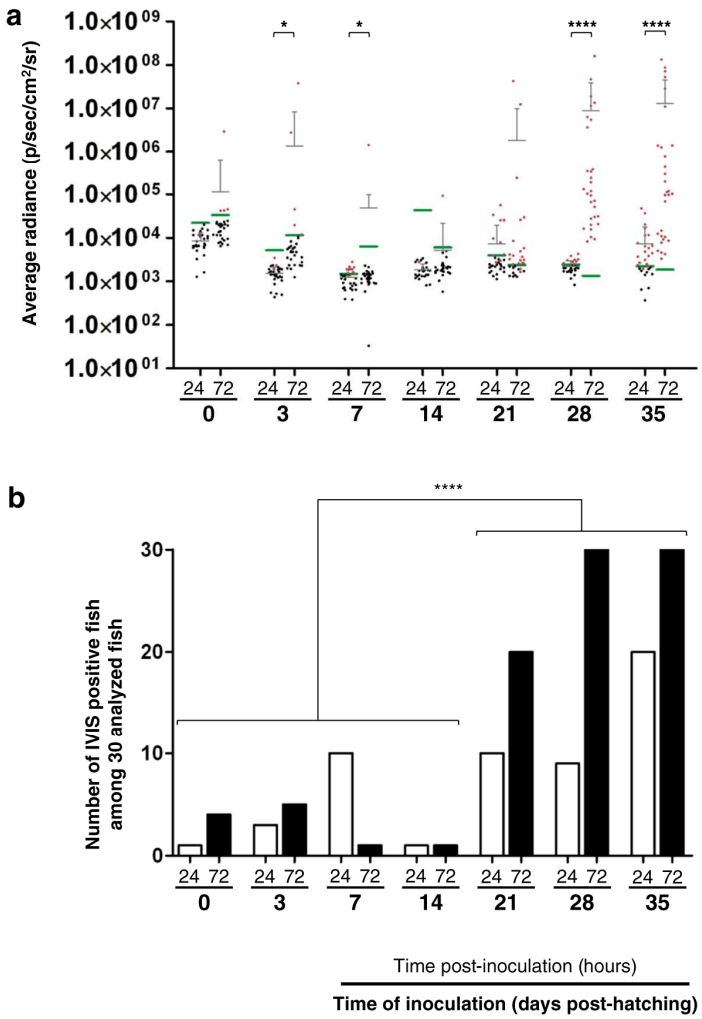


Figure 3 Quantitative measurements of the sensitivity and permissivity of carp to CyHV-3 during the early stages of development. Fish were mock-inoculated or inoculated at the indicated times post-hatching and then analyzed by IVIS at 24 and 72 hpi. **(a)** The average radiance (p/sec/cm^2/sr) emitted by individual infected fish ($n = 30$ per group) corrected for the background of each image is presented by dots. For each group of inoculated fish (according to time of inoculation and time post-inoculation), the mean + SD is presented as grey bars. For each time point, a group of mock-inoculated fish was analyzed to define the threshold of positivity (green bars), defined as the mean + 3 SD ($p < 0.00135$). Red dots represent infected fish defined as positive based on their average radiance being higher than the threshold or based on the expression of a spot of light (even if their average radiance was below the threshold). The data obtained at different times post-inoculation (24 versus 72 hpi) for each time of inoculation were compared by two-way ANOVA. **(b)** The number of positive fish among 30 analyzed inoculated fish. The number of positive fish according to time of inoculation was compared by logistical analysis. Inoculations performed at the embryonic and larval stages (between 0 and 14 days post-hatching) were compared to inoculations at the post-larval stages (between 21 and 35 days post-hatching). *$p < 0.05$, ****$p < 0.0001$.

Ronsmans *et al. Veterinary Research* 2014, **45**:100
http://www.veterinaryresearch.org/content/45/1/100

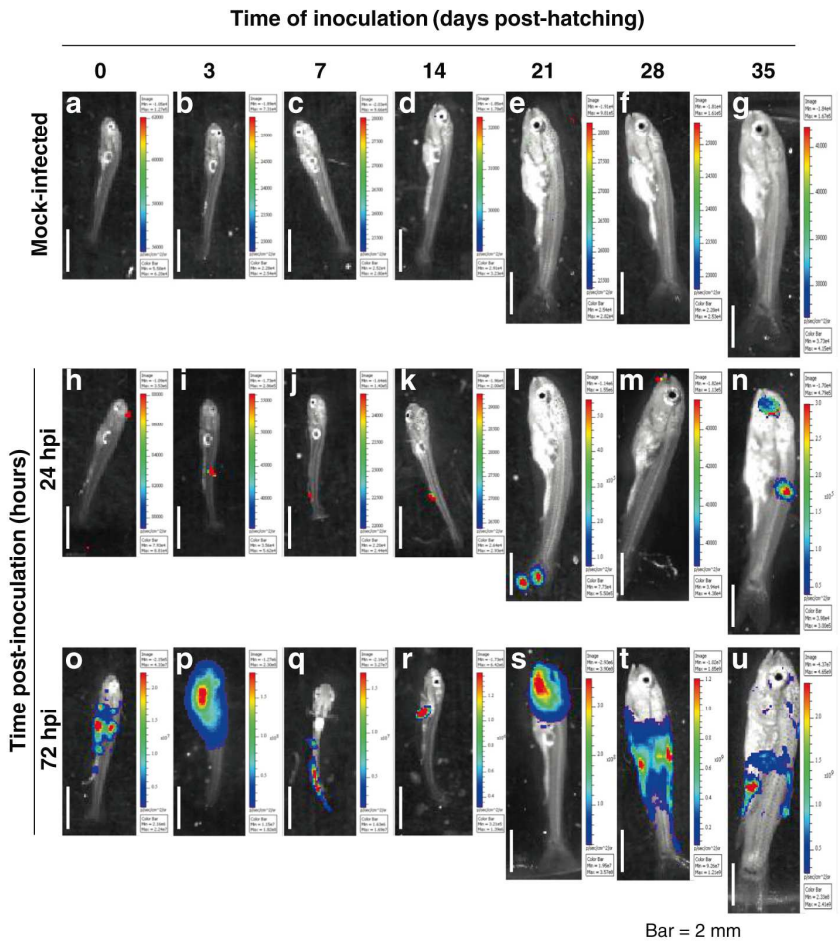


Figure 4 IVIS images of the sensitivity and permissivity of carp to CyHV-3 during the early stages of development. The fish inoculation methods and IVIS analysis are described in the legend of Figures 1 and 3. Mock-infected fish **(panels a to g)** and representative positive infected fish **(panels h to u)** as described in Figure 3 are shown for each time point of analysis. Images are presented with a relative photon flux scale automatically adapted to each image in order to use the full dynamic range of the pseudocolor scale. Scale bars = 2 mm.

the threshold of positivity revealed only a few fish, exclusively in the inoculated groups, that expressed a focal source of light. Consequently, these fish were also classified as positive (Figure 3a, see red dots below the threshold represented by a green bar). Images illustrating such a positive fish with an average radiance below the threshold are presented in Figure 4 (see panels h, i, and k). Interestingly, examination of images from all positive fish revealed that spots of light were distributed randomly on the body, head, and fins. On one occasion, a LUC signal was observed on the eye ball (Figure 4, panel n). None of the spots had a position compatible with the gills. These data suggest that, independent of the developmental stage at which the inoculation was performed, the skin is the major portal of entry after inoculation by immersion in water containing the virus.

Independent of the developmental stage at the time of inoculation and the time of analysis post-inoculation (24 or 72 hpi), at least one positive fish was detected (Figure 3), demonstrating that carp are sensitive to CyHV-3 infection at all stages of development. Photon emission was significantly higher 72 hpi compared to 24 hpi for the following times of inoculation (Figure 3A): 3 ($p < 0.05$), 7 ($p < 0.05$), 28 ($p < 0.0001$), and 35 ($p < 0.0001$) days post-hatching. These data suggest that carp are permissive to CyHV-3 at all stages of development, which was further supported by the IVIS images (Figure 4). Compared to images collected 24 hpi, those collected at 72 hpi systematically revealed more spots of light with a larger area and greater maximum radiance independent of the time of inoculation (Figure 4, second vs. third rows of panels).

Ronsmans *et al. Veterinary Research* 2014, **45**:100
http://www.veterinaryresearch.org/content/45/1/100

These data demonstrate that all developmental stages of carp are sensitive and permissive to CyHV-3 infection. However, the percentage of positive fish was significantly lower after inoculation 0 – 14 days post-hatching compared to inoculation at later stages (Figure 3b, $p < 0.0001$). No significant difference was found in the average radiance (p/sec/cm^2/sr) of positive fish (independent of the stage at which inoculation was performed) ($p < 0.05$), implying that, once infected, replication occurs similarly in fish, at least at the portal of entry.

Effect of epidermal mucus on the sensitivity of *Cyprinus carpio* to CyHV-3 during the early stages of development

Taken together, the data demonstrate that the embryo and larval stages are less sensitive to CyHV-3 infection than the juvenile and fingerling stages. The lower sensitivity of these early stages of development could be due to an absence of host expression of a component essential for completion of the viral infection (e.g., a cell surface binding/entry receptor) and/or the expression of an innate immune mechanism capable of preventing infection. Recently, we demonstrated that epidermal mucus acts as an innate immune barrier against CyHV-3 entry in fingerlings and adults [15]. In the present study, we investigated whether the lower sensitivity observed for the early developmental stages can be explained by a higher anti-viral activity of the epidermal mucus. To test this hypothesis, the different developmental stages of carp were inoculated using three different modes of inoculation (Figure 5): (i) immersion in water containing the virus (Bath, B), (ii) immersion in infectious water after removal of epidermal mucus (Mucus, M), and (iii) IP injection (IP). Inoculation times had to be restricted to at least 7 days post-hatching because younger fish did not support the stress induced by the process of mucus removal or IP inoculation. Independent of the stage at which IP inoculation was performed, nearly all inoculated fish expressed an intense and comparable LUC signal 24 hpi (Figure 6a). Visual examination of IVIS images revealed that fish inoculated intraperitoneally expressed an intense signal throughout the peritoneal cavity. This observation further supports the hypothesis that the lower sensitivity observed for early developmental stages is the consequence of a restriction of viral entry at the portal of entry. Some signals located on the fins likely reveal skin contamination that occurred during the IP inoculation (Figure 7, see panels s and t).

Independent of the developmental stage tested, removal of the epidermal mucus prior to inoculation by immersion in infectious water drastically increased the sensitivity of carp to CyHV-3. Fish inoculated by immersion after the removal of mucus had a significantly higher LUC signal than untreated control fish (Figure 6, compare M and B). In terms of the number of positive fish, removal of mucus prior to inoculation led to the percentage of positive fish being close to maximum and similar to that observed after IP inoculation (with the exception of inoculations performed 7 days post-hatching), and systematically higher than those observed for control inoculated fish (Figure 6b). The IVIS images presented in Figure 7 demonstrate that

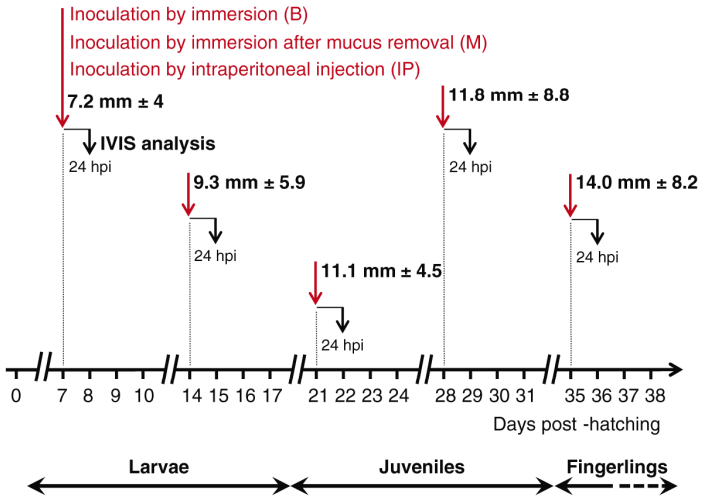


Figure 5 Timeline of the experiments performed to investigate the effect of the mode of inoculation on the sensitivity of carp to CyHV-3 infection during the early stages of development. At different times post-hatching (indicated by red arrows), carp were inoculated with the FL LUC strain according to three modes of inoculation: by immersion in infectious water (B), by immersion in infectious water just after removing the epidermal mucus (M), and by IP injection of the virus (IP). At 24 hpi, 30 carp were analyzed individually by IVIS. The two-headed arrows below the time scale represent the period of time during which the indicated developmental stage represented at least 80% of the fish population. The size of fish (mean ± SD, based on 15 measurements) at the time of infection (indicated by vertical red arrows) is indicated.

Ronsmans *et al. Veterinary Research* 2014, **45**:100
http://www.veterinaryresearch.org/content/45/1/100

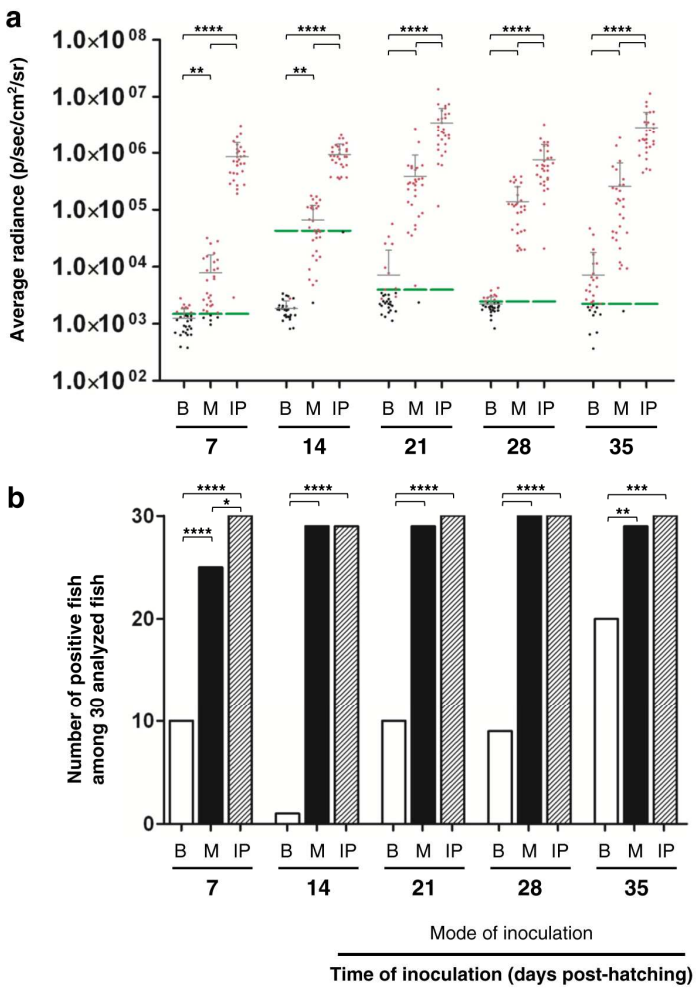


Figure 6 Quantitative measurements of the effect of the mode of inoculation on the sensitivity of carp to CyHV-3 infection during the early stages of development. The timeline of this experiment has been described in Figure 5. Fish were mock-inoculated or inoculated with the FL LUC strain using three modes of infection: immersion in infectious water (B), immersion in infectious water just after removing the epidermal mucus (M), and by IP injection of the virus (IP). Fish were analyzed by IVIS at 24 hpi. **(a)** The average radiance (p/sec/cm^2/sr) emitted by individual infected fish ($n = 30$ per group) corrected for the background of each image is presented by dots. For each group of inoculated fish (according to time of inoculation and mode of inoculation), the mean + SD is presented as grey bars. For each time point, a group of mock-inoculated fish was analyzed to define the threshold of positivity (green bars), defined as the mean + 3 SD ($p < 0.00135$). Red dots represent inoculated fish defined as positive based on their average radiance being higher than the threshold or based on the expression of a spot of light (even if their average radiance was below the threshold). The data obtained for the different modes of inoculation for a considered time of inoculation were compared by two-way ANOVA. **(b)** The number of positive fish among 30 analyzed infected fish. The data obtained for the different modes of inoculation for each time of inoculation were compared using the chi-squared test. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, and ****$p < 0.0001$.

fish inoculated after mucus removal expressed more spots of light with a larger area and higher intensity than control infected fish (Figure 7, compare the second and third rows of panels). Finally, we controlled the effect of the treatment to remove the mucus by histological examination of the skin epidermis (Figure 8). Despite the use of fixation conditions developed for visualization of the mucus, the mucus layer covering the surface of the epidermis was barely visible in some slides (Figure 8, upper panels). However, unicellular mucous glands containing mucus were easily identified throughout the epidermis at all stages of development. In contrast, these cells were no longer identifiable when the mucus was removed. The effect of the treatment was also visible on the surface of the uppermost layer of epidermal cells, which had a hairy appearance (Figure 8, panels h-j). The observed evolution of the structure of the epidermis according to ontogenesis was consistent with an earlier report [16].

Ronsmans *et al. Veterinary Research* 2014, **45**:100
http://www.veterinaryresearch.org/content/45/1/100

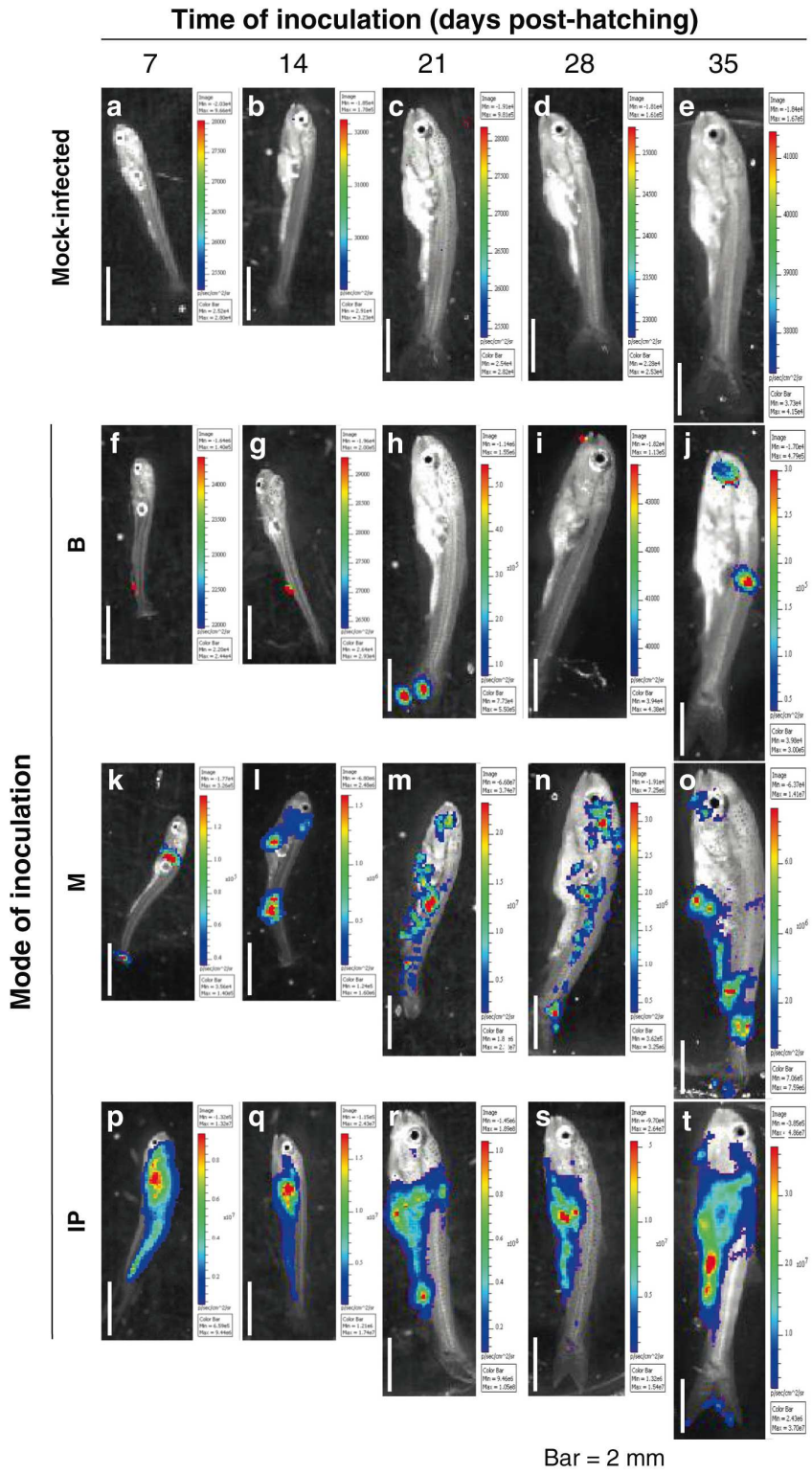


Figure 7 (See legend on next page.)

(See figure on previous page.)
Figure 7 IVIS images of the effect of the mode of inoculation on the sensitivity of carp to CyHV-3 during the early stages of development. The fish inoculation methods and IVIS analysis are described in the legend of Figures 5 and 6. Mock-infected fish **(panels a to e)** and representative positive infected fish **(panels f to t)** as described in Figure 6 are shown for each time point of analysis. Images are presented with a relative photon flux scale automatically adapted to each image in order to use the full dynamic range of the pseudocolor scale. Scale bars = 2 mm.

Taken together, the results demonstrate that the lower sensitivity to CyHV-3 observed for the early developmental stages of carp is due at least in part to a strong inhibition of viral entry into the host by the epidermal mucus.

Susceptibility of *Cyprinus carpio* to CyHV-3 disease during the early stages of development

Carp were mock-inoculated or inoculated with the FL LUC strain at the indicated times post-hatching. Survival rates were measured over a period of 30 days post-inoculation (dpi). For most groups, surviving fish were counted on days 15 and 30 post-inoculation.

The data were analyzed as follows. First, the survival rate of each infected group was compared to the control mock-inoculated group. Independent of the age at which the inoculation was performed or the time post-inoculation at which the survival rate was measured, each infected group had a survival rate lower than the control mock-inoculated group ($p < 0.0001$ at 15 and 30 dpi). Second, statistical analyses were performed after grouping the data related to the developmental stages at which inoculation was performed (Figure 9) as follows: embryo-larval stage (fish inoculated from 0 to 10 days post-hatching), juvenile stage (fish inoculated at day 21), and fingerling stage (fish inoculated at day 35). These analyses demonstrate that the mortality rate was significantly different between the three developmental stages tested at 15 ($p < 0.0001$) and 30 dpi ($p < 0.0001$), but mainly between the embryo-larval and two older stages.

Discussion

The goal of the present study was to investigate the ability of CyHV-3 to infect common carp during the early stages of its development (from embryos to fingerlings) after inoculation by immersion in infectious water. Using IVIS, we demonstrated that carp are sensitive and permissive to CyHV-3 infection at all stages of development. However, the sensitivity of the two early stages (embryo and larval stages) was limited compared to the older stages (juvenile and fingerling stages). Inoculation after removal of the epidermal mucus demonstrated that the reduced sensitivity of the early developmental stages was caused by a stronger inhibition of viral entry into

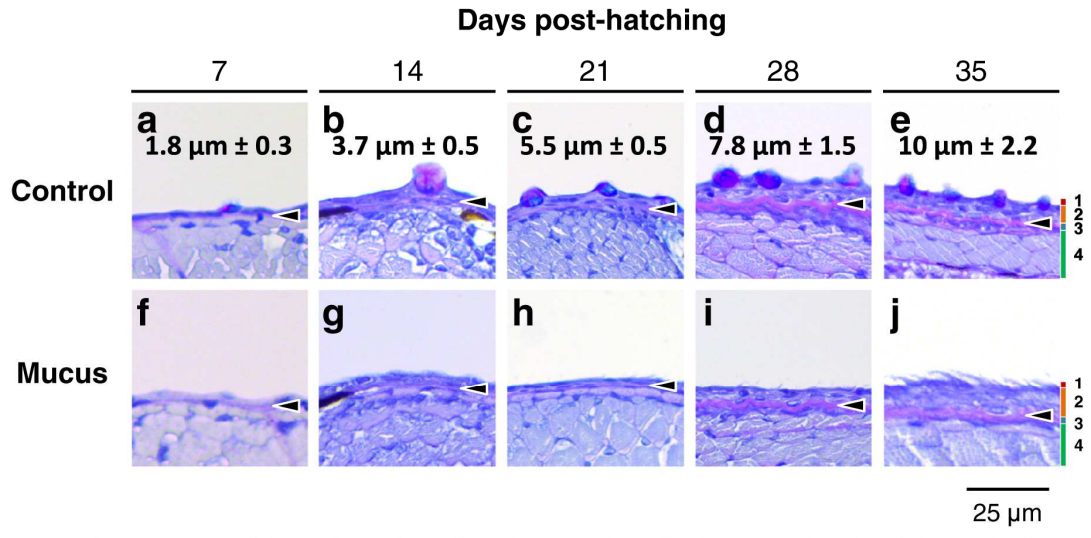


Figure 8 Histological structure of skin epidermis during the early stages of carp development. At the indicated days post-hatching, carp were harvested and either left untreated (Control, **panels a to e**) or treated for removal of the epidermal mucus (Mucus, **panels f to j**). The fish were then euthanized and processed for histological examination of the skin epidermis. The numbers in the upper panels represent the thickness of the epidermis (mean ± SD, based on 15 measurements). Histological structures were identified for the samples collected 35 days post-hatching **(panels e and j)**: 1, mucus; 2, epidermis; 3, basement membrane (arrow head); 4, dermis and dorsal muscles. Scale bar = 25 µm.

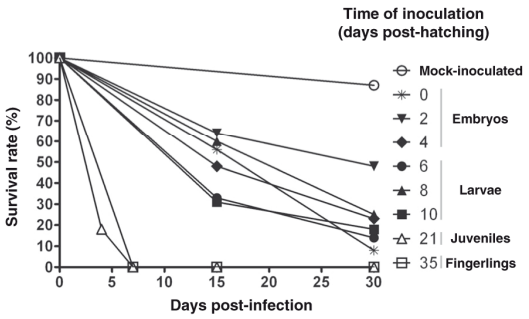


Figure 9 Susceptibility of *Cyprinus carpio* to CyHV-3 disease during the early stages of its development. Carp ($n = 80$) were mock-inoculated or inoculated with the FL LUC strain at the indicated times post-hatching by immersion in infectious water. Survival rates were measured over a period of 30 days post-infection.

the host by the epidermal mucus. Finally, the susceptibility of carp to CyHV-3 disease was tested by inoculating carp at different times after hatching and measuring the survival rate over a period of one month after inoculation. The data are consistent with the conclusion that carp are susceptible to CyHV-3 at all stages of development and that the sensitivity to CyHV-3 infection increases during ontogenesis.

Ito et al. reported that the larvae from two independent strains of common carp were resistant to CyHV-3 infection but juveniles from the same strains were highly susceptible [19]. The results of the present study contradict this earlier report by demonstrating that carp are sensitive and permissive to CyHV-3 at all stages of development. Importantly, the results of the present study are representative of two experiments performed with unrelated breeders. All of the breeders used in this study were proved to be free of CyHV-3. In their study, Ito et al. did not mention whether they controlled the serological status of the carp used for reproduction. One interesting hypothesis that could explain the paradox between the two studies is that the previous study used seropositive genitors. These immune genitors could have transferred transient humoral immune protection against CyHV-3 to their offspring. Teleost fish lay telolecithal eggs in which passive transfer of maternal antibodies occurs [22,23]. Whether embryos from immune versus naive breeders exhibit different sensitivity to CyHV-3 would be interesting to determine in the future, as well as how long this passive protection lasts. Experiments to test this hypothesis are currently in progress.

Epidermal mucus acts as an innate immune barrier against CyHV-3 entry into the host [15]. The results of the present study suggest that the lower sensitivity to CyHV-3 of the early developmental stages is due to more efficient protection by the mucus, which is conferred through two types of complementary mechanisms. First, the mucus forms an efficient mechanical barrier that constantly moves downstream along the fish and off of the trailing edges. Similar to the muco-ciliary escalator of the respiratory tract of pulmonate animals, fish mucus reduces pathogen access to epithelial cells. Second, the mucus contains numerous proteins, such as immunoglobulins, enzymes, and lytic agents, capable of neutralizing microorganisms [24-28]. Several hypotheses, which are not mutually exclusive, could explain the more efficient inhibition of CyHV-3 entry by the epidermal mucus in the early developmental stages. One hypothesis is that the mucus layer of the early stages of development forms a more uniform mechanical barrier on the fish body, possibly due to its biochemical composition, the hydrodynamic parameters of the fish, or as a consequence of reduced physical interactions between the fish and an object or other fish [29]. Another hypothesis is that the mucus produced by early stages of development contains a higher concentration of biologically active molecules capable of neutralizing CyHV-3. Even if technically difficult, it would be very interesting to compare the neutralization activity of soluble mucus extracts from larvae versus older stages that are more sensitive to CyHV-3. These experiments could reveal that the early stages of development express biologically active innate immune molecules in their mucus that are not expressed, or expressed at lower levels at later stages. In addition to their interest to fundamental science, such conclusions could be very interesting for application-oriented research, such as that trying to enhance fish resistance against pathogens by up-regulating innate immunity.

In conclusion, the present study demonstrates that carp are sensitive and permissive to CyHV-3 infection at all stages of development, even if the sensitivity of the early stages is reduced due to efficient inhibition of viral entry by the epidermal mucus. This study further supports the importance of the skin as the major portal of entry of CyHV-3 after inoculation by immersion in infectious water. It stresses the role of the epidermal mucus as an innate immune barrier against pathogen even and especially during the early stages of development.

Abbreviations

BAC: Bacterial artificial chromosome; CCB: Common carp brain; CyHV-3: Cyprinid herpesvirus 3; dpi: days post-infection; hpi: hours post-infection; IP: Intraperitoneal; IVIS: In vivo imaging system; KHV: Koi herpesvirus; LUC: Luciferase gene; PBS: Phosphate-buffered saline; pfu: plaque-forming unit; pi: post-infection; p/s/cm^2/sr: photon/second/centimeter square/steradian; SD: Standard deviation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MR did most of the experiments. MR, MB, KR, and AV contributed to the design of the study. MR and MB drafted the figures. DD performed the

histological analysis of carp epidermis. MR, MV, and FE developed the procedure for microinjection into carp embryos and larvae. CM and FL performed carp reproduction. Statistical analyses were performed by FF. MR and AV conceived the study and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgments
MR is a research fellow of the "Fonds National Belge de la Recherche Scientifique" (FNRS). This work was supported by a grant from the University of Liège and by grants from the "Fonds National Belge de la Recherche Scientifique" (FNRS). KR and AV are members of the BELVIR consortium (IAP, phase VII) granted by the Belgian Science Policy Office (BELSPO) (Belgium).

Author details
[1]Immunology-Vaccinology, Department of Infectious and Parasitic Diseases (B43b), Fundamental and Applied Research for Animals & Health, Faculty of Veterinary Medicine, University of Liège, 4000 Liège, Belgium. [2]Biostatistics and Bioinformatics applied to Veterinary Science (B43), Fundamental and Applied Research for Animals & Health, Faculty of Veterinary Medicine, University of Liège, 4000 Liège, Belgium. [3]Pathology, Department of Morphology and Pathology (B43), Fundamental and Applied Research for Animals & Health, Faculty of Veterinary Medicine, University of Liège, 4000 Liège, Belgium. [4]Transgenic Platform, Groupe Interdisciplinaire de Génoprotéomique Appliquée, Centre Hospitalier Universitaire B34, University of Liège, 4000 Liège, Belgium. [5]CEFRA-University of Liège, 10 Chemin de la Justice, 4500 Tihange, Belgium. [6]CERgroupe, 1 rue du Carmel, B-6900 Marloie, Belgium.

Received: 9 August 2014 Accepted: 24 September 2014
Published online: 04 October 2014

References

1. Balon E: **Origin and domestication of the wild carp, *Cyprinus carpio*: from Roman gourmets to the swimming flowers.** *Aquaculture* 1995, **129:**3–48.
2. Statistics and Information Service of the Fisheries and Aquaculture Department: **Fishery and Aquaculture Statistics.** In 2010 [http://www.fao.org/docrep/015/ba0058t/ba0058t.pdf].
3. Balon E: **The oldest domesticated fishes, and the consequences of an epigenetic dichotomy in fish culture.** *J Ichthyol Aquat Biol* 2006, **11:**47–86.
4. Huttenhuis HBT, Grou CPO, Taverne-Thiele AJ, Taverne N, Rombout JHWM: **Carp (*Cyprinus carpio* L.) innate immune factors are present before hatching.** *Fish Shellfish Immunol* 2006, **20:**586–596.
5. Huttenhuis HT, Romano N, Oosterhoud C, Taverne-Thiele A, Mastrolia L, Muiswinkel W, Rombout JWM: **The ontogeny of mucosal immune cells in common carp (*Cyprinus carpio* L.).** *Anat Embryol* 2006, **211:**19–29.
6. Laurila S, Holopainen IJ: **Features of embryonic and larval development of crucian carp, *Carassius carassius* (L.) with a note on species identification.** *Ann Zool Fenn* 1990, **27:**361–367.
7. Huttenhuis HBT: **Ontogeny of the common carp (*Cyprinus carpio* L.) immune system. *PhD thesis.*** In Wageningen University; 2005.
8. Vadstein O, Bergh Ø, Gatesoupe F-J, Galindo-Villegas J, Mulero V, Picchietti S, Scapigliati G, Makridis P, Olsen Y, Dierckens K, Defoirdt T, Boon N, De Schryver P, Bossier P: **Microbiology and immunology of fish larvae.** *Rev Aquacult* 2013, **5:**S1–S25.
9. Zapata A, Diez B, Cejalvo T, Gutiérrez-de Frías C, Cortés A: **Ontogeny of the immune system of fish.** *Fish Shellfish Immunol* 2006, **20:**126–136.
10. Zhang S, Wang Z, Wang H: **Maternal immunity in fish.** *Dev Comp Immunol* 2013, **39:**72–78.
11. Li M, Leatherland JF: **The interaction between maternal stress and the ontogeny of the innate immune system during teleost embryogenesis: implications for aquaculture practice.** *J Fish Biol* 2012, **81:**1793–1814.
12. Aoki T, Hirono I, Kurokawa K, Fukuda H, Nahary R, Eldar A, Davison A, Waltzek T, Bercovier H, Hedrick R: **Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide.** *J Virol* 2007, **81:**5058–5065.
13. Costes B, Fournier G, Michel B, Delforge C, Raj VS, Dewals B, Gillet L, Drion P, Body A, Schynts F, Lieffrig F, Vanderplasschen A: **Cloning of the koi herpesvirus genome as an infectious bacterial artificial chromosome demonstrates that disruption of the thymidine kinase locus induces partial attenuation in *Cyprinus carpio koi*.** *J Virol* 2008, **82:**4955–4964.
14. Costes B, Raj VS, Michel B, Fournier G, Thirion M, Gillet L, Mast J, Lieffrig F, Bremont M, Vanderplasschen A: **The major portal of entry of koi herpesvirus in *Cyprinus carpio* is the skin.** *J Virol* 2009, **83:**2819–2830.
15. Raj V, Fournier G, Rakus K, Ronsmans M, Ouyang P, Michel B, Delforges C, Costes B, Farnir F, Leroy B, Wattiez R, Melard C, Mast J, Lieffrig F, Vanderplasschen A: **Skin mucus of *Cyprinus carpio* inhibits cyprinid herpesvirus 3 binding to epidermal cells.** *Vet Res* 2011, **42:**92.
16. Chang WJ, Hwang PP: **Development of zebrafish epidermis.** *Birth Defects Res C Embryo Today Rev* 2011, **93:**205–214.
17. Michel B, Fournier G, Lieffrig F, Costes B, Vanderplasschen A: **Cyprinid herpesvirus 3.** *Emerg Infect Dis* 2010, **16:**1835–1843.
18. Rakus K, Ouyang P, Boutier M, Ronsmans M, Reschner A, Vancsok C, Jazowiecka-Rakus J, Vanderplasschen A: **Cyprinid herpesvirus 3: an interesting virus for applied and fundamental research.** *Vet Res* 2013, **44:**85.
19. Ito T, Sano M, Kurita J, Yuasa K, Lida T: **Carp larvae are not susceptible to koi herpesvirus.** *Fish Pathol* 2007, **42:**107–109.
20. Neukirch M, Böttcher K, Bunnajrakul S: **Isolation of a virus from koi with altered gills.** *Bull Eur Assoc Fish Pathol* 1999, **19:**221–224.
21. Fournier G, Boutier M, Stalin Raj V, Mast J, Parmentier E, Vanderwalle P, Peeters D, Lieffrig F, Farnir F, Gillet L, Vanderplasschen A: **Feeding *Cyprinus carpio* with infectious materials mediates cyprinid herpesvirus 3 entry through infection of pharyngeal periodontal mucosa.** *Vet Res* 2012, **43:**6.
22. Mulero I, García-Ayala A, Meseguer J, Mulero V: **Maternal transfer of immunity and ontogeny of autologous immunocompetence of fish: a minireview.** *Aquaculture* 2007, **268:**244–250.
23. Swain P, Nayak SK: **Role of maternally derived immunity in fish.** *Fish Shellfish Immunol* 2009, **27:**89–99.
24. Fontenot DK, Neiffer DL: **Wound management in teleost fish: biology of the healing process, evaluation, and treatment.** *Vet Clin North Am Exot Anim Pract* 2004, **7:**57–86.
25. Ellis AE: **Innate host defense mechanisms of fish against viruses and bacteria.** *Dev Comp Immunol* 2001, **25:**827–839.
26. Shephard KL: **Functions for fish mucus.** *Rev Fish Biol Fish* 1994, **4:**401–429.
27. Subramanian S, Ross NW, MacKinnon SL: **Comparison of antimicrobial activity in the epidermal mucus extracts of fish.** *Comp Biochem Physiol B Biochem Mol Biol* 2008, **150:**85–92.
28. Palaksha KJ, Shin GW, Kim YR, Jung TS: **Evaluation of non-specific immune components from the skin mucus of olive flounder (*Paralichthys olivaceus*).** *Fish Shellfish Immunol* 2008, **24:**479–488.
29. Vilizzi L, Walker KF: **The onset of the juvenile period in carp, *Cyprinus carpio*: a literature survey.** *Environ Biol Fishes* 1999, **56:**93–102.

doi:10.1186/s13567-014-0100-0
Cite this article as: Ronsmans *et al.*: Sensitivity and permissivity of *Cyprinus carpio* to cyprinid herpesvirus 3 during the early stages of its development: importance of the epidermal mucus as an innate immune barrier. *Veterinary Research* 2014 **45**:100.

Experimental section

2nd chapter:

A herpesvirus alters the behavior of its vertebrate host to enhance its replication and transmission

Manuscript submitted for publication

Maygane Ronsmans [1], Krzysztof Rakus [1], Maria Forlenza, M. Carla Piazzon, Geert F. Wiegertjes, Maxime Boutier, Joanna Jazowiecka-Rakus, Alekos Athanasiadis, Frédéric Farnir, Thomas Michiels and Alain Vanderplasschen

[1] These authors contributed equally to this work

Preamble

The raise of body temperature is a frequent innate immune response expressed by endotherms when they are infected by pathogens (for a recent review on this topic please see (Roth & Blatteis, 2014)). This increase of temperature is part of an integrated physiological syndrome accompanied by neuroendocrine and behavioral modifications, which is named "fever" and aimed to increase host immune defenses and survival. Fever is mainly induced through a humoral pathway. Its signaling starts with the detection of pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs) by immune cells which release pyrogenic cytokines such as interleukin (Il) 1β, Il6, tumor necrosis factor α (Tnfα) and interferons. These cytokines, called endogenous pyrogens, migrate through the bloodstream to the brain where they penetrate the preoptic-anterior hypothalamic area (POA) either by active transendothelial transport or by diffusion through the sensory circumventricular organs (CVOs). The CVO encompass the *organum vasculosum laminae terminalis* (OVLT), the *area postrema* (AP), and the *subfornical organ* (SFO), which all lack a tight blood-brain-barrier but rather present a fenestrated endothelium. In the POA, endogenous pyrogens induce the expression of COX-2 and hence, prostaglandin E_2 (PGE_2) synthesis. As an alternative or parallel pathway, the CVOs but also some brain endothelial and perivascular cells bearing TLRs, can react to PAMPs or circulating cytokines and directly secrete PGE_2, cytokines or other mediators. In addition to the PGE_2 locally produced in the brain, PGE_2 can also be produced by macrophages at the periphery. Independently of its peripheral or brain origin, PGE_2 stimulates a group of neurons bearing the PGE_2-receptor subtype 3 (EP3) in a particular locus of the POA called the median preoptic nucleus (MnPO) which elicits the intrinsic thermogenesis and heat conservation mechanisms. Beside the classical humoral activation pathway described above, fever can also be induced by a neural pathway, mainly through PGE_2 stimulated vagal afferent nerves. This neural stimulation of fever is presumed to be responsible for short onset of fever, as compared to fever resulting from the humoral pathway.

Ectotherms, like endotherms, can increase their body temperature when they are infected by pathogens. Ectotherms lack intrinsic thermogenesis and as a consequence their body temperature reflects the one of their environment. In order to increase their temperature, ectotherms including invertebrates (Campbell *et al.*, 2010; Hunt & Charnley, 2011), amphibians (Kluger, 1977), reptiles (Vaughn *et al.*, 1974) and fish (Cabanac & Laberge, 1998; Grans *et al.*, 2012; Reynolds *et al.*, 1976) have been shown to move to warmer places, a process called "behavioral fever". Signaling pathway of behavioral fever has been studied exclusively at the level of the central nervous system. These studies suggest an evolutionary and functional relationship between behavioral fever in ectotherms and fever in endotherms (Cabanac & Le Guelte, 1980; Evans *et al.*, 2015; Hutchison & Erskine, 1981). No study

has yet demonstrated whether this evolutionary relationship extends to the cytokine mediators that inform the brain of infections detected in the body by immune cells.

Fever in endotherms and behavioral fever in ectotherms can increase host survival to infection. This salutary effect is the consequence of the elevation of body temperature, which increases the efficiency of both innate and adaptive (when existing) immune mechanisms and can restrict replication of invading pathogens (Boltaña *et al.*, 2013; Covert & Reynolds, 1977; Elliot *et al.*, 2002). To date, no pathogen has been shown to alter the expression of behavioral fever to increase its fitness.

When studying the pathogenesis of CyHV-3, we observed that carp infected at 24°C (the thermal preference of healthy carp) tended to concentrate around the tank heater when it was running. This observation led us to postulate that infected subjects could express behavioral fever in natural environments where temperature gradients exist. Moreover, as temperatures above 30°C inhibit CyHV-3 replication *in vitro* and the development of CyHV-3 disease *in vivo* (Gilad *et al.*, 2003), we hypothesized that common carp can express salutary behavioral fever in response to CyHV-3 infection.

A herpesvirus alters the behavior of its host to enhance its replication and transmission

Krzysztof Rakus[1,□], Maygane Ronsmans[1,□], Maria Forlenza[2], M. Carla Piazzon[2], Geert F. Wiegertjes[2], Maxime Boutier[1], Joanna Jazowiecka-Rakus[1], Alekos Athanasiadis[3], Frédéric Farnir[4], Thomas Michiels[5] and Alain Vanderplasschen[1,*]

[1] Immunology-Vaccinology, Fundamental and Applied Research for Animals & Health (FARAH), Faculty of Veterinary Medicine, University of Liège, Liège, Belgium

[2] Cell Biology and Immunology Group, Department of Animal Sciences, Wageningen University, Wageningen, The Netherlands

[3] Instituto Gulbenkian de Ciência, Oeiras, Portugal

[4] Biostatistics and Bioinformatics, FARAH, Faculty of Veterinary Medicine, University of Liège, Liège, Belgium

[5] de Duve Institute, Université Catholique de Louvain, Brussels, Belgium

□ These authors contributed equally to this work

* Corresponding author. Mailing address: Immunology-Vaccinology (B43b), Quartier Vallée 2, Avenue de Cureghem 10, B-4000 Liège, Belgium. Phone: 32-4-366 42 64 - Fax: 32-4-366 42 61

E-mail: A.vdplasschen@ulg.ac.be

Abstract

When infected by pathogens, endotherms and ectotherms can both increase their body temperature to limit the infection. Ectotherms do so by moving to warmer places, hence the term “behavioral fever”. The function of cytokines in the regulation of this process is unknown. It is also unknown whether pathogens can alter this behavior to increase their fitness. Here, we show that cyprinid herpesvirus 3 promotes its replication and transmission by delaying the migration of infected fish to warmer environments. The mechanism of this inhibition was found to rely on the expression of a virally encoded soluble Tnfα-receptor. Our results demonstrate that fever in endotherms and behavioral fever in ectotherms are evolutionarily and functionally related through cytokine mediators that originated more than 400 million years ago. Remarkably, this study demonstrates for the first time the ability of a vertebrate virus to alter host behavior through the expression of a single gene.

Main Text

In endotherms, a primary symptom of infection is a rapid increase of body temperature known as fever. In the classical model of pathogenesis (Fig. 1A), pathogen-associated molecular patterns (PAMPs) acting as exogenous pyrogens are detected by immune cells, which react by secreting pyrogenic cytokines, such as tumor necrosis factor alpha (Tnfα), interleukin (Il)1, Il6, and interferons (*1-4*). These endogenous pyrogens then act at the level of the central nervous system where they induce synthesis of prostaglandins (PG), ultimately leading to fever (*5*).

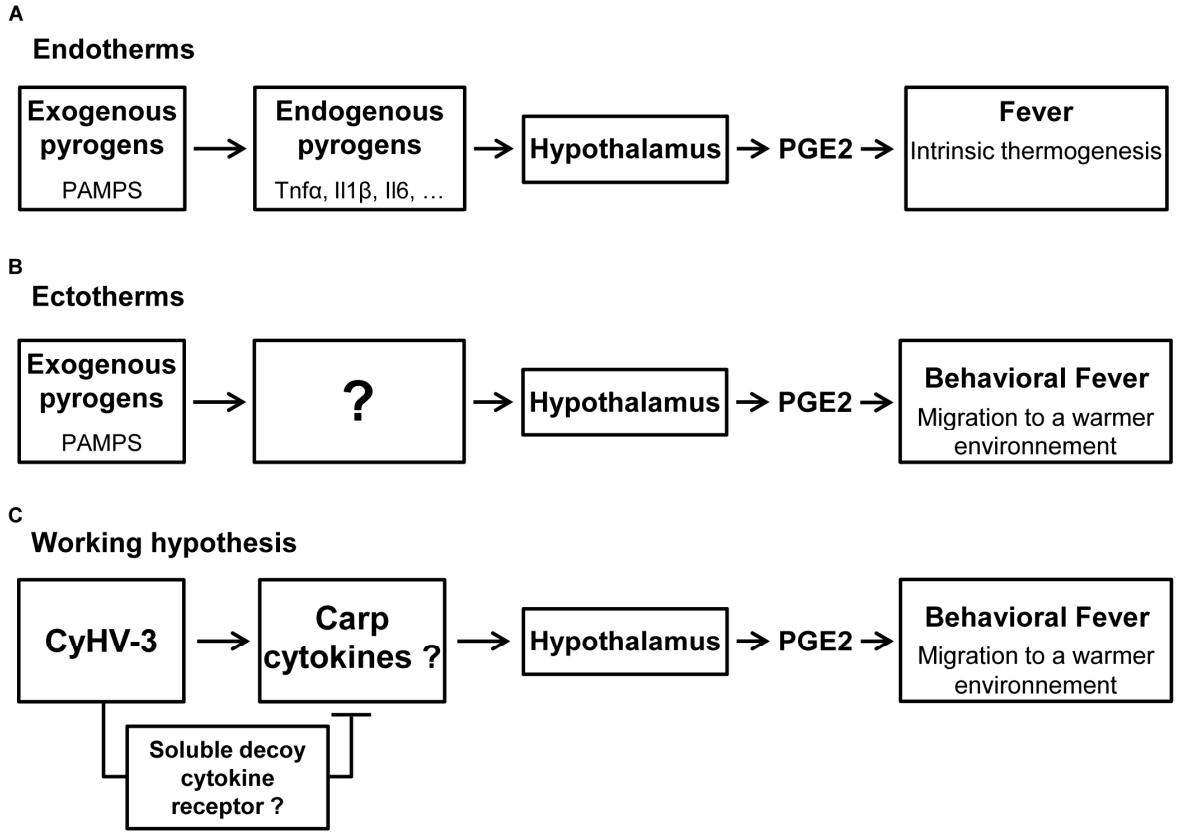


Fig. 1 – Schematic representation of signaling pathways of fever in endotherms (A) and behavioral fever in ectotherms (B). Schematic representation of the working hypothesis addressed in this study (C).

Ectotherms, including invertebrates (*6, 7*), amphibians (*8*), reptiles (*9*) and fish (*10-12*), have also been shown to increase their body temperature in response to infection or injection of exogenous pyrogens. As they lack intrinsic thermogenesis, ectotherms upregulate their body temperature by moving to warmer places. This process is known as “behavioral fever” (Fig. 1B). Previous studies of the regulation of behavioral fever in ectotherms at the level of the central nervous system have demonstrated its evolutionary relationship with fever in endotherms (*4, 13, 14*). However, no study has yet determined whether this evolutionary relationship extends to the cytokine mediators that inform the brain of the infections detected by immune cells throughout the body. Importantly, the identification of homologous pyrogenic cytokines in ectotherms and endotherms would further

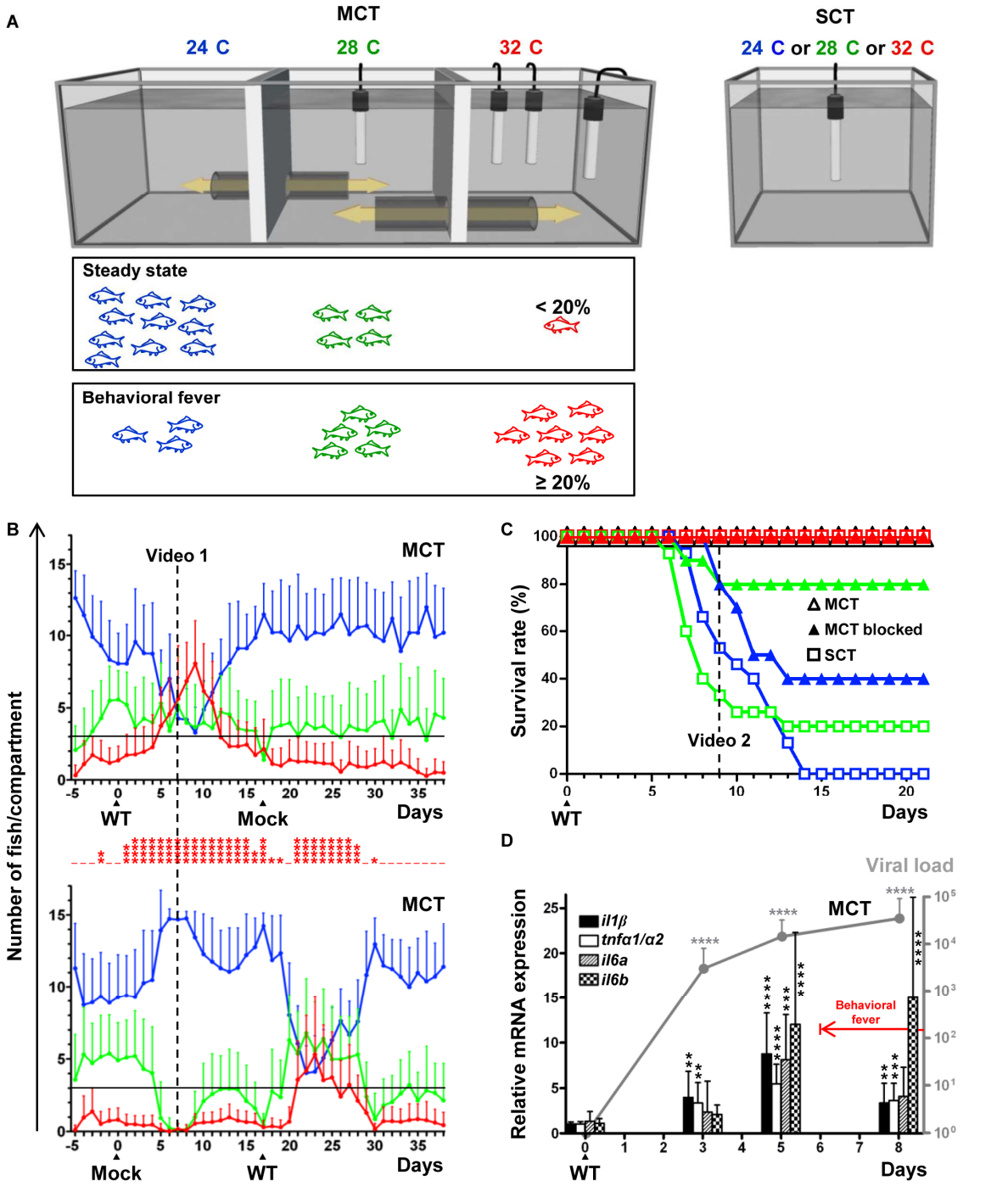


Fig. 2 – Common carp express salutary behavioral fever in response to CyHV-3 infection. (**A**) The experimental setup tank systems were as follows: multi-chamber tank (MCT) and single chamber tank (SCT). Throughout this manuscript, a color code was adopted to illustrate temperature with blue, green and red representing 24°C, 28°C and 32°C, respectively. Behavioral fever was defined as the migration of at least 20% of the fish to the 32°C compartment. (**B**) Distribution of fish (n=15) in the different compartments of the MCT according to time post-CyHV-3 infection. On day 0, fish were infected with wild-type CyHV-3 (WT) (upper graph) or were mock-infected (Mock) (lower graph). On day 17, the tank that was initially infected was mock-infected, and the previously mock-infected tank was infected. The number of fish in each compartment was counted every 30 min and expressed as a mean per day + SD. The days at which the numbers of fish in the 32°C compartment were different between the two tanks are indicated according to the level of significance (red asterisks). See also Supplementary Video 1 recorded at 7 dpi. (**C**) The effect of temperature on survival rate after CyHV-3 infection. Carp were housed in SCTs (n =15; at 24°C, 28°C and 32°C), in a MCT (n=15) (MCT) and in each compartment (n=10) of a MCT in which the tunnels were blocked by grids (MCT blocked). Survival rates were measured according to time post-infection with CyHV-3 (WT) (See also Supplementary Video 2 recorded 9 dpi). (**D**) Viral load (8 fish analyzed per time point, mean + SD) and cytokine gene expression (8 fish analyzed per time point, mean + SD) according to time post-CyHV-3 infection in MCT. Significant differences observed between CyHV-3 infected fish collected at different post-infection times and mock-infected fish sampled at day 0 are indicated by asterisks. The red arrow indicates the period during which behavioral fever was observed.

demonstrate the evolutionary and functional relationship between behavioral fever in ectotherms and fever in endotherms.

Fever in endotherms and behavioral fever in ectotherms can increase host survival to infection. This salutary effect is the consequence of the elevation of body temperature, which increases the efficiency of both innate and adaptive (when existing) immune mechanisms and can restrict replication of invading pathogens (*15-17*). To date, no pathogen has been shown to alter the expression of behavioral fever to increase its fitness.

When studying the pathogenesis of cyprinid herpesvirus 3 (CyHV-3), the causative agent of an emerging and deadly disease in common and koi carp (*Cyprinus carpio*), we observed that carp infected at 24°C (the thermal preference of healthy carp) tended to concentrate around the tank heater when it was running. This observation led us to postulate that infected subjects could express behavioral fever in natural environments where temperature gradients exist. Moreover, as temperatures above 30°C inhibit CyHV-3 replication *in vitro* and the development of CyHV-3 disease *in vivo (18)*, we hypothesized that common carp can express salutary behavioral fever in response to CyHV-3 infection.

To test this hypothesis common carp were housed in multi-chamber tanks (MCTs; Fig. 2A), where they could freely move between three chambers maintained at 24, 28 and 32°C. Fish distribution in the three compartments was recorded over time (Fig. 2B). Based on multiple observations of mock-infected and infected carp, behavioral fever was defined as the migration of at least 20% of the fish to the 32°C compartment. In the absence of infection, the majority of carp were distributed in the 24°C compartment and to a lesser extent in the 28°C compartment, with variations between experiments. Independently of this variation, the fish began to reside more frequently in the 32°C compartment between 4 and 6 days post-infection (dpi) (range defined based on all experiments performed), with a peak at approximately 6-9 dpi. At 12 dpi, the distribution of the fish was usually returned to normal, with only the occasional fish in the 32°C compartment. To confirm these observations, at 17 dpi, the tank that was initially infected was mock-infected (Fig. 2B, upper graph), and the previously mock-infected tank was infected (Fig. 2B, lower graph). We again observed that CyHV-3 infection induced behavioral fever. Interestingly, none of the fish infected with CyHV-3 in the MCTs died during the course of these experiments, leading to the hypothesis that expression of behavioral fever could be salutary for carp. The results presented in Fig. 2C confirmed this hypothesis. None of the fish infected in the MCTs died from the infection, whereas survival rates of 0% and 20% were recorded when fish were infected in single chamber tanks (SCTs; Fig. 2A) maintained at 24°C and 28°C, respectively. Significantly higher survival rates of 40% and 80% were observed when infected fish were blocked by grids in the 24°C and 28°C compartments of the MCT, respectively. The higher survival rates observed in the chambers of the MCT compared with their respective SCT are likely explained by the temperature variations existing in front of the entry of the tunnels. Consistent

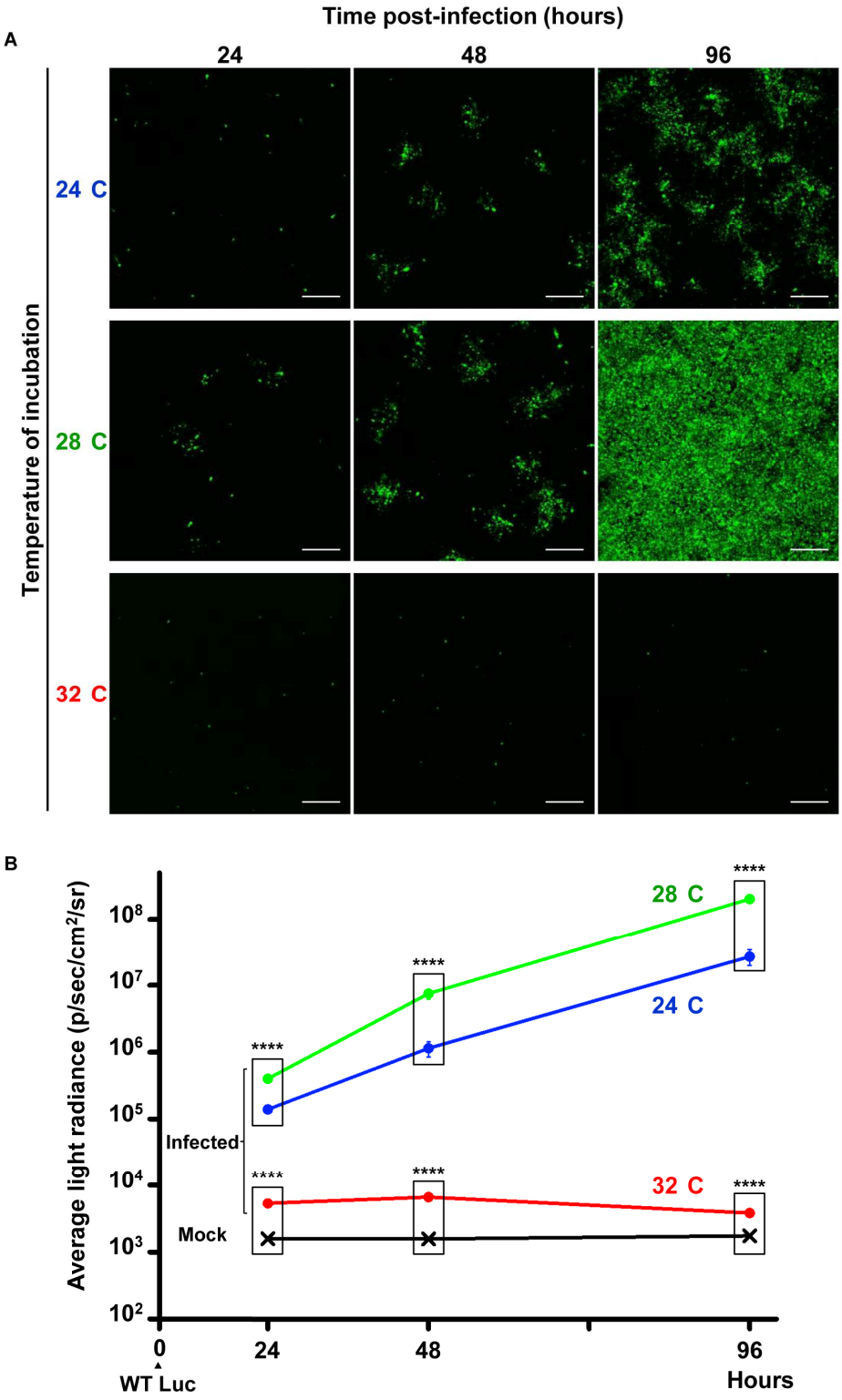


Fig. S1 – Effect of temperature on CyHV-3 replication *in vitro*. CCB monolayers were infected at a MOI of 0.01 pfu/cell for 2 h at 24°C with the CyHV-3 EGFP strain (**A**) or WT Luc strain (**B**). Cells were then incubated at 24, 28 or 32°C. At the indicated post-infection times, cells were analyzed to estimate the level of viral replication. (**A**) EGFP expression was visualized by confocal microscopy. Scale bars = 500 µm. (**B**) Luc expression was quantified by IVIS. Mock-infected cells were used as negative controls. The data presented are the average light radiance ± SD of quadruplicate analyses. The significant differences of framed data are marked with asterisks.

with an earlier report (*18*), infected fish in the SCT at 32°C or blocked in the 32°C compartment of the MCT did not develop CyHV-3 disease. The effect of temperature on the development of CyHV-3 disease is also illustrated by Video 2. It shows that clinical signs expressed by fish blocked in MCT chambers were inversely related to temperature. To test whether the correlation observed between the reduction of clinical signs and the elevation of temperature could be explained by a reduction of viral replication, CyHV-3 growth was measured *in vitro* at different temperatures using recombinant strains expressing reporter genes (Fig. S1). Viral growth was efficient at both 24°C and 28°C but was significantly higher at the latter temperature. By contrast, viral replication was completely blocked at 32°C. Collectively, these results indicate that the lower mortality observed at 28°C compared with 24°C was likely due to an enhancement of anti-viral immune mechanisms rather than an inhibition of viral growth, whereas the absence of mortality at 32°C was due to both inhibition of viral growth and enhancement of anti-viral immunity.

Clinical observation of fish in the MCTs revealed that their migration to the hottest compartment only occurred after they developed severe clinical signs. Viral load and carp proinflammatory cytokine gene expression confirmed that the onset of behavioral fever observed in this experiment at 6 dpi (Fig. 2D) occurred days after systemic replication of the virus (significant at 3 dpi) and upregulation of proinflammatory cytokines (significant for *il1β* and *tnfα1/α2* at 3 dpi, and for all cytokines tested at 5 dpi) (Fig. 2D). The relatively late onset of behavioral fever with respect to viral replication and cytokine upregulation led us to postulate that this phenomenon could be delayed by the virus to retain its host at a permissive temperature thereby favoring its replication and spreading (Fig. 1C). As some herpesviruses have been shown to express soluble decoy cytokine receptors (*19, 20*), we hypothesized that CyHV-3 could express such receptor(s) able to neutralize putative pyrogenic cytokines produced by the fish (Fig. 1C). According to this hypothesis, the onset of behavioral fever would only occur after the concentration of pyrogenic cytokine was sufficiently high to outcompete the neutralizing activity of the viral protein.

Bioinformatic analyses, including homology modeling, were performed on the CyHV-3 genome to identify viral gene candidates compatible with this model. Based on these analyses, ORF12, which encodes a putative soluble Tnfα-receptor (Fig. S2), was selected. The selection of ORF12 as a viral gene candidate for this model was also supported by the observation that its expression product is the most abundant viral protein in the CyHV-3 secretome (*21*) and the fact that Tnfα is an endogenous pyrogen in endotherms (*22-25*). Therefore, we hypothesized that Tnfα could act as a pyrogenic cytokine in ectotherms, such as in endotherms, and that CyHV-3 could neutralize this cytokine and, thus, the behavioral fever response through secretion of a soluble decoy Tnfα-receptor (Fig. 1C).

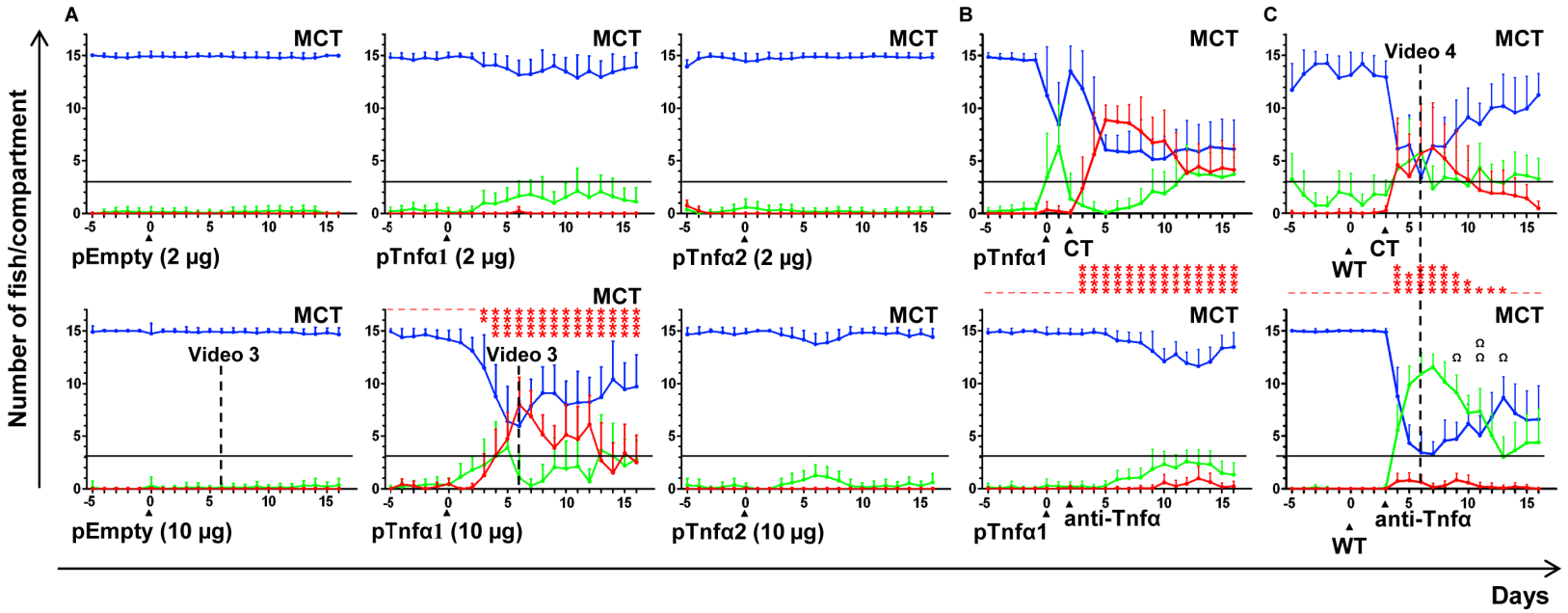


Fig. 3 – Tnfα mediates the expression of behavioral fever. Distribution of fish (n=15) in the compartments of the MCT was analyzed every 30 min and expressed as a mean per day + SD. The days at which the number of fish in the 32°C compartment was significantly different between the experimental tank and its respective control are indicated according to the level of significance (red asterisks). (**A**) Tnfα1 mediates the expression of behavioral fever. Carp were injected intramuscularly with the indicated plasmids at the dose of 2 and 10 µg/g fish (See also Supplementary Video 3 recorded on day 6). (**B**) Anti-Tnfα antibodies neutralize behavioral fever induced by injection of the pTnfα1 plasmid. Carp were injected with the pTnfα1 plasmid (10 µg/g fish) and then injected two days later with irrelevant control (CT) (upper graph) or anti-Tnfα (lower graph) antibodies. (**C**) Anti-Tnfα antibodies inhibit the expression of behavioral fever. Carp were infected with CyHV-3 (WT) and then injected three days later with CT (upper graph) or anti-Tnfα (lower graph) antibodies. The Ω symbol illustrates fish that died from the infection (See also Supplementary Video 4 recorded 6 dpi). The results presented in this figure are representative of duplicate experiments.

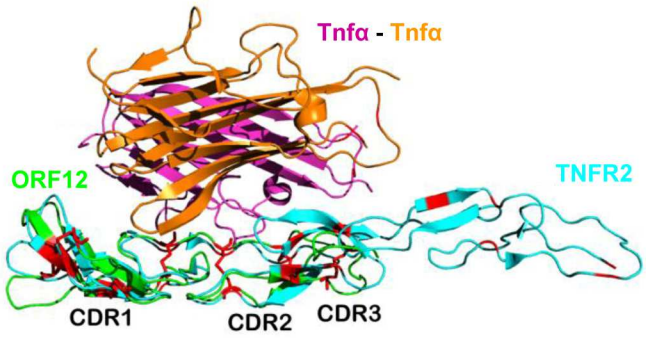


Fig. S2 – ORF12 structure prediction. Model of ORF12 protein (green) superimposed on the human TNFR2 structure (cyan) presented in complex with two TNFα molecules (magenta/orange). ORF12 cysteine residues involved in disulfide bonding are shown as sticks, cysteine rich domains are indicated as CRD 1-3.

To explore this hypothesis, we first tested whether expression of carp Tnfα induces behavioral fever. Vectors encoding carp Tnfα1 or Tnfα2 were injected into naïve animals housed in MCTs (Fig. 3A). Injection of an expression vector encoding carp Tnfα1 (Video 3), but not carp Tnfα2 or the empty vector, induced behavioral fever (Fig. 3A). The induction of behavioral fever by the vector encoding Tnfα1 was neutralized by injection of anti-Tnfα antibodies but not by injection of irrelevant control antibodies (Fig. 3B). These results indicate that Tnfα1 expression is sufficient to induce behavioral fever and that the anti-Tnfα antibodies used in this experiment are capable of neutralizing Tnfα *in vivo*.

Next, we used these antibodies to determine whether Tnfα is a key mediator of the behavioral fever response in CyHV-3 infected carp (Fig. 3C). CyHV-3 infected carp injected with anti-Tnfα antibodies migrated to the 28°C but not to the 32°C compartment; consequently, only 73.3% of the fish survived. By contrast, infected fish injected with irrelevant antibodies migrated to the 32°C compartment, and all survived the infection. These results demonstrate that carp Tnfα is a major mediator of behavioral fever in our model and support the hypothesis that CyHV-3 ORF12 acts as a decoy receptor for Tnfα, thereby neutralizing the cytokine and delaying the expression of behavioral fever in carp.

To test these hypotheses, a CyHV-3 ORF12 deletion (Δ12) mutant and a revertant (12Rev) virus (in which ORF12 was restored) were derived from the parental wild-type (WT) strain (Fig. S3). The genome structure and the transcription of the ORF12 region in all virus strains were controlled (Fig. 4A and B). The three strains replicated comparably in cell culture at 24°C and induced similar clinical signs and survival rates in carp housed at 24°C in the SCT (Fig. 4C and D). Therefore, ORF12 deletion does not exhibit a phenotype under these laboratory experimental conditions.

To test the hypothesis that ORF12 encodes a soluble Tnfα-receptor, concentrated supernatants were produced from cell cultures infected with CyHV-3 WT, Δ12 and 12Rev strains as well as from mock-infected cultures. Importantly, silver staining of total supernatant proteins confirmed that

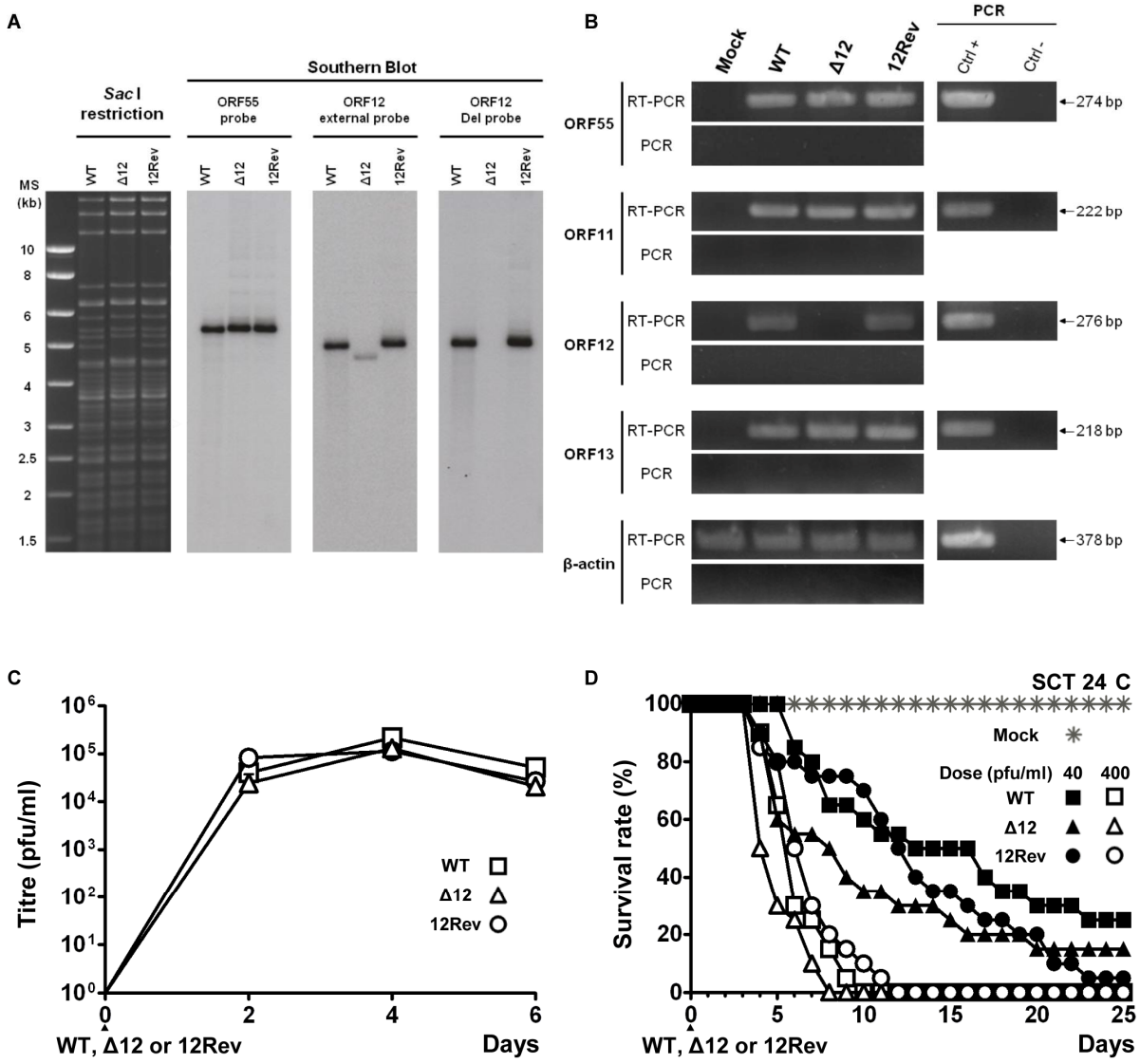


Fig. 4 – Characterization of CyHV-3 ORF12 recombinant strains. (**A**) Structural analysis of the genome of ORF12 recombinant strains by *Sac* I restriction and Southern blotting. Marker sizes (MS) are indicated on the left. (**B**) RT-PCR analysis of the ORF12 genome region. On the left, RT-PCR or PCR represent PCR products generated when the RT was performed or omitted from the reactions, respectively. On the right, control PCR reactions were performed using CyHV-3 DNA as template (Ctrl+) or no template (Ctrl-). (**C**) Effect of ORF12 deletion on viral growth *in vitro*. Replication kinetics of CyHV-3 ORF12 recombinant strains were compared with those of the WT strain using a multi-step growth assay at 24°C. The data presented are the means + SD of duplicate measurements. (**D**) Cumulative survival rates of common carp (n=20) mock-infected or infected with two different doses of CyHV-3 ORF12 recombinant strains.

ORF12 is the most abundant viral protein in the CyHV-3 secretome (*21*) (Fig. 5A). To test the ability of ORF12 to bind carp Tnfα, proteins of concentrated supernatants were coated on ELISA plates before incubation with carp Tnfα1 and Tnfα2 (Fig. 5B). Quantification of Tnfα binding demonstrated that WT and 12Rev supernatants contain Tnfα-binding activity in contrast to the Δ12 and mock-infected supernatants (Fig. 5B). Next, a bioluminescence reporter assay was used to determine whether ORF12 binding to Tnfα could neutralize its ability to activate NF-κB signaling (Fig. 5C). When incubated with concentrated supernatants from mock- or CyHV-3 Δ12-infected cells, carp Tnfα1 and Tnfα2 similarly induced activation of NF-κB. This activation was completely inhibited when the cytokines were pre-incubated with WT or 12Rev supernatants (Fig. 5C). Collectively, these data demonstrated that ORF12 encodes a soluble Tnfα-receptor able to neutralize both carp Tnfα1 and Tnfα2.

Finally, we tested whether ORF12 can delay the behavioral fever response. Carp housed in the MCT were infected with CyHV-3 Δ12 and 12Rev strains (Fig. 6). The onset of behavioral fever occurred at least two days earlier in fish infected with the Δ12 strain compared with fish infected with the 12Rev strain (Fig. 6A). None of the fish died from the infection during the course of these experiments. However, as fish infected with the Δ12 strain migrated to the hottest compartment earlier than those infected with the 12Rev strain, they expressed less severe clinical signs. To verify this phenotypic difference between the Δ12 and 12Rev strains (not observed in the SCT at 24°C; Fig. 4D), fish were infected with the two viral strains and housed either in the SCT at 24°C or in the MCT (Fig. 6B). At different post-infection times, viral load was measured. Interestingly, although the two strains exhibited comparable replication kinetics in the SCT throughout the course of the experiment and in the MCT before expression of behavioral fever, they differed drastically once the fish migrated to the 32°C compartment of the MCT. As a consequence of their early migration to a non-permissive temperature, fish infected with the Δ12 strain showed a faster and more drastic decrease of viral load in the gills than those infected with the 12Rev strain. As the viral load in the gills has been shown to be correlated with CyHV-3 excretion and CyHV-3 transmission to naïve cohabitant fish (*26*), these results demonstrate that CyHV-3 is able to alter the behavior of its host to favor its replication and transmission through the expression of a single gene.

In the present study, we used a relevant biological model in which CyHV-3 infects its natural host through a natural route. The data obtained highlight the importance of the environment in the pathogen-host-environment interplay (*27*). They also show the role of a cytokine as a mediator of behavioral fever in ectotherms and demonstrate the ability of a vertebrate virus to alter the behavior of its host through the expression of a single gene.

Depending on the environment, CyHV-3 infection can generate mortality rates ranging from 0 to 100%. In environments that allow the expression of behavioral fever, no mortality was observed. In

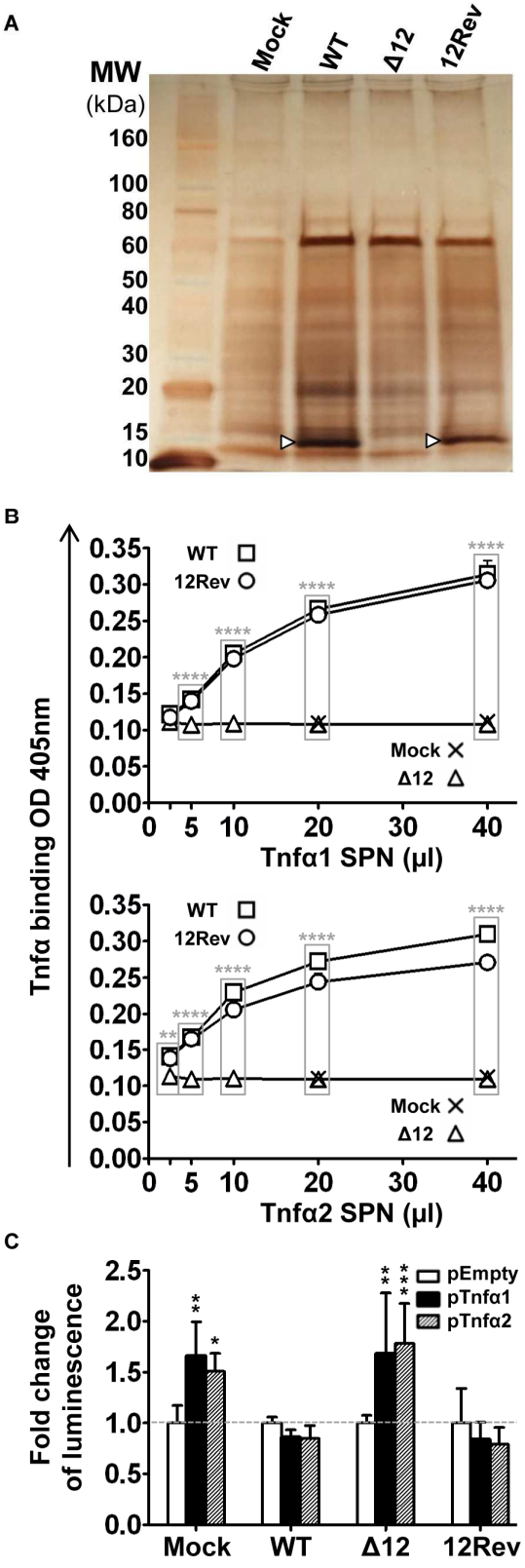


Fig. 5 – CyHV-3 ORF12 is a secreted protein that neutralizes carp Tnfα. (**A**) Silver staining of total proteins found in concentrated supernatants of CCB cells infected with CyHV-3 (WT, Δ12 and 12Rev strains) or mock-infected (Mock). Arrowheads indicate the band corresponding to ORF12 protein (expected molecular weight of the secreted form without glycosylation is 12.1 kDa). (**B**) ELISA binding assay of carp Tnfα1 and Tnfα2 to CyHV-3 secreted proteins. The data are the mean + SD of duplicate measurements. Results for which a significant difference was observed between WT/12Rev and Δ12/Mock groups are marked by asterisks. The data are representative of two independent experiments. (**C**) Neutralization of carp Tnfα1 and Tnfα2 by CyHV-3 secreted proteins was tested using a Tnfα bioluminescent reporter assay. The data are expressed as fold change of luminescence relative to the respective controls and represent the mean + SD of quadruplicate measurements. Results for which a significant difference was observed between pTNFα1 or pTNFα2 and pEmpty are marked by asterisks. These data are representative of three independent experiments.

contrast, in some conditions where this behavior cannot be expressed, mortality reached 100%. These observations demonstrate that the virulence of pathogens infecting ectotherms can be exacerbated by environmental changes that prevent their host from expressing behavioral fever. For example, the artificial introduction of an ectothermic species into a new environment where the range of temperatures is incompatible with the expression of behavioral fever can turn a benign host-adapted virus into a lethal virus. Our study highlights the importance of the environment in the pathogen-host-environment interplay and identifies a new mechanism through which environmental temperature changes contribute to disease emergence.

A few studies performed on the regulation of behavioral fever in ectotherms at the level of the central nervous system have suggested an evolutionary relationship between behavioral fever in ectotherms and fever in endotherms. However, whether peripheral cytokine mediators are involved in this evolutionary relationship has never been explored. Here, for the first time, we identified the role of Tnfα as a key mediator of behavioral fever in an ectotherm. Our results demonstrate that fever in endotherms and behavioral fever in ectotherms are evolutionarily and functionally related through common cytokine mediators that originated more than 400 million years ago. The results of this study suggest that the ancestral signaling pathway of behavioral fever regulation in ectotherms also evolved in endotherms to regulate the expression of fever. Our results also support the importance of the interplay between the immune system and the central nervous system (*28, 29*).

Viruses are able to manipulate, through the expression of dedicated genes, virtually all physiological processes of their host that can affect their replication and transmission. However, there are extremely few reports of viral genes that alter host behavior. To the best of our knowledge, there are only two such reports and both examined altered host behavior caused by invertebrate viruses. Tyrosine phosphatase (*30*) and ecdysteroid uridine 5'-diphosphatase-glucosyltransferase (*31*) are encoded by baculoviruses and have been shown to increase the locomotory activity and climbing behavior of infected caterpillar hosts, respectively. Here, we demonstrated that the CyHV-3 genome contains a gene that delays the expression of behavioral fever, demonstrating that this innate immune response has been acting as a selection pressure on pathogens throughout evolution. Interestingly, this function of the ORF12 gene was only observed under conditions that mimicked temperature gradients of natural environments, and no phenotypic expression of the ORF12 gene was observed under standard laboratory conditions. These results add a new dimension to environment enrichment for laboratory animals. By delaying the migration of infected fish to warmer environments, CyHV-3 enhances its transmission in two ways. First, it enhances viral replication and excretion by retaining infected fish at a permissive temperature. Second, it favors viral transmission by retaining the infected fish at the temperature preferred by non-infected fish. ORF12 delays the behavioral fever response

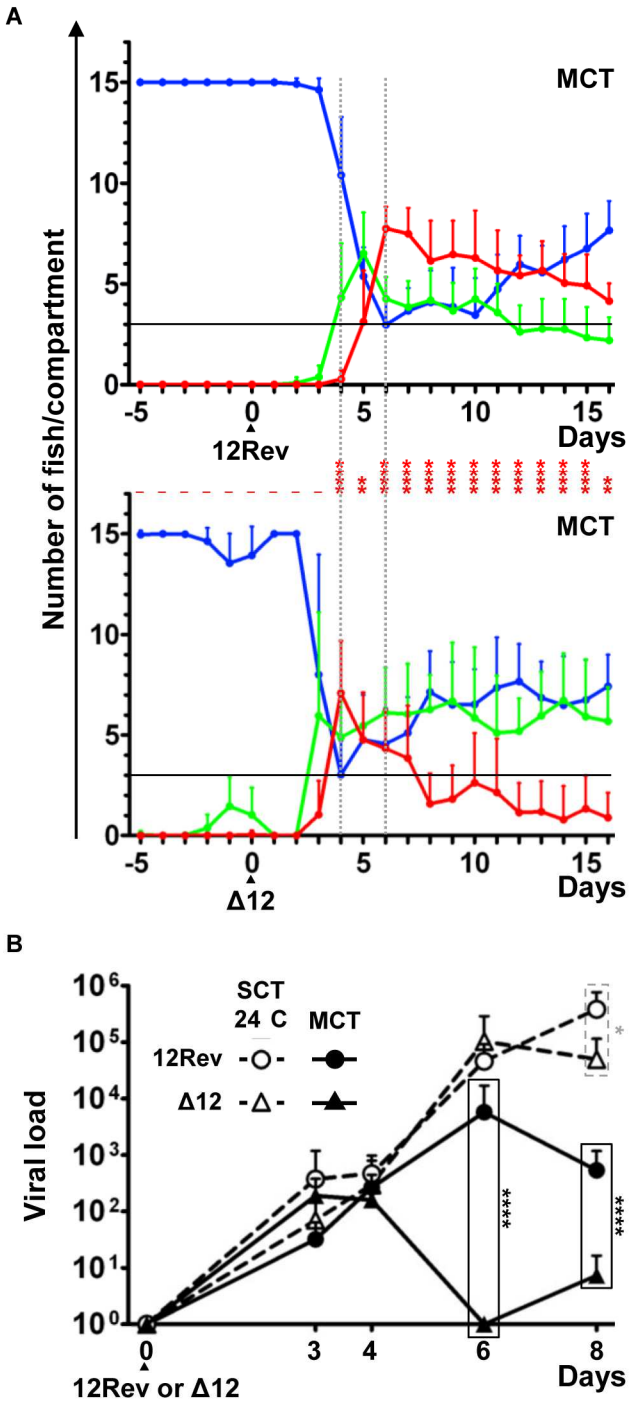


Fig. 6 – CyHV-3 ORF12 delays the expression of behavioral fever. (**A**) Distribution of fish (n=15) in the different compartments of the MCT according to time post-infection (days). Black arrowheads indicate the time at which fish were infected with 12Rev or Δ12 strains of CyHV-3. The number of fish in each compartment was counted every 30 min and expressed as a mean per day + SD. The days at which the number of fish in the 32°C compartment were significantly different between the two tanks are indicated according to the level of significance (red asterisks). The data are representative of three independent experiments. (**B**) Kinetics of viral load according to time post-infection. Fish housed in the SCT at 24°C and in the MCT were infected with 12Rev or Δ12 strains of CyHV-3. At the indicated post-infection times, viral load was measured in gills (6 fish analyzed per time point, mean + SD). For each tank system, the results obtained for the 12Rev and Δ12 strains were analyzed for significant differences (marked by asterisks).

rather than completely inhibiting it. This time-dependent effect of ORF12 likely reflects a selective advantage for CyHV-3. As indicated above, by delaying the behavioral fever response, ORF12 enhances viral spread through a fish population; furthermore, by allowing the fish to finally express behavioral fever and, thus, survive the infection, the virus promotes the appearance of latently infected fish that will carry and spread the herpesvirus infection throughout their lives (*32*).

In conclusion, this study demonstrates the ability of a vertebrate virus to alter the behavior of its hosts through the expression of a single gene. Remarkably, this viral gene illustrates the "*Central Theorem of the Extended Phenotype"*: the viral gene promotes its spread through a phenotypical effect on an independent biological entity (the infected fish) (*33*).

Materials and Methods

Cells and viruses

Epithelioma papulosum cyprini (EPC) and *Cyprinus carpio* brain (CCB) cells were cultured as described previously (*26, 34*). Two CyHV-3 recombinant strains described previously were used in the present study (*35, 36*). Both strains were derived from the CyHV-3 FL BAC plasmid and encode a reporter gene under control of the HCMV IE promoter. The FL BAC recovered strain (called CyHV-3 EGFP in the present manuscript) encodes an EGFP expression cassette inserted in the 3' end of ORF55 (*35*). The FL BAC revertant ORF136 Luc strain (called CyHV-3 Luc in the present manuscript) encodes a firefly (*Photinus pyralis*) luciferase expression cassette inserted between ORF136 and ORF137 (*36*).

Tnfα eukaryotic expression vectors and anti-Tnfα antibodies

The previously described eukaryotic expression plasmids pIRES-tnfα1-EGFP and pIRES-tnfα2-EGFP (hereafter referred to as pTnfα1 and pTnfα2) (*34*) were used to express carp Tnfα1 and Tnfα2, respectively. The empty vector, pIRES-EGFP (*34*) (hereafter referred to as pEmpty), was used as a negative control. These plasmids were used both *in vitro* (transfection of cell cultures) and *in vivo* (intramuscular injection of carp) as described previously (*34*). Affinity-purified polyclonal rabbit anti-carp Tnfα IgG binding to both carp Tnfα1 and Tnfα2 (anti-Tnfα) (*34*) as well as purified polyclonal rabbit irrelevant control IgG (CT) (BD Biosciences) were used in this study.

Homology modeling

Homology modeling of CyHV-3 ORF12 (NCBI ID: 131840042) was performed using the PHYRE2 server (*37*) (Fig. S2). Among several models of comparably high quality (confidence over 99%) based on different Tnf and Tnf-like receptor homologues, we selected a model based on the HVEM/TNFRSF14 structure (PDB ID: 1JMA) because it showed the highest score (confidence of 99.6%, 35% identity) and allowed for disulphide bonding of all cysteine residues of ORF12. A model of interaction of ORF12 with Tnfα was created by superimposing the ORF12 model onto the human TNFR2/TNFα complex structure (PDB ID: 3ALQ).

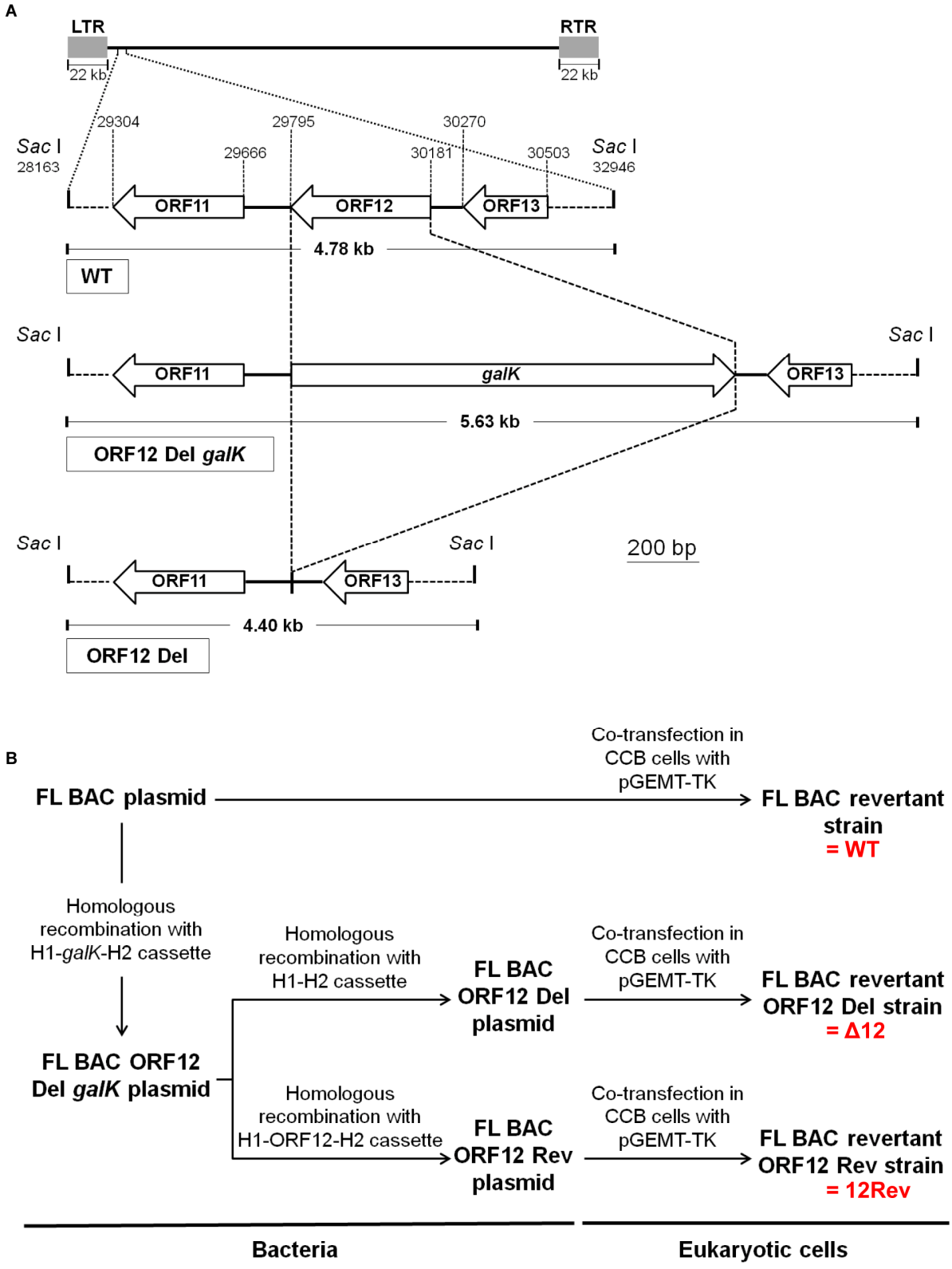


Fig. S3 – Production of CyHV-3 ORF12 recombinants. (**A**) The region of the CyHV-3 genome encoding ORF12 is illustrated for wild type (WT), ORF12 Del *galK* and ORF12 Del genotypes. ORFs are represented by white arrows. Restriction *Sac* I sites and predicted restriction fragments (in kb) are shown. All coordinates correspond to the reference CyHV-3 sequence available in GenBank (NC_009127.1). (**B**) Flow chart of steps performed to produce ORF12 recombinant plasmids and to reconstitute virus strains. To simplify the reading of the manuscript, abbreviations of the full names of the recombinants are provided (in red), and these abbreviations have been used throughout the manuscript.

Production of CyHV-3 ORF12 recombinant strains using BAC cloning and prokaryotic recombination technologies

The CyHV-3 FL BAC clone was used as the parental plasmid (*35*). Recombinant plasmids were produced using galactokinase (*galK*) positive/negative selection in bacteria (*26, 35*) (Fig. S3B). The first recombination process (*galK* positive selection) replaced ORF12 by *galK*, resulting in the FL BAC ORF12 Del *galK* plasmid. Recombination was achieved using the H1-*galK*-H2 recombination cassette, which consisted of the *galK* gene flanked by 50 bp sequences of the CyHV-3 genome flanking ORF12. This recombination cassette was produced by PCR using the p*galK* vector as a template and the primers listed in table S1. The second recombination process (*galK* negative selection) removed the *galK* gene (FL BAC ORF12 Del plasmid) or replaced the *galK* gene by the CyHV-3 wild type ORF12 sequence (FL BAC ORF12 Rev plasmid). The FL BAC ORF12 Del plasmid was obtained by recombination with the H1-H2 cassette. This cassette consisted of 50 bp of the CyHV-3 genome upstream and downstream of ORF12. The FL BAC ORF12 Rev plasmid was produced by recombination with the H1-ORF12-H2 cassette. This cassette was produced by PCR using the primers listed in table S1 and CyHV-3 FL DNA as the template. To reconstitute infectious virus, the recombinant BAC plasmids were co-transfected with the pGEMT-TK plasmid (molecular ratio of 1:75) (*35*) in CCB cells. Transfection with the pGEMT-TK plasmid induced recombination upstream and downstream of the BAC cassette, leading to its complete removal and consequent reversion to a wild-type ORF55 locus (FL BAC revertant strains) (*35*). Plaques negative for enhanced green fluorescent protein (EGFP) expression (the BAC cassette encodes an EGFP expression cassette) were picked and amplified.

Genetic characterization of CyHV-3 ORF12 recombinants

CyHV-3 ORF12 recombinants were characterized by sequencing the regions used to target recombination and by combined *Sac* I restriction fragment length polymorphism (RFLP)-Southern blot analyses (Fig. 4A) (*26, 35*). Probes for Southern blot analyses were produced by PCR using the primers listed in table S1 and the CyHV-3 FL genome as the template (*35*).

Transcriptional analysis by RT-PCR

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen) with on-column DNase I digestion. cDNA was synthesized from 1 µg of RNA using the iScript cDNA Synthesis Kit (Bio-Rad) and random hexamer/oligo(dT) primers. Finally, PCRs were performed with the primers listed in table S1.

Table S1. Primers

	Primer name	Sequence (5'- 3')	Coordinates*/ Accession number
Synthesis of recombination cassettes			
Cassette name			
H1-*galK*-H2	ORF12 *galK* F	AGGCTGCACTGCTGCGCACAGTGACGAGTAGACGGTGGGA GGTCGGTGAACCTGTTGACAATTAATCATCGGCA	29745-29794
	ORF12 *galK* R	CTTGTTTTACTATACTCTATCACATCTCCGACTTGATTTCTT CTCAAACCTCAGCACTGTCCTGCTCCTT	30231-30182
H1-ORF12-H2	ORF12 Fw RVT	ATGAAGAGTTTGTGTCGAGC	29668-29687
	ORF12 Rev RVT	GGCTACGTATAACTGTCATG	30312-30293
Synthesis of probes for Southern blot analysis			
Probe name			
CyHV-3 ORF55	ORF55InF	AGCGCTACACCGAAGAGTCC	95990-96009
	ORF55stopR	TCACAGGATAGATATGTTACAAG	96516-96494
CyHV-3 ORF12 Del	ORF12 Int Fw 5'	TCGTAGTCGCCCTGACATCC	29847-29866
	ORF12 Int Rev 3'	AACTAGACACTCATCATGCGG	30122-30102
CyHV-3 ORF12 external	ORF12 Fw 5'	GAATTTATATGCAGCGAGTG	29723-29742
	ORF12 Rev 3'	TATTGTCTGTTTCTGTGCTC	30261-30242
Transcriptional analysis			
Gene amplified			
CyHV-3 ORF11	ORF11 Fw	CAACCCACAACAGCAGTACC	29595-29576
	ORF11 Rev	TTGCCCTGTCTCATCTTGGT	29374-29393
CyHV-3 ORF12	ORF12 Int Fw 5'	TCGTAGTCGCCCTGACATCC	29847-29866
	ORF12 Int Rev 3'	AACTAGACACTCATCATGCGG	30122-30102
CyHV-3 ORF13	ORF13 Fw	TGTGAGTCATGACAGTTATACGT	30286-30308
	ORF13 Rev	ATGACTGACTGGACATCGGC	30503-30484
CyHV-3 ORF55	ORF55 ATG Fw	ATGGCTATGCTGGAACTGG	95866-95884
	ORF55 In Rev	GGCGCACCCAGTAGATTATG	96467-96448
Carp *β-actin*	Actin-Fw	ATGTACGTTGCCATCCAGGC	M24113
	Actin-Rev	GCACCTGAACCTCTCATTGC	
qPCR analysis for quantification of virus load			
Gene amplified			
CyHV-3 ORF89	KHV-86F	GACGCCGGAGACCTTGTG	AF411803
	KHV-163R	CGGGTTCTTATTTTTGTCCTTGTT	
	KHV-109P	(6FAM) CTTCCTCTGCTCGGCGAGCACG (BHQ1)	
Carp *glucokinase*	CgGluc-162F	ACTGCGAGTGGAGACACATGAT	AF053332
	CgGluc-230R	TCAGGTGTGGAGCGGACAT	
	CgGluc-185P	(6FAM) AAGCCAGTGTCAAAATGCTGCCCACT (BHQ1)	
RT-qPCR analysis for quantification of cytokine expression			
Gene amplified			
Carp *40S*	40S-F	CCGTGGGTGACATCGTTACA	AB012087
	40S-R	TCAGGACATTGAACCTCACTGTCT	
Carp *il1β*	IL-1β-F	AAGGAGGCCAGTGGCTCTGT	AJ245635
	IL-1β-R	CCTGAAGAAGAGGAGGCTGTCA	
Carp *tnfα1* and *tnfα2*	TNF-α1 and 2-F	GCTGTCTGCTTCACGCTCAA	AJ311800 and AJ311801
	TNF-α1 and 2-R	CCTTGGAAGTGACATTTGCTTTT	
Carp *il6a*	IL-6a-F	CAGATAGCGGACGGAGGGGC	KC858890
	IL-6a-R	GCGGGTCTCTTCGTGTCTT	
Carp *il6b*	IL-6b-F	GGCGTATGAAGGAGCGAAGA	KC858889
	IL-6b-R	ATCTGACCGATAGAGGAGCG	

*Coordinates based on the reference CyHV-3 genome (Accession number: NC_009127.1)
Underlined: 50 bp corresponding to the CyHV-3 sequence
Red: sequence corresponding to *galK*

Multi-step growth curves

Duplicate cultures of CCB cells were inoculated with CyHV-3 at a multiplicity of infection (MOI) of 0.5 plaque forming unit (pfu)/cell. After an incubation period of 2 h at 24°C, cells were washed with PBS and overlaid with Dulbecco's modified essential medium (DMEM, Sigma) containing 4.5 g of glucose/L and 10% foetal calf serum (FCS). Supernatants were collected from the infected cultures at successive intervals (0, 2, 4, and 6 dpi) and stored at -80°C. The titration of infectious viral particles was determined by duplicate plaque assays in CCB cells as described previously (*21, 36*).

Microscopy analysis

CCB cells were fixed with PBS containing 4% (w/v) paraformaldehyde for 15 min on ice followed by 10 min at room temperature (RT). Confocal microscopy analysis was performed with a Nikon A1R hybrid resonant.

Bioluminescent imaging

Firefly (*Photinus pyralis*) luciferase activity in the cell culture was imaged using an "*in vivo* imaging system" (IVIS) (IVIS spectrum, Caliper LifeSciences) (*36, 38-40*). The culture medium was replaced with fresh medium containing D-luciferin (150 µg/ml) (Caliper LifeSciences) 5 min before the analysis. Images were acquired using a field of view C, a maximum auto-exposure time of 1 min, a binning factor of 4, and an f/stop of 1. ROIs were drawn using Living Image 3.2 software, and the average light radiance (p/sec/cm²/sr) was taken as the final measure of the bioluminescence emitted over the ROI.

Production of concentrated cell supernatants

Cultures of CCB cells were infected with CyHV-3 at a MOI of 0.1 pfu/cell or mock-infected at 24°C for 2 h. After infection, cells were washed with PBS and overlaid with serum-free DMEM. Cell supernatants were collected 72 h post-inoculation and subjected to two cycles of centrifugation at 4°C (clarification at 2,000 g for 30 min followed by pelleting of viral particles at 100,000 g for 2 h through a 30% sucrose cushion). The supernatants were then concentrated 25-fold by centrifugation (2,000 g, 50 min, 4°C) through an Amicon Ultra-15 centrifugal filter unit (10K NMWL, Merck Millipore) and stored at -80°C until use. Total protein concentration was measured using the Pierce BCA Protein Assay kit (ThermoScientific).

Tnfα binding assay

Binding of carp Tnfα to the CyHV-3 secretome was analyzed by ELISA. ELISA plates (96-well; Greiner Bio-One) were coated with 5 µg of total protein of concentrated CCB supernatants diluted in coating buffer (0.016 M anhydrous sodium carbonate and 0.034 M sodium hydrogen carbonate) at pH 9.6 and incubated overnight at 4°C. Plates were washed with washing buffer (0.05% Tween in PBS; PBST) then blocked with blocking buffer (3% BSA in PBST) for 1 h at RT. After washing, plates were incubated for 1 h at RT with the supernatant of EPC cells, which were previously transfected with pTnfα1, pTnfα2 or pEmpty, diluted in 1% BSA in PBST. After extensive washing, bound Tnfα was quantified using rabbit anti-Tnfα antibodies (1:500 dilution) and goat anti-rabbit IgG-HRP (Dako, 1:1000 dilution) (*34*).

Tnfα bioluminescent reporter assay

EPC cells stably transfected with the pNiFty2-Luc plasmid (InvivoGen) (*41*), hereafter referred to as EPC-NFκB-Luc cells, were used to measure Tnfα bioactivity. The supernatants of EPC cells, which were previously transfected with pTnfα1 or pTnfα2 or pEmpty (*34*), were pre-incubated with concentrated supernatants of CyHV-3 infected or mock-infected CCB cells (corresponding to 40 µg of total protein) for 30 min at RT to allow for ORF12-Tnfα binding. EPC-NFκB-Luc cells were seeded into 96-well plates, incubated overnight at 27°C and subsequently stimulated with 50 µl of the pre-incubated mixtures described above. After incubation for 6 h at 27°C, cells were lyzed using Bright glow (Promega), and bioluminescence was measured using a Filter Max F5 Multi-Mode Microplate Reader (Molecular Devices). The fold change of luminescence was calculated by dividing the light units obtained for each sample by the result obtained for the respective control sample.

Ethics statement

The experiments, maintenance and care of fish complied with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (CETS n° 123). The animal studies were approved by the local ethics committee of the University of Liège, Belgium (Laboratory accreditation No. 1610008, protocol No. 1327). With respect to the 3Rs rule, all efforts were made to minimize suffering (definition of ending points) and to reduce the number of animals used. For example, the *in vitro* and *in vivo* experiments (Fig. 4 and 5) systematically led to comparable results for the CyHV-3 WT and 12Rev strains. Thus, only the latter strain was used as a control for subsequent *in vivo* experiments (Fig. 6).

Table S2. Temperature recorded in the compartments of MCTs

Figure	Tank	Virus/antibody/plasmid	24°C compartment	28°C compartment	32°C compartment
Fig. 1B	MCT	WT then mock-infected	22.6 ± 0.78°C	27.7 ± 0.49°C	33.9 ± 0.55°C
	MCT	Mock then WT infected	23.4 ± 0.57°C	27.9 ± 0.41°C	33.9 ± 0.42°C
Fig. 1C	MCT	WT	22.6 ± 0.78°C	27.7 ± 0.49°C	33.9 ± 0.55°C
	MCT blocked	WT	24.6 ± 0.40°C	28.1 ± 0.14°C	32.2 ± 0.25°C
Fig. 1D	MCT	WT	24.6 ± 0.32°C	28.1 ± 0.21°C	31.7 ± 0.50°C
Fig. 3A	MCT	Δ12	23.8 ± 0.35°C	28.2 ± 0.18°C	32.5 ± 0.20°C
	MCT	12Rev	23.6 ± 0.16°C	27.6 ± 0.26°C	32.3 ± 0.24°C
Fig. 3B	MCT	Δ12	24.8 ± 0.21°C	28.1 ± 0.17°C	32.2 ± 0.23°C
	MCT	12Rev	24.5 ± 0.20°C	27.9 ± 0.17°C	32.3 ± 0.38°C
Fig. 4A	MCT	WT/ anti-Tnfα	23.8 ± 0.29°C	28.5 ± 0.24°C	33.0 ± 0.27°C
	MCT	WT/ CT	23.5 ± 0.22°C	28.2 ± 0.31°C	32.6 ± 0.10°C
Fig. 4B	MCT	pEmpty (2 µg)	24.1 ± 0.24°C	28.1 ± 0.11°C	31.7 ± 0.07°C
	MCT	pEmpty (10 µg)	24.2 ± 0.50°C	27.8 ± 0.30°C	31.6 ± 0.46°C
	MCT	pTnfα1 (2 µg)	24.4 ± 0.39°C	27.9 ± 0.29°C	31.4 ± 0.42°C
	MCT	pTnfα1 (10 µg)	24.2 ± 0.38°C	28.1 ± 0.37°C	31.7 ± 0.55°C
	MCT	pTnfα2 (2 µg)	24.7 ± 0.24°C	28.1 ± 0.23°C	31.6 ± 0.24°C
	MCT	pTnfα2 (10 µg)	23.9 ± 0.11°C	27.7 ± 0.10°C	31.6 ± 0.09°C
Fig. 4C	MCT	pTnfα1 (10 µg)/ anti-Tnfα	23.9 ± 0.25°C	28.0 ± 0.28°C	31.9 ± 0.18°C
	MCT	pTnfα1 (10 µg)/ CT	24.0 ± 0.93°C	27.6 ± 0.46°C	32.1 ± 0.61°C

Fish

European common carp (*Cyprinus carpio carpio*) with an average weight of 10 g were kept in 180 L tanks at 24°C. Water parameters were checked twice per week. Microbiological, parasitic, and clinical examinations of the fish just before the experiments demonstrated that they were healthy. All experiments were preceded by an acclimation period of at least 3 weeks.

Tank systems

A single chamber tank (SCT) system and a multi-chamber tank (MCT) system were used in this study (Fig. 2A). The SCT system consisted of an 80 L single compartment tank (width x depth x height: 0.5 x 0.4 x 0.4 m) with a mean constant temperature of approximately 24, 28 or 32°C. The MCT system consisted of a 144 L tank (width x depth x height: 1.2 x 0.3 x 0.4 m) subdivided into three equal compartments by two thermal insulation rigid panels (6 cm thick and made of polyurethane foam). Neighboring compartments were connected by a 0.4 m long transparent tunnel with a square cross-section (8 x 8 cm) placed at the bottom of the tank. Each of the three chambers had independent aeration and circulation/filtering systems. A thermal gradient (24°C - 28°C - 32°C) was established between the three chambers by cooling the first compartment and by increasing heating of the second and third compartments. Temperatures in all three compartments were controlled by measurements every 30 min. The observed temperatures of the MCTs used for the experiments of this manuscript are presented as the mean ± SD in table S2. To simplify reading of the manuscript, the theoretical gradient of 24°C - 28°C - 32°C is presented in the manuscript using the color code described in the legend of Fig. 2. The MCTs were placed in the experimental room by pairs using a central axis of symmetry, with the 32°C compartments being close to this axis and the 24°C compartments being furthest from this axis. When repeating the experiments, the positions of the experimental groups were systematically swapped between tanks. In the MCTs, daily feeding was performed in the 24°C compartment independently of the position of the fish. We observed that fish in the other compartments migrated rapidly into the 24°C compartment soon after distribution of the food and then returned to their initial location after feeding.

Inoculation and injection of fish

For inoculation with CyHV-3, fish were collected from the tank and immersed for 2 h in water (volume adapted based on fish weight and fish number to use a biomass of 10%) containing the virus under constant aeration. All inoculations were performed at the dose of 100 pfu/ml, except for the experiment described in Fig. 4D, for which doses of 40 and 400 pfu/ml were used. At the end of the incubation period, the fish were returned to their tank. For antibody and plasmid injections, fish were collected from the tank and anaesthetised by immersion in water containing benzocaïne (25 mg/L). Antibodies were injected intraperitoneally at a dose of 1 µg/g of fish body weight. Plasmids (2 or 10 µg/g of fish body weight diluted in PBS) were injected using myojector U-100 insulin 29G syringes (Terumo) in the centre of the epaxial muscle mass at the level of the anterior limit of the dorsal fin. The dose of plasmid to be administered was delivered by performing one injection of 50 µl in each flank of the fish. For the experiments in the MCT, fish were returned to the 24°C compartment after inoculation or injection.

Monitoring of fish position in the MCTs

Fish were initially introduced into the 24°C chamber of the MCT, in which the tunnels were obstructed by grids to prevent fish migration out of the 24°C compartment. After an acclimation period of 3 weeks, the grids were removed, and the distribution of fish into the three compartments was monitored over time. A digital camera (Logitech HD Webcam c310) placed in front of each MCT recorded pictures every 3 min. The number of fish in each chamber was counted manually from the pictures recorded every 30 min, resulting in 48 measurements per day. When the positions of the fish were not compatible with an accurate count, the measures were made from the previous or the next picture (collected 3 min before or after the time point). The results are presented as the mean + SD (n=48) of the number of fish observed per day in each compartment.

Sampling of fish tissues

Fish were euthanized by immersion in water containing benzocaine (100 mg/L). Tissue samples were collected, placed in RNAlater (Invitrogen) and stored at -80°C until further analyses.

Quantification of virus genome copies by qPCR

The virus genome was quantified by real-time TaqMan qPCR in gills by amplifying fragments of the CyHV-3 ORF89 and carp *glucokinase* genes as described previously (*21*, *26*). The primers and probes are listed in table S1.

Quantification of carp gene expression by RT-qPCR

Total RNA was isolated from spleens using TRI reagent (Ambion, Invitrogen), including DNase I digestion and RNA purification using the RNeasy MinElute Cleanup Kit (Qiagen). cDNA was synthesized from 1 µg of RNA using the iScript cDNA Synthesis Kit (Bio-Rad), and the expression of analyzed genes was measured by RT-qPCR by amplifying fragments of carp *il1β*, *tnfα1/tnfα2*, *il6a* and *il6b* as described previously (*21, 42*). The primers used are listed in table S1. Gene expression was normalized to the *40S* ribosomal protein *s11*. Normalized data were expressed relative to non-infected fish at time point 0.

Statistical analyses

Most statistical analyses were performed using linear models proposed in SAS (v 9.3). Migration of fish to the 32°C compartment of the MCT following CyHV-3 infection (Fig. 2B) was analyzed using two-way ANOVA. The number of fish present in this compartment was used as the dependent variable and was modeled using a linear model involving time, tank, and their interactions. Post-hoc comparisons using least square means were used to compare the number of fish in the 32°C compartment of the MCTs every day. The same type of analysis was used to compare the migration of fish infected with the ORF12 recombinant strains (Fig. 6A) or injected with plasmids (Fig. 3A), antibodies (Fig. 3C), or both (Fig. 3B). For Fig. 3A, a third factor (the dose effect) was added into the model, and the corresponding two- and three-way interactions were also included. Differences in virus load (data after log transformation) and cytokine expression (data after log transformation) (Fig. 2D) between CyHV-3 infected fish collected at different post-infection times and mock-infected fish sampled at day 0 were analyzed using a one-way ANOVA. Post-hoc comparisons between days were performed using Tukey's test. These analyses were performed independently for each cytokine. The Tnfα binding assay (Fig. 5B), Tnfα neutralization assay (Fig. 5C), virus load (Fig. 6B), IVIS experiment (Fig. S1B) and multi-step growth curve (Fig. 4C) were analyzed using two- or three-way ANOVA. More precisely, for the binding assay of Tnfα, the level of binding was modeled using a linear model involving cell supernatants, Tnfα1 or Tnfα2 volumes as well as their interactions. For the neutralization assay of Tnfα biological activity, the fold change of luminescence (ratio of the Tnfα1 or Tnfα2 levels respective to the control supernatant level) was modeled using a linear model involving cell supernatants, cytokine (Tnfα1, Tnfα2 or control) supernatants, and their interactions. For virus load, the viral genome copies (data after log transformation) were modeled using a linear model involving virus strains, tanks, and their interactions. For the IVIS experiment, the logarithm of the average light radiance was modeled using a linear model involving the post-infection times, incubation temperatures, and their interactions. For multi-step growth curve, the logarithm of the titre was modeled using a linear model involving the day, viral strains, and their interactions. For the survival analyses (Fig. 2C and 4D), a purpose-made permutation procedure was performed in MS Excel. Survival curves were compared pairwise. First, the surface between two observed curves was

measured (observed surface), and the numbers of dead fish observed per day for the two groups to be compared were allocated randomly between them using a uniform distribution. This random allocation procedure generated pairs of simulated curves, and the surface between these curves was calculated. After repeating this procedure 1,000 times, the distribution of surfaces under the null hypothesis of no survival difference was approximated using the shuffled surface dataset. For each comparison, the p-value was estimated as the proportion of situations in which the surface between the two simulated curves was larger than the one actually observed. Statistical significance is represented as follows: -, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; and ****, $p<0.0001$. P-values less than 0.05 were considered significant.

References and Notes

1. J. Roth, C. M. Blatteis, Mechanisms of fever production and lysis: lessons from experimental LPS fever. *Compr. Physiol.* **4**, 1563-1604 (2014).
2. M. G. Netea, B. J. Kullberg, J. W. M. Van der Meer, Circulating cytokines as mediators of fever. *Clin. Infect. Dis.* **31**, 178-184 (2000).
3. C. A. Dinarello, Cytokines as Endogenous Pyrogens. *J. Infect. Dis.* **179**, 294-304 (1999).
4. S. S. Evans, E. A. Repasky, D. T. Fisher, Fever and the thermal regulation of immunity: the immune system feels the heat. *Nature Rev. Immunol.*, (2015).
5. C. B. Saper, A. A. Romanovsky, T. E. Scammell, Neural circuitry engaged by prostaglandins during the sickness syndrome. *Nat. Neurosci.* **15**, 1088-1095 (2012).
6. J. Campbell, B. Kessler, C. Mayack, D. Naug, Behavioural fever in infected honeybees: parasitic manipulation or coincidental benefit? *Parasitology* **137**, 1487-1491 (2010).
7. V. L. Hunt, A. K. Charnley, The inhibitory effect of the fungal toxin, destruxin A, on behavioural fever in the desert locust. *J. Insect Physiol.* **57**, 1341-1346 (2011).
8. M. J. Kluger, Fever in the frog *Hyla Cinerea*. *J. Therm. Biol.* **2**, 79-81 (1977).
9. L. K. Vaughn, H. A. Bernheim, M. J. Kluger, Fever in the lizard *Dipsosaurus dorsalis*. *Nature* **252**, 473-474 (1974).
10. W. W. Reynolds, M. E. Casterlin, J. B. Covert, Behavioural fever in teleost fishes. *Nature* **259**, 41-42 (1976).
11. M. Cabanac, F. Laberge, Fever in goldfish is induced by pyrogens but not by handling. *Physiol. Behav.* **63**, 377-379 (1998).
12. A. Grans, M. Rosengren, L. Niklasson, M. Axelsson, Behavioural fever boosts the inflammatory response in rainbow trout *Oncorhynchus mykiss*. *J. Fish Biol.* **81**, 1111-1117 (2012).
13. M. Cabanac, L. Le Guelte, Temperature regulation and prostaglandin E1 fever in scorpions. *J. Physiol.* **303**, 365-370 (1980).
14. V. H. Hutchison, D. J. Erskine, Thermal selection and prostaglandin E1 fever in the salamander *Necturus maculosus*. *Herpetologica* **37**, 195-198 (1981).
15. S. Boltaña, S. Rey, N. Roher, R. Vargas, M. Huerta *et al.*, Behavioural fever is a synergic signal amplifying the innate immune response. *Proc. R. Soc. B: Biol. Sci.* **280**, (2013).
16. S. L. Elliot, S. Blanford, M. B. Thomas, Host-pathogen interactions in a varying environment: temperature, behavioural fever and fitness. *Proc. R. Soc. B: Biol. Sci.* **269**, 1599-1607 (2002).
17. J. B. Covert, W. W. Reynolds, Survival value of fever in fish. *Nature* **267**, 43-45 (1977).
18. O. Gilad, S. Yun, M. A. Adkison, K. Way, N. H. Willits *et al.*, Molecular comparison of isolates of an emerging fish pathogen, koi herpesvirus, and the effect of water temperature on mortality of experimentally infected koi. *Journal of General Virology* **84**, 2661-2667 (2003).
19. M. L. Epperson, C. A. Lee, D. H. Fremont, Subversion of cytokine networks by virally encoded decoy receptors. *Immunological Reviews* **250**, 199-215 (2012).
20. J. R. Sedy, P. G. Spear, C. F. Ware, Cross-regulation between herpesviruses and the TNF superfamily members. *Nature review* **8**, 361-373 (2008).
21. P. Ouyang, K. Rakus, M. Boutier, A. Reschner, B. Leroy *et al.*, The IL-10 homologue encoded by Cyprinid herpesvirus 3 is essential neither for viral replication *in vitro* nor for virulence *in vivo*. *Vet. Res.* **44**, 53 (2013).
22. C. A. Dinarello, J. G. Cannon, S. M. Wolff, H. A. Bernheim, B. Beutler *et al.*, Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. *J. Exp. Med.* **163**, 1433-1450 (1986).
23. M. Mabika, H. Laburn, The role of tumour necrosis factor-alpha (TNF-alpha) in fever and the acute phase reaction in rabbits. *Pflugers Arch.* **438**, 218-223 (1999).
24. H. Nakamura, Y. Seto, S. Motoyoshi, T. Kadokawa, N. Sunahara, Recombinant human tumor necrosis factor causes long-lasting and prostaglandin-mediated fever, with little tolerance, in rabbits. *J. Pharmacol. Exp. Ther.* **245**, 336-341 (1988).
25. I. C. Kettelhut, A. L. Goldberg, Tumor necrosis factor can induce fever in rats without activating protein breakdown in muscle or lipolysis in adipose tissue. *J. Clin. Invest.* **81**, 1384-1389 (1988).

26. M. Boutier, M. Ronsmans, P. Ouyang, G. Fournier, A. Reschner *et al.*, Rational development of an attenuated recombinant Cyprinid herpesvirus 3 vaccine using prokaryotic mutagenesis and *In Vivo* bioluminescent imaging. *PLoS Pathog.* **11**, e1004690 (2015).
27. A. Engering, L. Hogerwerf, J. Slingenbergh, Pathogen-host-environment interplay and disease emergence. *Emerg. Microbes Infect.* **2**, e5 (2013).
28. L. Imeri, M. R. Opp, How (and why) the immune system makes us sleep. *Nat. Rev. Neurosci.* **10**, 199-210 (2009).
29. M. R. Irwin, S. W. Cole, Reciprocal regulation of the neural and innate immune systems. *Nature Rev. Immunol.* **11**, 625-632 (2011).
30. S. G. Kamita, K. Nagasaka, J. W. Chua, T. Shimada, K. Mita *et al.*, A baculovirus-encoded protein tyrosine phosphatase gene induces enhanced locomotory activity in a lepidopteran host. *Proc. Natl. Acad. Sci. USA* **102**, 2584-2589 (2005).
31. K. Hoover, M. Grove, M. Gardner, D. P. Hughes, J. McNeil *et al.*, A Gene for an Extended Phenotype. *Science* **333**, 1401 (2011).
32. A. N. Reed, S. Izume, B. P. Dolan, S. LaPatra, M. Kent *et al.*, Identification of B cells as a major site for cyprinid herpesvirus 3 latency. *J Virol* **88**, 9297-9309 (2014).
33. R. Dawkins, *The Extended Phenotype*. (Oxford Univ. Press, Oxford, 1982).
34. M. Forlenza, S. Magez, J. P. Scharsack, A. Westphal, H. F. J. Savelkoul *et al.*, Receptor-mediated and lectin-like activities of carp (*Cyprinus carpio*) TNF-α. *J. Immunol.* **183**, 5319-5332 (2009).
35. B. Costes, G. Fournier, B. Michel, C. Delforge, V. S. Raj *et al.*, Cloning of the koi herpesvirus genome as an infectious bacterial artificial chromosome demonstrates that disruption of the thymidine kinase locus induces partial attenuation in *Cyprinus carpio koi*. *J. Virol.* **82**, 4955-4964 (2008).
36. B. Costes, V. S. Raj, B. Michel, G. Fournier, M. Thirion *et al.*, The major portal of entry of Koi herpesvirus in *Cyprinus carpio* is the skin. *J. Virol.* **83**, 2819-2830 (2009).
37. L. A. Kelley, M. J. E. Sternberg, Protein structure prediction on the web: a case study using the Phyre server. *Nat. Protocols* **4**, 363-371 (2009).
38. V. Raj, G. Fournier, K. Rakus, M. Ronsmans, P. Ouyang *et al.*, Skin mucus of *Cyprinus carpio* inhibits Cyprinid herpesvirus 3 binding to epidermal cells. *Vet. Res.* **42**, 92 (2011).
39. G. Fournier, M. Boutier, V. Stalin Raj, J. Mast, E. Parmentier *et al.*, Feeding *Cyprinus carpio* with infectious materials mediates Cyprinid herpesvirus 3 entry through infection of pharyngeal periodontal mucosa. *Vet. Res.* **43**, 6 (2012).
40. M. Ronsmans, M. Boutier, K. Rakus, F. Farnir, D. Desmecht *et al.*, Sensitivity and permissivity of *Cyprinus carpio* to Cyprinid herpesvirus 3 during the early stages of its development: importance of the epidermal mucus as an innate immune barrier. *Vet. Res.* **45**, 100 (2014).
41. M. C. Piazzon, H. S. Savelkoul, D. Pietretti, G. F. Wiegertjes, M. Forlenza, Carp Il10 has anti-inflammatory activities on phagocytes, promotes proliferation of memory T cells, and regulates B cell differentiation and antibody secretion. *J. Immunol.* **194**, 187-199 (2015).
42. K. Rakus, I. Irnazarow, M. Adamek, L. Palmeira, Y. Kawana *et al.*, Gene expression analysis of common carp (*Cyprinus carpio* L.) lines during Cyprinid herpesvirus 3 infection yields insights into differential immune responses. *Dev. Comp. Immunol.* **37**, 65 - 76 (2012).

Acknowledgments

We thank A. Alcami, P. Boudinot, F. Bureau, A. Chariot, M. Georges and V. Mulero for discussions. The authors are grateful to the following people: C. Becco for his help in the setting of the automated picture recording system; C. Delforge, E. Deglaire, J. Javaux, and L. Dams for excellent technical assistance; and L. Leinartz for video editing. This work was supported by the University of Liège (Post-doc IN program), the Belgian Science Policy (Belspo) (BELVIR IAP7/45), the "Fonds National Belge de la Recherche Scientifique" (FNRS) (T.0153.13 and 1.5.176.12), an Investigator FCT development grant (IF/00641/2013), the European Community´s Seventh Framework Programme (FP7/2007-2013) under Grant agreement PIEF-GA-2011-302444 FISHIL10 (MCP) and the Netherlands Organisation for Scientific Research (NWO) under Veni project number 11200 (MF). M.R. is a research fellow of the FNRS.

Author information

The authors declare no competing interests. Correspondence and requests for materials should be addressed to A.vdplasschen@ulg.ac.be.

Author contributions

K.R., M.R. and A.V. conceived the study, designed most of the experiments and wrote the manuscript. A.V. made the preliminary observation at the origin of this project and obtained funding for its completion. K.R. built the MCT and demonstrated the expression of behavioral fever (Fig. 2A and B). K.R. and M.R. contributed to all experiments. M.R. designed, constructed and characterized the ORF12 recombinant strains of CyHV-3. M.B. contributed to the experiments presented in Fig. S1 and Fig. 4. J.J.-R. contributed to the experiments presented in Fig. 1D. M.F., M.C.P. and G.F.W. designed and performed the experiments presented in Fig. 5, and they also provided the plasmids and the antibodies used in Fig. 3. A.A. performed the *in silico* analyses that led us to postulate that ORF12 is a soluble Tnfα-receptor. F.F. performed statistical analyses. TM designed the experiment presented in Fig. 6B.

Supplementary Materials

Materials and Methods

References (*34-42*)

Figures S1-S3

Tables S1 and S2

Videos 1-4

Supplementary Video legends

Supplementary Video 1 – Video related to Fig. 2B. Common carp express behavioral fever in response to CyHV-3 infection. This video starts with an animated cartoon illustrating the structure of the MCT used in this study and how the MCTs were positioned in the room along a central axis of symmetry (hottest compartments being the closest to the center of the room). The video then presents movies of fish that were mock-infected (left and first presented MCT) or infected (right and second presented MCT) 7 days earlier with wild-type CyHV-3.

Supplementary Video 2 – Video related to Fig. 2C. Effect of water temperature on the development of CyHV-3 disease. This video starts with an animated cartoon illustrating the structure of a MCT in which the tunnels were blocked by grids. The video then presents a movie of fish that were infected 9 days earlier with wild-type CyHV-3 before their distribution into the compartments of the MCT.

Supplementary Video 3 – Video related to Fig. 3A. Tnfα1 mediates the expression of behavioral fever. This video starts with an animated cartoon explaining the flow chart of this experiment. Fish were injected intramuscularly with a control empty plasmid (pEmpty, left and first presented MCT) or with a derived plasmid (pTnfα1, right and second presented MCT) inducing the expression of carp Tnfα1 in fish. Movies of the fish were recorded 6 days post-plasmid injection.

Supplementary Video 4 – Video related to Fig. 3C. Anti-Tnfα antibodies inhibit the expression of behavioral fever induced by CyHV-3 infection. This video starts with an animated cartoon explaining the flow chart of this experiment. Fish were first infected with CyHV-3 and then injected three days later with anti-Tnfα antibodies (left and first presented MCT) or irrelevant control antibodies (right and second presented MCT). Movies of the fish were recorded at 6 dpi.

Discussion and perspectives

The Order *Herpesvirales* classifies an important group of viruses into three families: the *Herpesviridae* family comprising viruses infecting mammals, birds and reptiles; the *Malacoherpesviridae* family comprising viruses infecting molluscs; and finally, the *Alloherpesviridae* family encompassing viruses from fish and amphibians. The separation of the different families is thought to have occurred around 500 million years ago (McGeoch *et al.*, 2006; van Beurden & Engelsma, 2012). Even if alloherpesviruses share common features (Ackermann, 2004; Pellett *et al.*, 2011) with the more studied *Herpesviridae* family members, such as their virion structure, their replication cycle (Mettenleiter, 2004) and their ability to establish lifelong persistent/latent infection, they also exhibit peculiarities such as the poikilothermic physiology of their host, their wider host range and their tendency to cause severe acute infection that can be lethal. All together these features of the alloherpesviruses make them an original subject of both fundamental and applied research. Cyprinid herpesvirus 3 (CyHV-3), a member of the *Alloherpesviridae* family, is a highly contagious and virulent pathogen which has been inducing severe economic losses in common and koi carp industries worldwide since its emergence in the late 1990s. Due to its economic importance, CyHV-3 became rapidly a subject of applied research after its isolation. However, besides its importance for applied research, CyHV-3 possesses several characteristics which make this virus an interesting object of fundamental studies. First, the accumulation of research and knowledge on CyHV-3 far exceeds that of any other member of the *Alloherpesviridae* family making this virus an archetype of this family. Moreover, CyHV-3 natural host, the common carp *Cyprinus carpio*, is a traditional species for research on fish immunology. Thereby, the CyHV-3/carp homologous model represents an interesting and original field of fundamental research for host-virus interactions (Adamek *et al.*, 2014; Rakus *et al.*, 2013). Second, the CyHV-3/carp model is one of the very few laboratory animal models that allow the study of the complete infectious cycle of a herpesvirus, including transmission from infected to naive subjects (Boutier *et al.*, 2015). Finally, CyHV-3 possesses the largest genome (295 kb, 156 ORFs) described amongst *Herpesvirales* with several ORFs predicted to encode for proteins involved in immune evasion mechanisms (Aoki *et al.*, 2007; Davison *et al.*, 2013), e.g. 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 Il10 homolog, ORF139 encoding a poxvirus B22R protein homolog (Aoki *et al.*, 2007). Altogether, these facts explain the interest of the scientific community for the CyHV-3/carp model that allows the study of all aspects of host-pathogen interactions. In the present manuscript, we used this model to study the roles of two unrelated innate immune mechanisms of carp in anti-CyHV-3 immunity.

Teleosts develop both innate and adaptive immune responses. The innate immune system is the initial line of defense and is of primary importance in combating fish infections (Magnadóttir, 2006). It recognizes common structures of microorganisms through germline-encoded pattern recognition receptors (PRR). Facing an infection, recognition of pathogen-associated molecular patterns (PAMPs) by PRR expressed on cell membranes (extra or intracellularly) or in cytoplasm of

various cell types, i.e. phagocytes, leucocytes, dendritic cells or non-immune cell types, can trigger different reactions aiming at pathogen elimination. Beside phagocytosis, cells of the innate immune system are also potent producers of cytokines and chemokines notably involved in the regulation of adaptive immune response. Different PRR are involved in viral sensing; (i) Several Toll-like receptors (TLRs) localized on cell surface (i.e. TLR4 or TLR22) or on cytoplasmic vesicles (i.e. TLR3, TLR7 or TLR9) are able to recognize viral envelope components or viral nucleic acids (Pietretti & Wiegertjes, 2014; Workenhe *et al.*, 2010). These receptors present a cytoplasmic Toll/interleukin-1 receptor homology (TIR) domain which, upon ligand activation, interacts with adaptor proteins (MyD88, TRIF, MAL, TRAF). These signaling pathways trigger the activation of the transcription nuclear factor-κB (NF- κB) or the activator protein-1 (AP-1), both leading to the transcription of proinflammatory cytokines. On the other hand, this signaling could activate production of type I interferon (Ifn) through activation of Ifn regulatory factors (IRF) 3/7 (Pietretti & Wiegertjes, 2014). A total of 11 TLRs have been identified in teleost fish (Workenhe *et al.*, 2010) and even if fish exhibit a diverse repertoire, the TLR signaling pathways and functions seem well-conserved with their mammalian orthologs (Langevin *et al.*, 2013). (ii) Three retinoic acid inducible protein-I (RIG-I) like helicases (RLHs) are described in humans, i.e. retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). While RIG-I has been retrieved only in some groups of fish including salmonids and cyprinids, LGP2 and MDA5 seem to be conserved in all fish species (Langevin *et al.*, 2013). These receptors are cytoplasmic sensors of dsRNA and upon ligand binding, their caspase recruitment domain (CARD) interacts with the mitochondrial antiviral signaling protein (MAVS, also known as CARDIF, VISA, or IPS-1) which induces downstream signaling pathway and activation of NF-κB and IRF3/7 (Langevin *et al.*, 2013; Workenhe *et al.*, 2010). The adaptor STING is a transmembrane protein located in the endoplasmic reticulum which senses cytoplasmic viral DNA and connects the signaling pathway downstream of MAVS leading to IRF3 activation. STING has been identified in fish and plays an important role in the RLR/IRF3-dependent signaling (Langevin *et al.*, 2013). Altogether, detection of viral infection by PRRs could induce expression of proinflammatory cytokines such as Il1β, Il6 and Tnfα, as well as type I Ifn which could transactivate a vast repertoire of Ifn-stimulated genes (ISGs) (Langevin *et al.*, 2013; Pietretti & Wiegertjes, 2014; Workenhe *et al.*, 2010). Finally, these cytokines are involved both in the innate immune defenses against viral infection but also, in the regulation of the subsequent adaptive immune response. While innate immune response enables early non-specific pathogen detection, adaptive immune response develops later and relies on specific recognition of antigen motifs by the mean of two cellular receptors, B cell receptor (BCR) and T cell receptor (TCR). This specific response is under several constraints in fish due to their poikilothermic nature, the limited antibodies repertoire, i.e. teleosts develop IgM, IgD and IgT/Z antibodies, the limited affinity maturation and memory, i.e. absence of isotypic switch, and relatively slow lymphocyte proliferation (Reyes-Cerpa *et al.*, 2012; Whyte, 2007; Workenhe *et al.*, 2010).

In the first study, we investigated the sensitivity of carp to CyHV-3 infection according to their developmental stage, with a special interest on the role of epidermal mucus as an innate immune barrier against CyHV-3 entry. An age dependent susceptibility has been described for some alloherpesviruses (van Beurden & Engelsma, 2012). In most cases, younger animals are more susceptible to diseases. Nevertheless, studies on this age dependent susceptibility are frequently restricted to the clinical outcome and do not address virological read-outs. However, investigating the viral and/or host basis underlying this age dependent susceptibility is of great importance for the control of infectious diseases. Indeed, determining the host factors associated with higher (or lower) resistance to the disease would help developing prophylactic tools. Also, determining the sensitivity of developmental stages can be essential for the development of safe and efficacious attenuated vaccines.

CyHV-3 can affect carp at all ages but a higher sensitivity to the disease has been reported for younger fish (1-3 months, equivalent to 2.5-6 g) compared to mature fish (1 year, equivalent to 230 g) (Michel *et al.*, 2010; Perelberg *et al.*, 2003; Rakus *et al.*, 2013). However, the only study which addressed the susceptibility of carp at early stages of development concluded that carp larvae were not susceptible to CyHV-3 infection (Ito *et al.*, 2007). Susceptibility of a cell (and by extension of an organism) to a viral infection can be defined by two properties, the cell sensitivity (defined as the ability to support viral entry) and the cell permissivity (defined as the ability to support viral replication) to infection. By investigating the clinical outcome, Ito *et al.* (2007) suggested a low (potentially null) permissivity of carp larvae to infection. However, the sensitivity of carp larvae has never been investigated. Moreover, the PCR assay performed on surviving fish is puzzling as it was done at 21 or 23 days post-infection (dpi), far later than the clinical signs and mortalities. The probability to reveal an abortive infection without viral replication in carp larvae was therefore low.

In 2008, the host laboratory cloned the CyHV-3 genome as a bacterial artificial chromosome (BAC) which allowed production of viral recombinants using prokaryotic mutagenesis (Costes *et al.*, 2008). Notably, a luciferase (LUC) recombinant strain was produced by inserting the LUC cassette between the ORF136 and ORF137 under the control of the human cytomegalovirus (HCMV) immediate-early promoter (Costes *et al.*, 2009) thereby leading to expression of the reporter gene even in absence of viral replication. Using *in vivo* imaging system (IVIS), this recombinant allows for highly sensitive detection and localization of the viral infection as early as 12 to 24 hours post-infection (Costes *et al.*, 2009).

In the first experimental chapter of this thesis, we revisited the question of the sensitivity of carp to CyHV-3 during the early developmental stages using the CyHV-3 LUC recombinant and IVIS technology. We showed that carp are sensitive to CyHV-3 infection since hatching as one embryo already expressed a positive signal at day 0; the positive signal tends to increase between 24 and 72 hours post-infection which suggests that infected fish were supporting a replicative infection and so are permissive to the infection. Concerning the number of positive individuals, it remains relatively low during the first stages of development. A significant number of positive subjects was observed

from day 21 (juvenile stage) onwards. At the same time, we measured the mortalities after inoculation performed at the different stages of development. The results further support that carp undergo a replicative infection, as CyHV-3 infection induces mortalities at all stages of life. As a first conclusion, we demonstrated that carp are permissive to CyHV-3 since hatching, even if their permissivity tends to increase with development (100% mortalities by day 7 post-infection when fish are infected at days 21 and 35 post-hatching).

The epidermis of adult teleost fish is a living stratified squamous epithelium divided into three layers which all contain cells capable of mitotic division. The surface and basal layers are single-cell layers composed of keratinocytes and undifferentiated basal cells, respectively. The pluristratified intermediate layer is composed of unicellular glands (mucous cells and club cells), ionocytes and undifferentiated cells. The scales are dermal structures covered by the epidermis (Roberts & Ellis, 2012). The epidermis has previously been shown to be the major portal of entry of CyHV-3 when the infection is performed by immersion in infectious water (Costes *et al.*, 2009). Compared to adults, the epidermis of embryos has a simplified structure consisting of surface and basal layers (single-cell layers) interrupted by unicellular glands and ionocytes (Campinho *et al.*, 2007; Chang & Hwang, 2011). Our study reinforces the role of the skin as a major portal of entry of CyHV-3 in young carp, similarly to adults. Indeed, (i) once we performed infections by immersion in water containing the LUC recombinant strain, the IVIS positive signal was randomly distributed on the body surface (and not preferentially localized around the gills); (ii) the gills are not fully developed at hatching and gas exchanges occur through the blood vessels of the yolk sac and the caudal fin in embryos (Huttenhuis, 2005), excluding virus entry through gills at that stage; (iii) as a side project, I produced during my thesis a CyHV-3 recombinant expressing betagalactosidase (CyHV-3 LacZ strain). Infection of larvae with this recombinant demonstrated the presence of positive epidermal cells in the skin after X-gal staining and histological sections. Despite the robustness and the large number of proofs supporting the role of the skin in CyHV-3 entry, some authors claim that the gills (and the gut) could be additional portals of entry (Monaghan *et al.*, 2015). As mentioned in the preamble, the completion of this thesis gave me the opportunity to work on an applied project aiming to develop a safe and efficacious attenuated vaccine against CyHV-3 (Boutier *et al.*, 2015) (see Annex). This project provided one additional argument in favor of the entry through the skin. Indeed, the Δ56-57 vaccine strain developed presents a slower spread within infected fish compared to wild type strain which allows a better discrimination between the portal(s) of entry and the secondary sites of infection. Though the skin of all fish was positive as early as 2 days after inoculation with the vaccine LUC strain, all of the other tested organs (including gills and gut) were positive in the majority of fish only after 6 dpi, suggesting that these organs represent secondary sites of replication.

The sensitivity of the two earliest stages of development (embryo and larval stages) was limited compared to the older stages (juveniles and fingerlings). The sensitivity only increased once fish metamorphosed in juveniles, and that was correlated with a lower survival rate of later stages.

Several hypotheses could explain the lower sensitivity of the early developmental stages. Firstly, teleost fish lay telocithal eggs in which some maternal factors are transmitted passively. Both innate and adaptive immune factors are transmitted such as complement factors (e.g. C3), enzymes (e.g. serine protease, lysozyme), α macroglobuline, serum amyloid A, lectins and immunoglobulins (Ig) (Swain & Nayak, 2009; Zhang *et al.*, 2013). These factors are transferred to immature oocytes during vitellogenesis, by active transcytose across follicle cells or incorporated together with the vitellogenin (Zhang *et al.*, 2013). Maternal IgM, structurally and antigenically identical to the maternal serum IgM, usually persist for a limited duration and are spent within completion of yolk sac absorption period (Swain & Nayak, 2009). Besides the protection conferred against surrounding infectious agents, maternal immunoglobulins could play a role against vertically transferred pathogens, in opsonisation to activate the classic complement pathway, or simply act as nutritional yolk proteins (Magnadottir *et al.*, 2005). In our study, we performed duplicate experiments with unrelated breeders proved to be free of CyHV-3 making the presence of maternally transferred IgM unlikely. On the contrary, Ito *et al.* (2007) did not mention the serological status of the breeders used, this could explain some discrepancies with our study if they used seropositive genitors conferring transient humoral protection to the offspring. Secondly, the skin surface correlates with fish size, making the main portal of entry for CyHV-3 smaller in young carp compared to adults. Thirdly, herpesvirus entry requires several cellular receptors mediating viral binding and entry (Spear & Longnecker, 2003). These receptors could be absent or not functional during the first stages of life and only develop during ontogenesis. A fourth hypothesis resides in the behavioral characteristics of young carp. In adults, several behaviors could increase the transmission of CyHV-3 between individuals, i.e. infected fish rubbing themselves against each other or against objects which could promote a “skin-to-skin” transmission between infected and naive carp (Boutier *et al.*, 2015; Raj *et al.*, 2011). Similarly, cannibalism expressed by healthy fish against sick carp could promote the spreading of the infection in the carp population (Fournier *et al.*, 2012). These behaviors, even if expressed during the earlier developmental stages, remain quite limited until the juvenile stage (Vilizzi & Walker, 1999). Last but not least, our results suggest that the early developmental stages could express innate immune mechanisms at a higher or more efficient level than older stages. At the time of hatching, fish rely entirely on cellular and humoral components of innate immunity as specific adaptive response mature later in life. For example, lymphoid organs (thymus, spleen and head kidney) start developing after hatching and antibody responses are only functional later in life, at 1-2 months, while the presence of antigenic tolerance is reported before (Huttenhuis *et al.*, 2006; Huttenhuis, 2005; Li & Leatherland, 2012). Among these innate defenses, physical barriers like the skin and notably its mucus layer are on the front line (Vadstein *et al.*, 2013).

Teleost skin behaves like a mammalian type I mucosal surface; it harbors abundant mucus-producing cells, lacks keratinization and, living epithelial cells are in direct contact with the water environment (Xu *et al.*, 2013b). This mucosal surface in aquatic species is in constant interaction with

numerous antigens from pathogenic or commensal microorganisms, called the microbiota (Gomez *et al.*, 2013). While commensal colonization brings physiological, metabolic and immunological benefits to the host, both pathogenic and commensal microorganisms share microbe-associated molecular patterns (MAMPs) recognized by PRR; this stresses the role of regulatory mechanisms (Gomez *et al.*, 2013). Among the three mucosa-associated lymphoid tissues (MALTs), teleost skin presents the skin-associated lymphoid tissue (SALT) which encompasses both innate and adaptive components (Angeles Esteban, 2012; Gomez *et al.*, 2013; Rombout *et al.*, 2014; Salinas & Miller, 2015). A primary innate barrier of the skin is formed by the mucus layer. Mucus confers protection through two main mechanisms. First, it is mainly composed of mucins which are highly glycosylated glycoproteins, along with other proteins, lipids and ions. Mucus composition determines its viscoelasticity, adhesiveness and protective properties (Angeles Esteban, 2012). Altogether, mucus represents an effective mechanical barrier that can trap invading pathogens, and slough them off due to the constant mucus production and the antero-posterior movement along the fish. These characteristics make the mucus a first barrier that can prevent pathogens from reaching epidermal cells. Second, mucus contains numerous biologically active proteins with biostatic and biocidal activities, that can neutralize pathogens (Ellis, 2001; Fontenot & Neiffer, 2004; Li & Leatherland, 2012; Palaksha *et al.*, 2008; Shephard, 1994; Subramanian *et al.*, 2008). Notably, skin mucus contains: (i) Enzymes (Angeles Esteban, 2012) such as lysozyme which is a bactericidal enzyme, acid and alkaline phosphatases which are important lysosomal enzymes, cathepsins, esterases or proteases. These latter are categorized according to their catalytic mechanisms into serine (e.g. trypsin), cysteine (e.g. cathepsin B and L), aspartic (e.g. cathepsin D), and metalloproteases. These proteases could act directly on pathogens or prevent their invasion indirectly by modifying mucus properties (Angeles Esteban, 2012). (ii) Complement components (Gomez *et al.*, 2013), i.e. C7, Factors P and D were demonstrated at the transcript level in the skin of carp (Gonzalez *et al.*, 2007b), and the expression of complement genes has been revealed upregulated in response to mucosal pathogens (Gonzalez *et al.*, 2007a). The complement system, present in both vertebrates and invertebrates, is responsible in mammals for the modulation of the adaptive immune response, the promotion of inflammatory reactions, the elimination of apoptotic and necrotic cells, and pathogens destruction. (iii) Lectins are also present in fish mucus. They can lead to opsonization or activation of the complement pathway (Angeles Esteban, 2012). (iv) Teleost skin mucus is also a major source of antimicrobial peptides (AMP) with antibacterial, antiviral and antifungal activities, and these AMP could have important applied implications (Angeles Esteban, 2012; Gomez *et al.*, 2013). For example Marel et al. (2012) demonstrated that adding β-glucan to the food of common carp increased the expression of mucin (*Muc5B*) and two β-defensin genes (*BD1 and BD2*) in the skin (Marel *et al.*, 2012). (v) Finally, mucosal secretions also contain components of humoral adaptive immunity, i.e. immunoglobulins (Igs) (Angeles Esteban, 2012; Gomez *et al.*, 2013; Rombout *et al.*, 2014). Indeed, diffuse IgM^+ and IgZ/T^+ B cells and plasma cells are scattered in the teleost SALT, without apparent organization such

as mammalian MALT lymph nodes or Peyer patches. These leucocytes secrete Igs which are transported to the mucus layer via the polymeric Ig receptor (pIgR) (Salinas & Miller, 2015). Moreover, rainbow trout IgM^+ and IgT^+ B cells were shown to have phagocytic and bactericidal capacities (Li *et al.*, 2006; Zhang *et al.*, 2010). Comparably to IgA in mammals, IgZ/IgT represents the teleost isotype specialized in mucosal immunity, mainly present in mucus secretion as a polymer (compared to the monomer found in plasma). Whereas the highest concentration of mucus Ig is IgM, the ratio IgM/IgT in mucosal secretion is significantly lower than that found in plasma. Moreover, even if both Igs are involved in responses against several pathogens, IgT is the main Ig coating bacteria from skin microbiota and following infections, a clear compartmentalization of IgT and IgM specific responses in skin mucosa and systemic areas, respectively, is described (Salinas & Miller, 2015; Xu *et al.*, 2013a). In carp, two IgZ isotypes are described with IgZ2 preferentially in mucosal tissues and IgZ1 preferentially associated with systemic organs (Rombout *et al.*, 2014). Underneath the mucus, several cellular components classified according to their innate or adaptive belonging are described. Among adaptive immune cells, IgM^+ and IgT^+ B cells have been already described above. Noteworthily, IgM^+ B cells have been recently shown as the major site of latency for CyHV-3 in carp (Eide *et al.*, 2011; Reed *et al.*, 2014). The localization of latently infected B cells in the SALT would allow the virus to persist and reactivate near a potential excretion site. Beside the B cells, teleost skin also present distinct subpopulations of T-cells like cytotoxic T cells, helper T cells or regulatory T cells but their role in mucosal immune responses and the cooperation between B and T cells has not been studied extensively yet (Gomez *et al.*, 2013; Rombout *et al.*, 2014). Cellular innate components in the epithelial barrier involve (i) the epithelial cells which express PRRs like TLR or nod-like receptors (NLR) and directly interact with pathogens or commensals. Moreover, their rapid turnover is an effective way of clearing pathogens (Gomez *et al.*, 2013). (ii) Innate immune cells encompass macrophages, granulocytes and, mast cells (sometimes referred to eosinophilic granule cells (EGCs)) which are tissue-resident cells particularly in proximity to surfaces that interface the external environment and were shown to contain AMP (Gomez *et al.*, 2013). Finally, some studies reported the characterization of dendritic-like cells in zebrafish and rainbow trout which could play similar role as mammalian Langerhans cells (Bassity & Clark, 2012; Lugo-Villarino *et al.*, 2010).

In 2011, Raj *et al.* demonstrated that the skin mucus can inhibit the binding of CyHV-3 on epidermal cells *in vivo* and can neutralize CyHV-3 *in vitro*. Indeed, by applying several physical treatments to remove the mucus layer, CyHV-3 entry was significantly increased (Raj *et al.*, 2011). In our first experimental chapter, we addressed the hypothesis that the mucus of larvae could act as an efficient innate immune barrier. To test this hypothesis, we performed two additional types of infection (i) by intraperitoneal (IP) injection of CyHV-3 and, (ii) by immersion of fish in infectious water after partial removal of the mucus layer with a swab. Independently of the age at infection, removal of the mucus increased significantly virus entry and the sensitivity of larvae reached a level comparable to older developmental stages. When fish were infected by IP injection, 100% of the fish

were positive for the infection. These results suggest that the lower sensitivity of early stages is mainly, but probably not exclusively, explained by a more effective inhibition of the virus binding by epidermal mucus. This higher efficiency of the mucus of young carp at inhibiting CyHV-3 binding to epidermal cells could be conferred by several complementary mechanisms. (i) The mucus barrier could be more uniform on the skin surface because of its lower clearance. Indeed, in adult fish, skin mucus acts as the muco-ciliary escalator of the respiratory tract of pulmonate animals, constantly moving downstream the fish body and off the trailing edges. Fish behaviors such as swimming, rubbing on objects or between each others were shown to increase its removal. As explained earlier, such behaviors are quite limited at the beginning of ontogenesis (Vilizzi & Walker, 1999). Moreover, the biochemical characteristics such as the mucus viscosity are of major importance in the clearance efficiency, but the viscoelasticity properties of young carp mucus need further investigations (Angeles Esteban, 2012). (ii) The mucus contains many active innate factors, i.e. enzymes, complement factors or lectins, that can neutralize microorganisms and possibly, these biologically substances are differentially expressed or more concentrated in larval mucus. Even if technically challenging, it will be interesting to identify the biologically active molecules expressed in epidermal mucus at the earliest stage that can be possibly studied. The evolution of proteomics is associated with a constant reduction of the quantity of protein sample required and a parallel increase of the dynamic range of concentrations that can be detected. This technical evolution should facilitate the comparative analysis of mucus proteome from adult and early developmental stage.

Understanding factors influencing the lower sensitivity at early stages of development and notably the epidermal mucus, is of great interest both for fundamental and applied research. Mucus analysis in young carp, even if collection would be technically challenging (Subramanian *et al.*, 2007), could reveal the presence of active molecules able to neutralize the CyHV-3 that are no longer expressed or at lower level in adults. Besides its fundamental interest, this knowledge could be useful for applied purpose. Indeed, in aquaculture, cost-effective mass vaccination is usually performed by bathing fish in water containing attenuated or even inactivated vaccines (Brudeseth *et al.*, 2013). Moreover, probiotics (entire or component(s) of a micro-organism which are beneficial to the health of the host), prebiotics (non-digestible food ingredient that have beneficial effects on the host by selectively stimulating the growth and/or the activity of specific health promoting bacteria) and symbiotics (co-administration of probiotic with appropriate prebiotic as substrates) are frequently administered in aquaculture in order to treat pathogen-related diseases or as preventive treatments (Cerezuela R. *et al.*, 2011). For example, combined administration of galacto-oligosaccharide (GOS) and *Pediococcus acidilactici* in rainbow trout increased significantly the bactericidal activity of trout skin mucus and their resistance to streptococcus (Hoseinifar *et al.*, 2015). Determining the mucus composition responsible for the greater resistance of carp larvae to CyHV-3 could help in developing nutritional or probiotic supply stimulating such innate components and thereby, could help in

conferring protection to vaccinated fish. Alternatively, these biologically active molecules could be produced as recombinant proteins for biotechnological applications.

The second experimental section was devoted to the study of a second innate defense mechanism, the behavioral fever. Based on their internal temperature state, animals are classified as poikilotherms, whose internal temperature are variable and adapt to their external ambient temperature, and homeotherms, which regulate their temperature and maintain a thermal homeostasis (Prosser & Nelson, 1981). Based on the mechanisms used for increasing their body temperature, animals could also be classified as ectotherms, which use mainly environmental heat and behavioral thermoregulation, and endotherms, which rely on behavioral and autonomic effector responses such as internal heat production and heat conservation mechanisms (Bicego *et al.*, 2007; Prosser & Nelson, 1981). Most fish species are ectotherms and regulate their temperature primarily through behavioral adaptations, which represent a lower metabolic cost (Bicego *et al.*, 2007). Some fish lineages are exceptions and express the ability to conserve some metabolic heat and increase the temperature of some specific tissues and organs, this capacity is called “regional endothermy” (Bicego *et al.*, 2007; Kathryn A. Dickson & Jeffrey B. Graham, 2004). Amongst teleost, regional endothermy was demonstrated in tunas and butterfly mackerel (*Scombridae* family), which warm their aerobic swimming musculature, and billfishes (*Istiophoridae* and *Xiphiidae* families), which warm the eye and brain region through cranial heater tissue specialized in thermogenesis. Even if the source of heat varies, all endothermic fish make use of a counter current heat exchanger (*retia mirabilia*) which decreases heat loss to surrounding environmental water by convection and conduction (Kathryn A. Dickson & Jeffrey B. Graham, 2004; Wegner *et al.*, 2015). Regional endothermy has probably been evolutionarily selected for thermal niche expansion and enhancement of aerobic swimming performance (Altringham & Block, 1997; Kathryn A. Dickson & Jeffrey B. Graham, 2004). Recently, Wegner *et al.* (2015) reported whole body endothermy in a mesopelagic fish, the opah (*Lampridae* family), allowing it to exploit cold and deep waters while maintaining high physiological performance (Wegner *et al.*, 2015). Our model species *Cyprinus carpio* is a strict poikilotherm whose body temperature, as in other ectotherms, reflects the ambient temperature of its environment and, based on optimal growth and propagation, its thermal preference is 20-25°C (FAO, 2015).

Following an infection, both endotherms and ectotherms are able to increase their body temperature. As they lack endogenous thermogenesis, ectotherms do so by moving to warmer places, hence the term behavioral fever. Behavioral fever has been described for decades in invertebrates like insects (Campbell *et al.*, 2010; Hunt & Charnley, 2011), amphibians (Kluger, 1977), reptiles (Vaughn *et al.*, 1974) and fish (Reynolds *et al.*, 1976). Even if behavioral fever has been mostly studied in ectothermic species, the behavioral thermoregulation in general is a widespread mechanism also present in endotherms (Bicego *et al.*, 2007). Indeed, it represents the most cost-effective response as it

requires lower energetic demands and can be sustained longer than autonomic response (Cabanac, 1998).

Behavioral adaptations of endotherms comprise both operant-conditioned behavior, e.g. turning on the heater for humans, and changes in ambient thermal preference similarly to ectotherms, e.g. basking in the sun for rewarming in mammals and birds after hibernation or daily torpors. (Cabanac & Laberge, 1998; Geiser *et al.*, 2004). In clinical situations, thermoregulatory behavior can also play a significant role in endotherms fever production, i.e. some authors reported a significant correlation between the magnitude of fever and the magnitude of warm ambient temperature selected by rats injected with LPS (Briese, 1997), and may even be crucial in some individuals whose capacity for physical thermoregulation is limited (Florez-Duquet *et al.*, 2001). For example, neonates have inadequate heat-producing and heat-conserving mechanisms so that they are not able to respond efficiently to thermal stresses. In that context, Satinoff *et al.* (1976) demonstrated that newborn rabbits injected with a pyrogen cannot develop fever if they rely solely on internal thermoregulatory mechanisms while they were able to do so behaviorally by selecting a higher environmental temperature in a gradient apparatus (Satinoff *et al.*, 1976). Similarly, old rats injected with LPS only developed fever if they were allowed to thermoregulate behaviorally in a thermal gradient (Florez-Duquet *et al.*, 2001). Our own personal experience of fever reminds us lying in bed under thick blankets, with heaters at maximum, all these behaviors helping to increase body temperature while reducing energetic costs inherent to thermogenesis. Altogether, the behavioral part of fever in endotherms should not be neglected and its similarity with behavioral fever of ectotherms suggests that their mechanisms could have been conserved throughout evolution.

In the CyHV-3/carp homologous model in which CyHV-3 infects its natural host *Cyprinus carpio* through a natural route of infection, preliminary observation of behavioral thermoregulation was made in experimental single chamber tanks (SCT), when infected carp tended to concentrate around the heater when it was running. This observation led us to consider that this behavior could represent the expression of behavioral fever. We first investigated whether CyHV-3 infection induces behavioral fever in carp. For that purpose, we built multi-chamber tank (MCT) systems with three compartments in which a temperature gradient was established. Carp were allowed to move freely between the three compartments according to their thermal preference. With this system, we showed that carp infected with CyHV-3 tended to migrate to the hotest compartment with a peak around days 6 to 9 post-infection. Notably, this behavior was salutary as none of the carp infected in MCT died from the infection. We confirmed this beneficial effect of behavioral fever by infecting groups of carp in the three blocked compartments of a MCT compared with infection in different SCTs at the three corresponding temperatures. Whatever the tank, infections at 32°C did not induce any mortality, and on contrary, mortality rates increased as temperature decreased. Compared to SCTs, mortalities in the blocked compartments of MCTs were lower, probably due to the temperature gradient near the gridded tunnels.

CyHV-3 replication is blocked *in vitro* at temperatures above 30°C and *in vivo*, outbreaks only occur when water temperatures are between 18°C and 28°C (Michel *et al.*, 2010). We confirmed this observation by infecting CCB cells at different temperatures with CyHV-3 recombinant strains expressing either EGFP or LUC reporter genes. We demonstrated that viral replication was efficient at both 24°C and 28°C but completely blocked at 32°C. Surprisingly, while mortalities of infected carp at 28°C were systematically lower than at 24°C *in vivo*, viral replication *in vitro* was higher at 28°C than 24°C. This apparent paradox between the *in vitro* and *in vivo* results could reveal either that the enhanced viral replication observed at 28°C *in vitro* does not perfectly reflect the *in vivo* situation or that the *in vivo* lower mortalities illustrate a positive balance, in favor of the host, between an enhancement of host immune response and a higher viral replication.

This adaptive value of fever as a beneficial reaction to an infection is well-known and explains its evolutionary conservation in endothermic species, i.e. the survival benefit conferred to the host exceeds the metabolic cost of elevating its temperature (Kluger, 1986; Kluger *et al.*, 1975). The improvement of host fitness could be explained by two factors. First, the raise of body temperature enhances immune system efficiency by promoting both innate, e.g. increase of the neutrophils recruitment with elevated respiratory burst and increased phagocytic potential of macrophages and dendritic cells, and adaptive immunity, e.g. higher rate of lymphocytes trafficking through lymphoid organs (Evans *et al.*, 2015). In ectotherms, as body temperature varies according to the ambient environment, immune responses must be functional over a wide range of temperatures. Nevertheless, lower environmental temperature was shown to affect negatively both cellular and humoral specific immune responses (Bly & Clem, 1992; Le Morvan *et al.*, 1998). In carp, temperature below the limit of 14°C was shown to be immunologically non-permissive for adaptive responses (Avtalion, 1969). Second, the increase of host temperature could decrease or inhibit pathogens replication (Anderson *et al.*, 2013; Fisher & Hajek, 2014). The survival value of fever has already been shown in ectotherms (Elliot *et al.*, 2002; Kluger *et al.*, 1975) among which several fish species. For example, fever significantly increases survival of goldfish *Carassius auratus* injected with live *Aeromonas hydrophila (Covert & Reynolds, 1977),* and zebrafish *Danio rerio* infected with the spring viraemia of carp virus (SVCV) do not exhibit any clinical signs of infection if they are allowed to express behavioral fever in contrast to fish held under constant conditions (Boltaña *et al.*, 2013). However, none of these studies relied on a relevant biological model due to the use of artificial route of infection or a non natural infection model.

Study of signaling pathways of behavioral fever in ectotherms suggested a functional and evolutionary relationship with fever in endotherms. Firstly, the role of many different exogenous pyrogens has been shown in ectothermic species (Cabanac & Laberge, 1998; Stahlschmidt & Adamo, 2013; Żbikowska *et al.*, 2013). As an example, Reynolds *et al.* (1978) induced a febrile response in goldfish through intraperitoneal injection of *Escherichia coli* endotoxin (Reynolds *et al.*, 1978). Secondly, performing discrete electrolytic lesions in the hypothalamic preoptic area (POA) abolished

the development of behavioral fever, showing that this particular region of the brain is, similarly to endotherms, the integration site of ectotherms for pyrogenic signal (Bicego & Branco, 2002; Nelson & Prosser, 1979). Thirdly, while PGE1 injections were shown to induce febrile behavior in several ectotherms (Cabanac & Le Guelte, 1980; Casterlin & Reynolds, 1978; Hutchison & Erskine, 1981; Myhre *et al.*, 1977), the injection of indomethacin, a COX inhibitor, was shown to impair the behavioral fever in toads (Bicego *et al.*, 2002), both observations demonstrating the role of prostaglandins as effector mediators of behavioral fever.

Even if some main steps in the induction pathway are conserved between behavioral fever in ectotherms and fever in endotherms, evolutionary relationship has never been demonstrated for their peripheral cytokine mediators and our study is the first to identify Tnfα as an endogenous pyrogen in ectotherms. We showed that Tnfα1 plasmid injection induced the migration to the 32°C compartment in a dose-dependent manner. This demonstrates that Tnfα1 is a sufficient mediator of behavioral fever in common carp. On the other hand, the injection of carp anti-Tnfα antibodies to CyHV-3 infected fish blocked partially the expression of behavioral fever. This demonstrates that Tnfα is an essential mediator of behavioral fever in the infectious model used. Our study proves that behavioral fever in ectotherms and fever in endotherms share an evolutionarily and functionally related peripheral mediator, which originated more than 400 million years ago. Besides Tnfα, several other cytokines are recognized as predominant pyrogens in endotherms, notably Il1β, Il6 and interferons (Roth & Blatteis, 2014). These cytokines have been shown to act in a cascade mode. Cells of the innate immune system produce Tnfα and Il1β when detecting some PAMPs. Tnfα induces the expression of both Il1β and Il6; Il1β induces the expression of Il6; and finally the Il6 produced induces the expression of PGE2 leading to fever. We observed that the infection of carp by CyHV-3 leads to the overexpression of Tnfα, Il1β and Il6 before the onset of behavioral fever supporting the hypothesis that these three cytokines could form a cascade of endogenous pyrogens in ectotherms like described in endotherms (which would further demonstrate the evolutionary relationship between behavioral fever of ectotherms and fever of endotherms).

During their evolution, vertebrates underwent several rounds (R) of whole genome duplication (WGD) events (Wiens & Glenney, 2011). The timing of 1R is thought to be at the base of the node leading to all vertebrates while 2R may have occurred either before or after the divergence of jawless and jawed vertebrates. Then, teleosts (a group of ray-finned fish) have also undergone a fish-specific WGD (FSGD) early in the basal lineage (3R) and another more recently in the salmonids and some cyprinids (4R) (Fillatreau *et al.*, 2013; Santini *et al.*, 2009; Wiens & Glenney, 2011). These genome duplication events led to polyploidization in teleosts and resulted in the presence of different paralogous genes for several mammalian cytokine genes. The stable retention of these cytokine isoforms throughout evolution suggests they could have divergent functions via subfunctionalization, neofunctionalization, or both (Fillatreau *et al.*, 2013; Hong *et al.*, 2013). Notably, different isoforms exist for the putative endogenous pyrogens orthologs in fish. (i) Tnfα is a proinflammatory cytokine

inducing various cell responses including proliferation, differentiation, necrosis, apoptosis and other cytokines expression, e.g. Il1β and Il6. Depending on the cellular context and the responding receptor, Tnfα can induce either NF-κB mediated survival and proinflammatory pathway, or apoptosis (Forlenza *et al.*, 2009). In teleost fish, two types of Tnfα exist and are supposed to have emerged from the FSGD. Among the nine fish species that possess more than one Tnfα gene, common carp is the only species to have its three Tnfα molecules in the same clade (Hong *et al.*, 2013). Forlenza *et al* (2009) demonstrated that Tnfα in carp primes but does not directly activate phagocytes and only promotes phagocytes activation indirectly, via stimulation of endothelial cells. Indeed, carp Tnfα directly stimulated the expression of proinflammatory cytokines, chemokines, and adhesion molecules in endothelial cells but not in phagocytes. On the other hand, supernatants from Tnfα-treated endothelial cells were able to promote leukocyte migration and respiratory burst activity and Tnfα-primed phagocytes responded faster to endothelial cell supernatants (Forlenza *et al.*, 2009). Between the isoforms of carp Tnfα, the most obvious difference is at the transcription level where Tnfα2 is expressed constitutively at very low levels but could be upregulated to a greater extent than Tnfα1. Moreover, Tnfα2 was a more potent inducer both *in vitro* and *in vivo* (Forlenza *et al.*, 2009). (ii) Il1β is a member of the Il1 family and is one of the earliest expressed proinflammatory cytokine. It is mainly produced by blood monocytes and tissue macrophages and is a major initiator of inflammatory and immune responses, i.e. activation of T and B cells, activation of NK cells, and stimulation of macrophages to secrete proinflammatory mediators (e.g. Il6) (Hong *et al.*, 2001; Reyes-Cerpa *et al.*, 2012). It is produced in an inactive pro-Il1β precursor that remains cytosolic and needs to be cleaved by the Il1β converting enzyme (ICE) (encompassing the proinflammatory protease caspase-1) to become biologically active (Hong *et al.*, 2001; Lopez-Castejon & Brough, 2011). Il1β homologs have been described and sequenced in several fish species including carp (Fujiki *et al.*, 2000; Hong *et al.*, 2001); however sequence alignment of fish genes didn't revealed the aspartic acid residue specific for the ICE cutting site (Reyes-Cerpa *et al.*, 2012). (iii) Il6 is a member of the Il6 family and is produced by different cell types including T lymphocytes, macrophages, fibroblasts, neurons, endothelial and glial cells (Reyes-Cerpa *et al.*, 2012). Il6 stimulates target cells via a membrane bound Il6 receptor (Il6R) which, upon ligand binding, associates with the signaling receptor protein gp130. While this gp130 receptor is ubiquitously expressed on all cell types, the membrane-bound Il6R is only expressed by few cells; cells that don't express such mIl6R then respond to a complex of Il6 and naturally occurring soluble Il6R (Scheller *et al.*, 2011). Il6 has been implicated in the control of Ig production, lymphocyte and monocyte differentiation, chemokine secretion and migration of leucocytes to inflammation sites. Moreover, while Il6 can stimulate itself expression in an autocrine or paracrine manner, it can significantly downregulate Tnfα and Il1β expression (Reyes-Cerpa *et al.*, 2012). (iv) Fish virus-induced Ifns could be classified in two groups according to the number of cysteine residues predicted to be engaged in disulfide bridges: two for Ifns of group I and four for Ifns of group II; both groups present characteristic type I Ifn architecture, nevertheless they signal via two distinct receptors

(Langevin *et al.*, 2013). Fish also possess clear orthologs of mammalian type II Ifns (γ) but as Ifnγ are not always induced by viral infections under conditions where type I Ifns are, they are probably not specialized antiviral cytokines. Fish Ifns are generally expressed by discrete, scattered cell populations either in an "Ifnβ" pattern, by fibroblasts and other tissue cells that may be direct targets of the viruses, or in an "Ifnα" fashion by more specialized immune cells. Once Ifns bind to their membrane receptors, this binding activates the JAK-STAT signaling pathway and induces a vast repertoire of ISGs, some of them exerting a direct antiviral activity, e.g. MX, VIPERIN or PKR (Langevin *et al.*, 2013; Reyes-Cerpa *et al.*, 2012).

Interestingly, several authors showed that the activation of defensive genes in plants by pathogen and herbivore attacks, or by other mechanical wounding, can result from the action of a variety of signaling molecules which could be transported in the atmosphere (Baldwin & Schultz, 1983; Farmer & Ryan, 1990). For example, Farmer *et al.* showed that when they applied methyl jasmonate, a common plant secondary compound, to surfaces of tomato plants, it induces the synthesis of defensive proteinase inhibitor proteins in the treated plants as well as in nearby non-treated plants. They also demonstrate this interplant communication from leaves of one species of plant to leaves of another species to activate the expression of defensive genes (Farmer & Ryan, 1990). In the context of CyHV-3 infection in carp, it would be interesting to investigate whether the production of antiviral interferon response in infected fish could induce a similar antiviral state in cohabitant fish before their own infection. This "interfish" communication through interferon mediator transported in water environment, would represent a danger signal warning naive fish to raise its defenses against potential viral aggressors. However, the epidemic outbreaks of CyHV-3 disease do not support this interesting hypothesis.

A perspective of the study performed in this thesis would consist to investigate whether the homology existing between the cytokine signaling pathways of behavioral fever in ectotherms and fever in endotherms extends to other cytokines than Tnfα. This hypothesis could be tested using functional approaches combining both "gain of function" and "loss of function" as we did to identify the role of Tnfα as a key mediator of behavioral fever in carp. The "gain of function" approach will consist to test each of the three cytokines (including paralogous sequences) for its ability to induce expression of behavioral fever (migration of fish to the warmer compartment of the MCTs). Empty plasmid and plasmid encoding Tnfα1 will be used as negative and positive control (induction of behavioral fever), respectively. These experiments will tell us whether the three cytokines tested are endogenous pyrogens in ectotherms like their orthologs in endotherms. The "loss of function" approach will consist to neutralize the three cytokines *in vivo* through injection of neutralizing antibodies and to determine whether or not this treatment affects the expression of behavioral fever. The "loss of function" approach using these antibodies will be used in two types of experiments aiming to answer two types of questions. Firstly, they will be used to determine the role of each of the three cytokines in the expression of behavioral fever induced by CyHV-3 infection. Secondly, they

will also be exploited to determine the position of each cytokine in the cascade of endogenous pyrogens. This question will require the combination of the "gain of function" and "loss of function" approaches, and will consist to inject naive fish with expression vector encoding paralogous cytokines shown to induce behavioral fever expression and determine whether injection of antibodies raised against another cytokine is able to block the phenomenon.

An emerging scientific field consists to study the interactions and reciprocal regulation between the innate immune system and the central nervous system (CNS) (Irwin & Cole, 2011). On one hand, CNS activation of the hypothalamic-pituitary-adrenal (HPA) axis and sympathic nervous system (SNS) can regulate transcription of proinflammatory (such as Il1β, Il6 or Tnf) and antiviral genes (such as Ifn); while HPA axis inhibits both, SNS suppresses antiviral genes transcription but stimulates the proinflammatory genes. On the other hand, proinflammatory cytokines could modulate activities of several neurotransmitters within the brain such as noradrenaline, dopamine and serotonine, and induce behavioral changes (Irwin & Cole, 2011). An increasing number of evidences suggest a role of cytokines as common mediators of altered CNS function during inflammatory states induced by bacterial and viral infections in the brain or the periphery (Galic *et al.*, 2012); any increase in peripheral proinflammatory cytokines causes a "mirror" inflammatory response with upregulation of cytokines in the CNS. Notably, this was shown to affect neuronal excitability and increase seizure susceptibility. Elevation within the brain of Tnfα due to peripheral inflammation increased excitability in a Tnfα-dependent manner and intracerebroventricular injection of Tnfα alone into the brain was able to enhance seizure susceptibility in healthy animals (Riazi *et al.*, 2008). In our experiments, Tnfα1-injected fish appear more nervous which could be interpreted as an increased neuronal excitability (see video 3). In addition, increased concentrations of proinflammatory cytokines were associated with fatigue, sleepiness and depression in humans; pharmacological antagonism of Tnfα has been shown to reduce depressive symptoms (Tyring *et al.*, 2006). As a conclusion, peripheral innate immune responses can influence CNS functions and behavioral processes (Irwin & Cole, 2011), and our results also support the importance of the interplay existing between the immune system and the CNS.

Tnfα is a key mediator of behavioral fever in a teleost species. However, CyHV-3 infected carp only expressed behavioral fever and started to migrate to the 32°C compartment at a late stage of the disease, when they exhibited already severe clinical signs and significant increases of viral load and proinflammatory cytokines expression. This observation let us postulate that the virus could be able to delay this salutary behavior. Noteworthily, we explained above that viral infection stimulates the production of various cytokines and chemokines that have crucial roles in antiviral defense and immune regulation. In this regard, viruses have evolved many strategies targeting these cytokine mediators and notably, large DNA viruses, e.g. poxviruses and herpesviruses, encode proteins that mimic cytokines (virokines) or cytokine receptors (viroceptors) (Alcami, 2003). For example, the Kaposi's sarcoma-associated herpesvirus (KSHV) encodes a viral Il6 homolog (Jones *et al.*, 1999) and

vaccinia virus has been shown to inhibit fever in mice through expression of a soluble Il1β receptor (Alcami & Smith, 1996). Tnfα is a potent pleiotropic inflammatory cytokine which can induce various cellular responses from apoptosis to inflammatory genes expression, and both Tnfα-induced signals can contribute to host defenses against pathogens (Walczak, 2011). Because of this important role in inflammation and immune response, the Tnf superfamily has been exerting a selection pressure on viruses which developed strategies, targeting virtually every step of Tnf signaling (Benedict, 2003; Rahman & McFadden, 2006; Sedy *et al.*, 2008). (i) Some viruses interact directly with the Tnf ligand, notably by producing viral decoy receptors that could bind the cytokine and prevent by competition its binding to cellular Tnf receptor. Best studied examples are poxviruses as most of them have been shown to encode at least one active Tnf-binding protein (Alejo *et al.*, 2011; Seet *et al.*, 2003). (ii) Some viruses modulate the expression of cellular Tnf receptor and associated factors. For example, Epstein-Barr virus (EBV) protein BZLF1 downregulates TNFR1 promoter activity and so, TNFR1 transcription (Morrison *et al.*, 2004). (iii) Other viruses block the caspase activation (Wolf & Green, 1999), and some well-characterized examples are the induction of expression of cellular FLICE/caspase-8 inhibitory proteins (FLIP) by herpes simplex virus 1 (HSV-1) (Medici *et al.*, 2003) or the production of a viral FLIP by KSHV (Thome *et al.*, 1997). (iv) Some viruses target the mitochondria and impair the mitochondrion-dependent cell death pathway (Wajant *et al.*, 2003). For example, KHSV produces a viral Bcl-2 homolog that can inhibit apoptosis through interaction with Bax and Bak (Sarid *et al.*, 1997). (v) Finally, many viruses modulate NF-κB pathway which is the proinflammatory and antiapoptotic Tnf-mediated pathway. For example, by activating NF-κB and upregulating antiapoptotic proteins, the protein gD of HSV-1 inhibits apoptosis (Medici *et al.*, 2003). Through bioinformatics analysis including modelling, we predicted that ORF12 encodes for a soluble Tnfα receptor and proposed that the expression of this putative viral decoy receptor could neutralize the Tnfα-mediated behavioral fever.

In our study, we demonstrate that the predicted TNFR homolog encoded by CyHV-3 ORF12 is able to bind and neutralize Tnfα which classifies CyHV-3 in the first category described above and encompassing viruses interacting with the Tnf ligand by production of decoy receptors. To challenge our hypothesis that CyHV-3 could delay the expression of behavioral fever in common carp through neutralization of a key endogenous pyrogen Tnfα, we produced an ORF12 deleted and derived revertant strain. While we could not observe any differences when infections were performed in SCTs, we showed that fish infected in MCT with the ORF12 deleted virus migrated significantly earlier than fish infected with the ORF12 revertant virus which confirmed our working hypothesis. As a consequence of their earlier migration, fish infected with the ORF12 deleted strain exhibited less severe clinical signs coupled with a faster and more drastic decrease of viral load. Altogether, these results demonstrate that CyHV-3 acquired a gene that delays the expression of behavioral fever, demonstrating that this innate immune response has been acting as a selection pressure on this pathogen throughout evolution. Interestingly, the ORF12 gene revealed a function only in conditions

mimicking temperature gradients consistent with natural environments but had no phenotype under standard laboratory conditions. This result adds a new dimension to environment enrichment for laboratory animals. Our study strongly supports the concept that environment enrichment for laboratory animals may be essential for clarifying the function of numerous viral and cellular genes that do not show a phenotype under standard laboratory conditions.

In addition to ORF12, the CyHV-3 encodes for other potential immune evasion mechanisms, notably ORF134 encoding an Il10 homolog and ORF4 encoding another member of the TNFR family. Il10 is generally described as an immunosuppressive cytokine that could inhibit expression of a large number of cytokines as, for example, Tnfα, Ifnγ, Il1β, Il2, Il3, Il6, and MHC class I (de Waal *et al.*, 1991; Fiorentino *et al.*, 1991). While the role of vIl10 has been demonstrated for example in the pathogenesis of the cytomegalovirus (Chang & Barry, 2010; Ouyang *et al.*, 2013), Ouyang *et al.* (2013) demonstrated that the Il10 homolog encoded by ORF134 is essential neither for replication *in vitro* nor for virulence *in vivo* (Ouyang et al., 2013). Similarly, some preliminary data from our lab tend to show that an ORF4 deleted strain and the derived revertant do not exhibit differences in their phenotype when infections are performed in SCT at 24°C (data not shown). However, the expression product of these two genes could play a role in the viral dysregulation of the behavioral fever response: the Il10 homolog by downregulating the expression of Tnfα and ORF4 through a still unidentified possible mechanism. In regard to this context, it would be interesting to test the different recombinant strains for ORF134 and ORF4, in an enriched environment where a temperature gradient exists and allows fish to express behavioral fever. This experiment could, as in the case of ORF12, reveal a phenotypic difference that was not detected in standard experimental conditions.

Overall, our study emphasizes the tripartite interplay between pathogen, host and environment (Engering *et al.*, 2013). The underlying causal factors of disease emergence could be categorized by their impact on each component of this triangle. (i) A pathogen could emerge by transmission from a reservoir into a novel host species, which is called spill-over. In human, this concerns notably the reported zoonoses but it has also been described for several emerging diseases in various species (Woolhouse *et al.*, 2005). For example, a chytrid fungus recently introduced in Europe induced a novel form of chytridiomycosis and a rapid decline in the population of European salamanders. This fungus, likely originating from a reservoir salamander species in Asia, was probably introduced through human and animal traffic into naive European amphibian populations (Martel *et al.*, 2014). (ii) A pathogen could develop novel traits, whereby inducing outbreaks within the same host species, by mutations increasing its virulence or acquisition of antibiotic/antiviral resistance. For example, a high polymorphism (HPR)-deleted or HPR0 variant of infectious salmon anaemia virus (ISAV; *Orthomyxoviridae* family) emerged in populations of farmed Atlantic salmon (*Salmo salar*) in Norway and was recently notified to the World Organisation for Animal Health (OIE) (OIE, 2015). (iii) A disease complex could emerge in a novel geographic area, so-called “geographic expanders” due to changes in landscapes or “geographic jumps”, which are usually governed by chance. Arboviruses

(arthropod-borne viruses) are great example of geographic expanders (Jimenez-Clavero, 2012) as global warming allows their vectors to encroach regions neighboring their actual distribution. On the other hand, the chytrid fungus described above (Martel *et al.*, 2014), possibly represents a geographical jump (Engering *et al.*, 2013). The results of this thesis suggest a new mechanism through which environmental temperature changes can contribute to disease emergence. Our data demonstrate that while an infection can be benign in conditions where the environment is compatible with the expression of behavioral fever, it can cause a high mortality rate when the environment does not allow the expression of this behavior. This finding has important ecological implications and should be taken into account when considering for example modifications of natural habitats, introduction of ectotherms in new biotopes and environmental conditions in aquaculture systems.

The point described above suggests that CyHV-3 disease could be the consequence of the artificial migration of CyHV-3/carp to environment where the host could not express salutary behavioral fever. The origin of the current distribution of common carp is doubtful. It is well-known that domestication of common carp created many different strains, crossbreeds and hybrids that are now somehow spread over the world in both cultured and wild populations (Chistiakov & Voronova, 2009). However, this widespread repartition of common carp is relatively ancient compared to the recent emergence of CyHV-3. This supports the hypothesis that the couple CyHV-3/carp could have been adapted in a particular region of the world where behavioral fever is possible during most seasons compatible with CyHV-3 disease. This adaptation could have been disrupted by recent movement of infected carp to regions where the behavioral fever for resident carp is impossible during part of the year. Notably, it is well-known that CyHV-3 is a seasonal disease, more frequently occurring during spring and autumn in temperate regions (Rakus *et al.*, 2013). Usually, this has been attributed to the temperature restriction of the viral replication. Our study suggests that this seasonal characteristic of CyHV-3 disease is also the consequence of the (in-)ability of common carp to express behavioral fever when facing infection.

Besides the importance of environment in regards to pathogen-host-environment interplay, viruses have developed mechanisms that interfere with almost all physiological processes of their host to increase their replication and transmission (Janeway *et al.*, 2001). In this study, we observed a virus able to modify the behavior of its host and we clarified the underlying mechanism. There are many reports on pathogens inducing changes in their host's behavior to increase their fitness (Brodeur & Mc Neil, 1989; Goulson, 1997; Kamita *et al.*, 2005; Poulin, 1995; van Houte *et al.*, 2013). Most of them concern parasites modifying the behavior of their arthropod vector or vertebrate host in vector-borne diseases (Lefèvre & Thomas, 2008). For example, *Plasmodium mexicanum* has evolved mechanisms to manipulate temperature preference of its host; indeed, infected sand flies are attracted by higher temperatures than uninfected ones, which are optimal for rapid parasite development but sub-optimal for sand flies eggs development (Fialho & Schall, 1995). The CyHV-3 that delays behavioral fever in its natural host, common carp, through expression of a single gene is really an original finding for two

main reasons. First, examples of viruses altering the behavior of their vertebrate host to increase their fitness are rare, and the most frequently cited example is rabies. In furious rabies, hosts exhibit aggressiveness and increased salivation which enhance viral transmission (Hemachudha *et al.*, 2002). As rabies virus infects the CNS of its host, its pathogeny seems to provide an easy explanation of this modified behavior; however rabies virus does not selectively target brain areas responsible for regulating aggression nor strikes exclusively, or even preferentially, the limbic system (Laothamatas *et al.*, 2003; Laothamatas *et al.*, 2008). To date, mechanisms for the rabies induced behavioral changes remain mainly unknown even if immune reactions provoked by the virus are suggested (Hemachudha *et al.*, 2002). Second, descriptions of viral genes that were proved to modify host behavior have only been reported twice and both concerned baculoviruses infecting invertebrate hosts. Tyrosine phosphatase (Kamita *et al.*, 2005; Katsuma *et al.*, 2012) and ecdysteroid uridine 5'-diphosphate-glucosyltransferase (Hoover *et al.*, 2011) encoded by baculoviruses have been shown to increase respectively the locomotory activity and the climbing behavior of infected caterpillar hosts, both predicted to increase viral transmission.

In the homologous CyHV-3/carp model, we demonstrated that CyHV-3 is able to delay behavioral fever through the expression of a single gene ORF12 and we demonstrated an adaptive value of this mechanism. By delaying behavioral fever, CyHV-3 maintains infected fish longer at permissive temperature, i.e. enhancing viral replication and excretion, which corresponds also to the temperature preferred by naive fish, thereby increasing its transmission. Nevertheless, ORF12 only delays the expression of behavioral fever rather than completely inhibiting its appearance which allows infected fish to survive and turn the infection to latency (Eide *et al.*, 2011; Reed *et al.*, 2014; Ronen *et al.*, 2003). This timing effect of ORF12 is likely to reflect also a long-term selective advantage conferred to the virus, as it allows infected fish to survive the infection and become latently infected by the virus whereby representing future source of viral transmission. Altogether, these results represent the perfect illustration of the "*Central Theorem of the Extended Phenotype"* of Richard Dawkins (Dawkins, 1982) according to which a viral gene, in this case the CyHV-3 ORF12, promotes its selection and spread through a phenotypical effect in an independent biological entity, the infected carp.

A nice perspective would be to investigate the long-term consequences of this ORF12 timing effect on the viral infection (establishment of latency, persistence of latency, reactivation and transmission to naive cohabitant subjects) and, from the immunological points of view, on the adaptive immune response developed by infected subjects. The rational of these questions is that the delay of behavioral fever could be important to reach a viral load compatible with successful establishment of latency and antigenic stimulation of the immune system (both innate and adaptive systems). Maintaining fish at permissive temperature increases viral replication and so, proportionally the antigenic stimulation. This could notably induce a better immune response in infected fish. Thereby, when fish migrate to the 32°C compartment and recover from lytic infection, they will become healthy

carriers of the virus, able to reactivate and spread the virus to naive fish but also survive any re-exposure through an effective immune protection. In other words, the ORF12 gene by delaying the migration of fish to a warmer environment could improve the adaptive immune response of the infected fish (through a better antigenic stimulation) and so enhance its survival to subsequent re-infections. This adaptive immune response could be particularly important if reactivation or super infection occur when the environment does not allow the expression of salutary behavioral fever (no restrictive temperature). These hypotheses are supported by vaccination protocols developed soon after the emergence of CyHV-3 (Ronen *et al.*, 2003). Fish were exposed to pathogenic virus for 3 to 5 days at permissive temperature before being transferred to non-permissive temperature for 30 days. However, the immune protection induced by this ingenious process was relatively low as 40% of the fish died when challenged. This could result from an insufficient immune stimulation due to a too early viral replication inhibition (Ronen *et al.*, 2003).

In conclusion, throughout this thesis, we travelled along innate immune mechanisms of CyHV-3 natural host, *Cyprinus carpio*. We first demonstrated that carp are sensitive and permissive to CyHV-3 infection since hatching but that their sensitivity remains relatively low at the beginning of ontogenesis, due to efficient epidermal mucus defense. Then, we showed that adult carp infected with CyHV-3 express salutary behavioral fever mediated by Tnfα, an essential and sufficient endogenous pyrogen in carp. Moreover, we showed that behavioral fever has been acting as a selection pressure on CyHV-3 which developed the capacity to delay this process by the expression of a single gene. This thesis on the whole gives insights in many fields such as virology, immunology, animal behavior, ecology, evolution or animal welfare, and emphasizes that a disease is not only a host infected by a pathogen but on contrary, involves many other contributors; As Lourens G. M. Baas Becking said: “Everything is everywhere, but, the environment selects”. Performing my PhD on these subjects stimulated my broad-mindedness and reminded me that in science, as in other life domains, you need to pay attention to what is going on around, feel free to challenge dogmas and keep the thirst for surprising discoveries. During a thesis, one could easily get lost on this way to knowledge but I was fortunately well surrounded by colleagues, friends and my promoter who encouraged me and put me back on the right track when required. Finally, I would like to end this dissertation with a quote of Steve Jobs: “Your time is limited, so don’t waste it living someone else’s life. Don’t be trapped by dogma, which is living with the results of other people’s thinking. Don’t let the noise of other’s opinions drown out your own inner voice. And most important, have the courage to follow your heart and intuition. They somehow already know what you truly want to become. Everything else is secondary.”

Summary - Résumé

Summary

Cyprinid herpesvirus 3 (CyHV-3) is a highly pathogenic virus responsible for a lethal disease in both common and koi carp (*Cyprinus carpio*). The common carp is one of the most important freshwater species cultivated for human consumption. Its colourful subspecies koi is grown for personal pleasure and exhibitions. Both common and koi carp are economically important and since its description in the late 1990s, the CyHV-3 has caused severe financial losses in these two carp industries worldwide. Because of its economic importance and its numerous original biological properties, CyHV-3 became rapidly an attractive subject for both applied and fundamental research.

The objectives of this thesis were to investigate the role of two unrelated innate immune mechanisms of carp in anti-CyHV-3 immunity. The first objective was to determine the role of epidermal mucus as an innate immune barrier against CyHV-3 entry during the early developmental stages of carp. To test this hypothesis, we investigated the sensitivity and the permissivity of carp to CyHV-3 during the early stages of its development. This hypothesis was tested using a recombinant CyHV-3 strain expressing luciferase as a constitutive reporter gene and *in vivo* bioluminescence imaging system. We demonstrated that carp are sensitive and permissive to CyHV-3 infection since hatching, but that their sensitivity remains relatively low in the two early developmental stages. Similarly to adults, we confirmed that the skin is the main portal of entry for the virus at early stages, and our results stress out the role of epidermal mucus as an innate immune defense of carp against pathogens even and especially at the early stages of development. The results of this study have been published in *Veterinary research.*

The second objective of this thesis consisted to investigate whether carp express behavioral fever when infected by CyHV-3; and if so, what could be the effect of this innate immune reaction on the development of CyHV-3 disease. When infected by pathogens, both endotherms and ectotherms can express a salutary reaction by increasing their body temperature. While in endotherms this reaction is called fever and depends on intrinsic thermogenesis, ectotherms like teleosts can only upregulate their body temperature by moving to warmer places, hence the term behavioral fever. When studying the pathogenesis of CyHV-3, we observed that carp infected at 24°C (the thermal preference of healthy carp) tended to concentrate around the tank heater when it was running. This observation led us to postulate that infected subjects could express behavioral fever in natural environments where temperature gradients exist. Using multi-chamber tanks encompassing a gradient from 24°C to 32°C, we observed that carp infected by CyHV-3 express a salutary behavioral fever that completely suppresses virus induced mortalities. The relatively late onset of behavioral fever with respect to clinical signs, viral replication and cytokine upregulation led us to postulate that this phenomenon could be delayed by the virus to retain its host at a permissive temperature thereby favoring its replication and spreading. As some herpesviruses have been shown to express soluble decoy cytokine

receptors, we hypothesized that CyHV-3 could express such receptor(s) able to neutralize putative pyrogenic cytokines produced by the fish. We found that CyHV-3 ORF12 encodes a soluble decoy receptor for carp Tnfα and that this viral protein makes the virus capable of delaying the migration of infected fish to warmer environments. Remarkably, the study of the molecular mechanism through which the virus alters its host's behavior led to the discovery of the first pyrogenic cytokine in ectotherms (Tnfα). This study is the first to report the ability of a vertebrate virus to alter the behavior of its host through the expression of a single gene. This second study was submitted for publication when this thesis was printed.

In conclusion, we investigated two innate immune mechanisms expressed by carp against CyHV-3 infection. The results generated in this thesis bring findings related to several scientific fields such as virology, immunology, animal behavior, evolution, ecology and even animal welfare.

Résumé

L'herpesvirus cyprin 3 (CyHV-3) est un virus hautement pathogène responsable d'une maladie mortelle chez les carpes commune et koi (*Cyprinus carpio*). La carpe commune est l'un des poissons d'eau douce les plus produits pour la consommation humaine. Sa sous-espèce colorée koi est élevée par loisir et pour des compétitions de collectionneurs. Les carpes commune et koi sont toutes deux importantes d'un point de vue économique et, depuis sa description fin des années 90, le CyHV-3 a induit des pertes sévères dans ces deux industries à travers le monde. En raison de son importance économique et de ses nombreuses propriétés biologiques originales, le CyHV-3 est rapidement devenu un sujet intéressant à la fois pour la recherche appliquée et fondamentale.

Les objectifs de cette thèse étaient d'étudier deux mécanismes indépendants de la réponse immunitaire innée de la carpe contre le CyHV-3. Le premier objectif était d'étudier le rôle du mucus cutané comme barrière immunitaire innée contre l'entrée du CyHV-3 au cours des premiers stades de développement de la carpe. Afin d'investiguer cette hypothèse, nous avons étudié la sensibilité et la permissivité des carpes au CyHV-3 au cours des premiers stades de développement. Pour ce faire, nous avons utilisé une souche recombinante du CyHV-3 exprimant la luciférase comme gène rapporteur constitutif et la technique de bioluminescence *in vivo*. Nous avons démontré que les carpes sont sensibles et permissives à l'infection par le CyHV-3 dès l'éclosion, mais que leur sensibilité reste relativement faible au cours des deux stades de développement les plus précoces. Nous avons confirmé que, comme chez les adultes, la peau est la porte d'entrée pour le virus au cours des stades précoces et nos résultats ont mis en exergue le rôle du mucus cutané comme défense immunitaire innée de la carpe contre les pathogènes, aussi et surtout au cours des premiers stades de développement. Les résultats de cette étude ont été publiés dans *Veterinary Research*.

Le second objectif de cette thèse visait à révéler l'expression d'une fièvre comportementale chez les carpes infectées par le CyHV-3; et si tel était le cas, étudier les effets de cette réaction immunitaire innée sur le développement de la maladie induite par le CyHV-3. Lors d'une infection par des pathogènes, les endothermes ainsi que les ectothermes peuvent exprimer une réaction salutaire en augmentant leur température. Alors que chez les endothermes, cette réaction s'appelle la fièvre et dépend de la thermogénèse endogène, les ectothermes, comme les téléostéens, ne peuvent augmenter leur température corporelle qu'en se déplaçant dans des eaux plus chaudes, d'où le terme « fièvre comportementale ». En étudiant la pathogénèse du CyHV-3, nous avons observé que des carpes infectées à 24°C (température préférentielle des carpes non-infectées) avaient tendance à se grouper autour du chauffage de l'aquarium pendant que celui-ci fonctionnait. Cette observation nous a amenés à postuler que les sujets infectés pourraient exprimer une fièvre comportementale dans les environnements naturels où un gradient de température existe. En réalisant une infection au sein

d'aquariums compartimentés où un gradient de température était établi de 24°C à 32°C, nous avons observé que les carpes infectées par le CyHV-3 exprimaient une fièvre comportementale salutaire, supprimant toutes les mortalités induites par le virus. Le déclenchement relativement tardif de la fièvre comportementale, au regard des signes cliniques, de la charge viral et de l'augmentation de l'expression des cytokines, nous a amenés à postuler que le virus pourrait retarder ce phénomène afin de maintenir plus longtemps son hôte à une température permissive et ainsi, favoriser sa réplication et sa transmission. Comme des récepteurs leurres ont été révélés chez certains herpesvirus, nous avons émis l'hypothèse que le CyHV-3 pourrait exprimer ce genre de récepteur(s) capable de neutraliser de potentielles cytokines pyrogènes produites par le poisson. Nous avons découvert que l'ORF12 du CyHV-3 code pour un récepteur soluble leurre du Tnfα et que cette protéine virale permet au virus de retarder la migration des poissons infectés dans les eaux plus chaudes. De façon remarquable, l'étude du mécanisme moléculaire par lequel le virus modifie le comportement de son hôte a conduit à la découverte de la première cytokine pyrogène chez les ectothermes (Tnfα). Cette étude est également la première à rapporter un virus capable de modifier le comportement de son hôte vertébré par l'expression d'un seul gène. Cette deuxième étude était soumise pour publication au moment de l'impression de cette thèse.

En conclusion, nous avons investigué deux mécanismes immunitaires innés de la carpe face à une infection par le CyHV-3. Les résultats de cette thèse ont généré des découvertes ayant trait à différents domaines scientifiques comme la virologie, l'immunologie, le comportement animal, l'évolution, l'écologie et même le bien-être animal.

References

Ackermann, M. (2004). Herpesviruses: a brief overview. *Methods Mol Biol* **256**, 199-219.

Adamek, M., Rakus, K. L., Brogden, G., Matras, M., Chyb, J., Hirono, I., Kondo, H., Aoki, T., Irnazarow, I. & Steinhagen, D. (2014a). Interaction between type I interferon and Cyprinid herpesvirus 3 in two genetic lines of common carp *Cyprinus carpio*. *Dis Aquat Organ* **111**, 107-118.

Adamek, M., Rakus, K. L., Chyb, J., Brogden, G., Huebner, A., Irnazarow, I. & Steinhagen, D. (2012). Interferon type I responses to virus infections in carp cells: *In vitro* studies on Cyprinid herpesvirus 3 and Rhabdovirus carpio infections. *Fish Shellfish Immunol* **33**, 482-493.

Adamek, M., Steinhagen, D., Irnazarow, I., Hikima, J., Jung, T. S. & Aoki, T. (2014b). Biology and host response to Cyprinid herpesvirus 3 infection in common carp. *Dev Comp Immunol* **43**, 151-159.

Adamek, M., Syakuri, H., Harris, S., Rakus, K. L., Brogden, G., Matras, M., Irnazarow, I. & Steinhagen, D. (2013). Cyprinid herpesvirus 3 infection disrupts the skin barrier of common carp (*Cyprinus carpio* L.). *Vet Microbiol* **162**, 456-470.

Adkison, M. A., Gilad, O. & Hedrick, R. P. (2005). An enzyme linked immunosorbent assay (ELISA) for detection of antibodies to the koi herpesvirus (KHV) in the serum of koi *Cyprinus carpio*. *Fish Pathol* **40**, 53-62.

Alcami, A. (2003). Viral mimicry of cytokines, chemokines and their receptors. *Nature review* **3**, 36-50.

Alcami, A. & Smith, G. L. (1996). A mechanism for the inhibition of fever by a virus. *Proc Natl Acad Sci U S A* **93**, 11029-11034.

Alejo, A., Pontejo, S. M. & Alcami, A. (2011). Poxviral TNFRs: properties and role in viral pathogenesis. *Adv Exp Med Biol* **691**, 203-210.

Altringham, J. D. & Block, B. A. (1997). Why do tuna maintain elevated slow muscle temperatures? Power output of muscle isolated from endothermic and ectothermic fish. *J Exp Biol* **200**, 2617-2627.

Anderson, R. D., Blanford, S., Jenkins, N. E. & Thomas, M. B. (2013). Discriminating fever behavior in house flies. *PLoS One* **8**, e62269.

Ángeles Esteban, M. (2012). An Overview of the Immunological Defenses in Fish Skin. *ISRN Immunology* **2012**, 1-29.

Antychowicz, J., Reichert, M., Matras, M., Bergmann, S. M. & Haenen, O. L. (2005). Epidemiology, pathogenicity and molecular biology of koi herpesvirus isolated in Poland. *Bulletin of the Veterinary Institute in Pulawy* **49**, 367-373.

Aoki, T., Hirono, I., Kurokawa, K., Fukuda, H., Nahary, R., Eldar, A., Davison, A. J., Waltzek, T. B., Bercovier, H. & Hedrick, R. P. (2007). Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide. *J Virol* **81**, 5058-5065.

Aoki, T., Takano, T., Unajak, S., Takagi, M., Kim, Y. R., Park, S. B., Kondo, H., Hirono, I., Saito-Taki, T., Hikima, J. & Jung, T. S. (2011). Generation of monoclonal antibodies specific for ORF68 of koi herpesvirus. *Comp Immunol Microbiol Infect Dis* **34**, 209-216.

Ariav, R., Tinman, S., Paperna, I. & Bejerano, I. (1999). First report of newly emerging viral disease of *Cyprinus carpio* species in Israel. In *the EAFP 9th International Conference*. Rhodes, Greece.

Athanasiadis, A. (2012). Zalpha-domains: at the intersection between RNA editing and innate immunity. *Semin Cell Dev Biol* **23**, 275-280.

Avarre, J. C., Madeira, J. P., Santika, A., Zainun, Z., Baud, M., Cabon, J., Caruso, D., Castric, J., Bigarre, L., Engelsma, M. & Maskur, M. (2011). Investigation of Cyprinid herpesvirus-3 genetic diversity by a multi-locus variable number of tandem repeats analysis. *J Virol Methods* **173**, 320-327.

Avarre, J. C., Santika, A., Bentenni, A., Zainun, Z., Madeira, J. P., Maskur, M., Bigarre, L. & Caruso, D. (2012). Spatio-temporal analysis of cyprinid herpesvirus 3 genetic diversity at a local scale. *J Fish Dis* **35**, 767-774.

Avtalion, R. R. (1969). Temperature effect on antibody production and immunological memory, in carp (*Cyprinus carpio*) immunized against bovine serum albumin (BSA). *Immunology* **17**, 927-931.

Baldwin, I. T. & Schultz, J. C. (1983). Rapid changes in tree leaf chemistry induced by damage: evidence for communication between plants. *Science* **221**, 277-279.

Balon, E. (2006). The oldest domesticated fishes, and the consequences of an epigenetic dichotomy in fish culture. *J Ichthyol Aquat Biol* **11**, 47-86.

Balon, E. K. (1995). Origin and domestication of the wild carp, *Cyprinus carpio*: from Roman gourmets to the swimming flowers. *Aquaculture* **129**, 3-48.

Bassity, E. & Clark, T. G. (2012). Functional identification of dendritic cells in the teleost model, rainbow trout (*Oncorhynchus mykiss*). *PLoS One* **7**, e33196.

Baumer, A., Fabian, M., Wilkens, M. R., Steinhagen, D. & Runge, M. (2013). Epidemiology of cyprinid herpesvirus-3 infection in latently infected carp from aquaculture. *Dis Aquat Organ* **105**, 101-108.

Benedict, C. A. (2003). Viruses and the TNF-related cytokines, an evolving battle. *Cytokine Growth Factor Rev* **14**, 349-357.

Bercovier, H., Fishman, Y., Nahary, R., Sinai, S., Zlotkin, A., Eyngor, M., Gilad, O., Eldar, A. & Hedrick, R. P. (2005). Cloning of the koi herpesvirus (KHV) gene encoding thymidine kinase and its use for a highly sensitive PCR based diagnosis. *BMC Microbiol* **5**, 13.

Bergmann, S. M. & Kempter, J. (2011). Detection of koi herpesvirus (KHV) after re-activation in persistently infected common carp (*Cyprinus carpio* L.) using non-lethal sampling methods. *B Eur Assoc Fish Pat* **31**, 92-100.

Bergmann, S. M., Kempter, J., Sadowski, J. & Fichtner, D. (2006). First detection, confirmation and isolation of koi herpesvirus (KHV) in cultured common carp (*Cyprinus carpio* L.) in Poland. *B Eur Assoc Fish Pat* **26**, 97-104.

Bergmann, S. M., Lutze, P., Schütze, H., Fischer, U., Dauber, M., Fichtner, D. & Kempter, J. (2010a). Goldfish (*Carassius auratus auratus*) is a susceptible species for koi herpesvirus (KHV) but not for KHV disease (KHVD). *B Eur Assoc Fish Pat* **30**, 74-84.

Bergmann, S. M., Riechardt, M., Fichtner, D., Lee, P. & Kempter, J. (2010b). Investigation on the diagnostic sensitivity of molecular tools used for detection of koi herpesvirus. *J Virol Methods* **163**, 229-233.

Bergmann, S. M., Sadowski, J., Kielpinski, M., Bartlomiejczyk, M., Fichtner, D., Riebe, R., Lenk, M. & Kempter, J. (2010c). Susceptibility of koi x crucian carp and koi x goldfish hybrids to koi herpesvirus (KHV) and the development of KHV disease (KHVD). *J Fish Dis* **33**, 267-272.

Bergmann, S. M., Schütze, H., Fischer, U., Fichtner, D., Riechardt, M., Meyer, K., Schrudde, D. & Kempter, J. (2009). Detection KHV genome in apparently health fish. *B Eur Assoc Fish Pat* **29**, 145-152.

Bicego, K. C., Barros, R. C. & Branco, L. G. (2007). Physiology of temperature regulation: comparative aspects. *Comp Biochem Physiol A Mol Integr Physiol* **147**, 616-639.

Bicego, K. C. & Branco, L. G. (2002). Discrete electrolytic lesion of the preoptic area prevents LPS-induced behavioral fever in toads. *J Exp Biol* **205**, 3513-3518.

Bicego, K. C., Steiner, A. A., Antunes-Rodrigues, J. & Branco, L. G. (2002). Indomethacin impairs LPS-induced behavioral fever in toads. *Journal of applied physiology (Bethesda, Md : 1985)* **93**, 512-516.

Bigarré, L., Baud, M., Cabon, J., Antychowicz, J., Bergmann, S. M., Engelsma, M., Pozet, F., Reichert, M. & Castric, J. (2009). Differentiation between Cyprinid herpesvirus type-3 lineages using duplex PCR. *J Virol Methods* **158**, 51-57.

Bly, J. E. & Clem, W. (1992). Temperature and teleost immune functions. *Fish Shellfish Immunol* **2**, 159-171.

Boltaña, S., Rey, S., Roher, N., Vargas, R., Huerta, M., Huntingford, F. A., Goetz, F. W., Moore, J., Garcia-Valtanen, P., Estepa, A. & MacKenzie, S. (2013). Behavioural fever is a synergic signal amplifying the innate immune response. *Proceedings of the Royal Society B: Biological Sciences* **280**.

Bondad-Reantaso, M. G., Subasinghe, R. P., Arthur, J. R., Ogawa, K., Chinabut, S., Adlard, R., Tan, Z. & Shariff, M. (2005). Disease and health management in Asian aquaculture. *Vet Parasitol* **132**, 249-272.

Booy, F. P., Trus, B. L., Davison, A. J. & Steven, A. C. (1996). The capsid architecture of channel catfish virus, an evolutionarily distant herpesvirus, is largely conserved in the absence of discernible sequence homology with herpes simplex virus. *Virology* **215**, 134-141.

Boutier, M., Ronsmans, M., Ouyang, P., Fournier, G., Reschner, A., Rakus, K., Wilkie, G. S., Farnir, F., Bayrou, C., Lieffrig, F., Li, H., Desmecht, D., Davison, A. J. & Vanderplasschen, A. (2015). Rational development of an attenuated recombinant cyprinid herpesvirus 3 vaccine using prokaryotic mutagenesis and in vivo bioluminescent imaging. *PLoS Pathog* **11**, e1004690.

Bretzinger, A., Fischer-Scherl, T., Oumouma, R., Hoffmann, R. & Truyen, U. (1999). Mass mortalities in koi, *Cyprinus carpio*, associated with gill and skin disease. *B Eur Assoc Fish Pat* **19**, 182-185.

Briese, E. (1997). Selected temperature correlates with intensity of fever in rats. *Physiol Behav* **61**, 659-660.

Brodeur, J. & McNeil, J. N. (1989). Seasonal microhabitat selection by an endoparasitoid through adaptive modification of host behavior. *Science* **244**, 226-228.

Brudeseth, B. E., Wiulsrod, R., Fredriksen, B. N., Lindmo, K., Lokling, K. E., Bordevik, M., Steine, N., Klevan, A. & Gravningen, K. (2013). Status and future perspectives of vaccines for industrialised fin-fish farming. *Fish Shellfish Immunol* **35**, 1759-1768.

Cabanac, M. (1998). Thermiatrics and Behavior In *Physiology and Pathophysiology of Temperature Regulation* p. 308. Edited by C. M. Blatteis.

Cabanac, M. & Laberge, F. (1998). Fever in goldfish is induced by pyrogens but not by handling. *Physiol Behav* **63**, 377-379.

Cabanac, M. & Le Guelte, L. (1980). Temperature regulation and prostaglandin E1 fever in scorpions. *J Physiol* **303**, 365-370.

Campbell, J., Kessler, B., Mayack, C. & Naug, D. (2010). Behavioural fever in infected honeybees: parasitic manipulation or coincidental benefit? *Parasitology* **137**, 1487-1491.

Campinho, M. A., Silva, N., Sweeney, G. E. & Power, D. M. (2007). Molecular, cellular and histological changes in skin from a larval to an adult phenotype during bony fish metamorphosis. *Cell Tissue Res* **327**, 267-284.

Casterlin, M. E. & Reynolds, W. W. (1978). Prostaglandin E1 fever in the crayfish Cambarus bartoni. *Pharmacol Biochem Behav* **9**, 593-595.

Cerezuela R., Meseguer J. & MA., E. (2011). Current knowledge in synbiotic use for fish aquaculture: A review *Journal of Aquaculture Research & Development*.

Chang, W. J. & Hwang, P. P. (2011). Development of zebrafish epidermis. *Birth Defects Res C Embryo Today* **93**, 205-214.

Chang, W. L. & Barry, P. A. (2010). Attenuation of innate immunity by cytomegalovirus IL-10 establishes a long-term deficit of adaptive antiviral immunity. *Proc Natl Acad Sci U S A* **107**, 22647-22652.

Chen, J., Chee, D., Wang, Y., Lim, G. Y., Chong, S. M., Lin, Y. N. & Huangfu, T. (2014). Identification of a novel cyprinid herpesvirus 3 genotype detected in koi from the East Asian and South-East Asian Regions. *J Fish Dis*.

Chistiakov, D. A. & Voronova, N. V. (2009). Genetic evolution and diversity of common carp *Cyprinus carpio* L. *Cent Eur J Biol* **4**, 304-312.

Cho, M. Y., Won, K. M., Kim, J. W., Jee, B. Y., Park, M. A. & Hong, S. (2014). Detection of koi herpesvirus (KHV) in healthy cyprinid seed stock. *Dis Aquat Organ* **112**, 29-36.

Costes, B., Fournier, G., Michel, B., Delforge, C., Raj, V. S., Dewals, B., Gillet, L., Drion, P., Body, A., Schynts, F., Lieffrig, F. & Vanderplasschen, A. (2008). Cloning of the koi herpesvirus genome as an infectious bacterial artificial chromosome demonstrates that disruption of the thymidine kinase locus induces partial attenuation in *Cyprinus carpio koi*. *J Virol* **82**, 4955-4964.

Costes, B., Raj, V. S., Michel, B., Fournier, G., Thirion, M., Gillet, L., Mast, J., Lieffrig, F., Bremont, M. & Vanderplasschen, A. (2009). The major portal of entry of koi herpesvirus in *Cyprinus carpio* is the skin. *J Virol* **83**, 2819-2830.

Covert, J. B. & Reynolds, W. W. (1977). Survival value of fever in fish. *Nature* **267**, 43-45.

Davidovich, M., Dishon, A., Ilouze, M. & Kotler, M. (2007). Susceptibility of cyprinid cultured cells to cyprinid herpesvirus 3. *Arch Virol* **152**, 1541-1546.

Davison, A. J. (1992). Channel catfish virus: a new type of herpesvirus. *Virology* **186**, 9-14.

Davison, A. J. (1998). The genome of salmonid herpesvirus 1. *J Virol* **72**, 1974-1982.

Davison, A. J., Cunningham, C., Sauerbier, W. & McKinnell, R. G. (2006). Genome sequences of two frog herpesviruses. *J Gen Virol* **87**, 3509-3514.

Davison, A. J. & Davison, M. D. (1995). Identification of structural proteins of channel catfish virus by mass spectrometry. *Virology* **206**, 1035-1043.

Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., McGeoch, D. J., Minson, A. C., Pellett, P. E., Roizman, B., Studdert, M. J. & Thiry, E. (2009). The order *Herpesvirales*. *Arch Virol* **154**, 171-177.

Davison, A. J., Kurobe, T., Gatherer, D., Cunningham, C., Korf, I., Fukuda, H., Hedrick, R. P. & Waltzek, T. B. (2013). Comparative genomics of carp herpesviruses. *J Virol* **87**, 2908-2922.

Davison, A. J., Trus, B. L., Cheng, N., Steven, A. C., Watson, M. S., Cunningham, C., Le Deuff, R. M. & Renault, T. (2005). A novel class of herpesvirus with bivalve hosts. *J Gen Virol* **86**, 41-53.

Dawkins, R. (1982). *The Extended Phenotype*. Oxford: Oxford Univ. Press.

de Waal, M., Abrams, J., Bennett, B., Figdor, C. & de Vries, J. (1991). Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* **174**, 1209 - 1220.

Deback, C., Boutolleau, D., Depienne, C., Luyt, C. E., Bonnafous, P., Gautheret-Dejean, A., Garrigue, I. & Agut, H. (2009). Utilization of microsatellite polymorphism for differentiating herpes simplex virus type 1 strains. *J Clin Microbiol* **47**, 533-540.

Dickson, K. A. & Graham, J. B. (2004). Evolution and consequences of endothermy in fishes. *Physiol Biochem Zool* **77**, 998-1018.

Dinarello, C. A. (1999). Cytokines as Endogenous Pyrogens. *J Infect Dis* **179**, 294-304.

Dinarello, C. A., Cannon, J. G., Wolff, S. M., Bernheim, H. A., Beutler, B., Cerami, A., Figari, I. S., Palladino, M. A., Jr. & O'Connor, J. V. (1986). Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. *J Exp Med* **163**, 1433-1450.

Dishon, A., Davidovich, M., Ilouze, M. & Kotler, M. (2007). Persistence of cyprinid herpesvirus 3 in infected cultured carp cells. *J Virol* **81**, 4828-4836.

Dishon, A., Perelberg, A., Bishara-Shieban, J., Ilouze, M., Davidovich, M., Werker, S. & Kotler, M. (2005). Detection of carp interstitial nephritis and gill necrosis virus in fish droppings. *Appl Environ Microbiol* **71**, 7285-7291.

Dixon, P. F., Joiner, C. L., Way, K., Reese, R. A., Jeney, G. & Jeney, Z. (2009). Comparison of the resistance of selected families of common carp, *Cyprinus carpio* L., to koi herpesvirus: preliminary study. *J Fish Dis* **32**, 1035-1039.

Dong, C., Li, X., Weng, S., Xie, S. & He, J. (2013). Emergence of fatal European genotype CyHV-3/KHV in mainland China. *Vet Microbiol* **162**, 239-244.

Dong, C., Weng, S., Li, W., Li, X., Yi, Y., Liang, Q. & He, J. (2011). Characterization of a new cell line from caudal fin of koi, *Cyprinus carpio koi*, and first isolation of cyprinid herpesvirus 3 in China. *Virus Res* **161**, 140-149.

Doszpoly, A., Benko, M., Bovo, G., Lapatra, S. E. & Harrach, B. (2011a). Comparative analysis of a conserved gene block from the genome of the members of the genus ictalurivirus. *Intervirology* **54**, 282-289.

Doszpoly, A., Kovacs, E. R., Bovo, G., LaPatra, S. E., Harrach, B. & Benko, M. (2008). Molecular confirmation of a new herpesvirus from catfish (*Ameiurus melas*) by testing the performance of a novel PCR method, designed to target the DNA polymerase gene of alloherpesviruses. *Arch Virol* **153**, 2123-2127.

Doszpoly, A., Papp, M., Deakne, P. P., Glavits, R., Ursu, K. & Dan, A. (2015). Molecular detection of a putatively novel cyprinid herpesvirus in sichel (*Pelecus cultratus*) during a mass mortality event in Hungary. *Arch Virol* **160**, 1279-1283.

Doszpoly, A., Somogyi, V., LaPatra, S. E. & Benko, M. (2011b). Partial genome characterization of acipenserid herpesvirus 2: taxonomical proposal for the demarcation of three subfamilies in *Alloherpesviridae*. *Arch Virol* **156**, 2291-2296.

Eide, K. E., Miller-Morgan, T., Heidel, J. R., Bildfell, R. J. & Jin, L. (2011a). Results of total DNA measurement in koi tissue by Koi Herpes Virus real-time PCR. *J Virol Methods* **172**, 81-84.

Eide, K. E., Miller-Morgan, T., Heidel, J. R., Kent, M. L., Bildfell, R. J., Lapatra, S., Watson, G. & Jin, L. (2011b). Investigation of koi herpesvirus latency in koi. *J Virol* **85**, 4954-4962.

El-Din, M. M. M. (2011). Histopathological studies in experimentally infected koi carp (*Cyprinus carpio koi*) with koi herpesvirus in Japan. *World Journal of Fish and Marine Science*, 252-259.

El-Matbouli, M., Saleh, M. & Soliman, H. (2007). Detection of cyprinid herpesvirus type 3 in goldfish cohabiting with CyHV-3-infected koi carp (*Cyprinus carpio koi*). *Vet Rec* **161**, 792-793.

El-Matbouli, M. & Soliman, H. (2011). Transmission of Cyprinid herpesvirus-3 (CyHV-3) from goldfish to naive common carp by cohabitation. *Res Vet Sci* **90**, 536-539.

Elliot, S. L., Blanford, S. & Thomas, M. B. (2002). Host-pathogen interactions in a varying environment: temperature, behavioural fever and fitness. *Proc Biol Sci* **269**, 1599-1607.

Ellis, A. E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. *Dev Comp Immunol* **25**, 827-839.

Engelsma, M. Y., Way, K., Dodge, M. J., Voorbergen-Laarman, M., Panzarin, V., Abbadi, M., El-Matbouli, M., Frank Skall, H., Kahns, S. & Stone, D. M. (2013). Detection of novel strains of cyprinid herpesvirus closely related to koi herpesvirus. *Dis Aquat Organ* **107**, 113-120.

Engering, A., Hogerwerf, L. & Slingenbergh, J. (2013). Pathogen-host-environment interplay and disease emergence. *Emerg Microbes Infect* **2**, e5.

Epperson, M. L., Lee, C. A. & Fremont, D. H. (2012). Subversion of cytokine networks by virally encoded decoy receptors. *Immunol Rev* **250**, 199-215.

Evans, S. S., Repasky, E. A. & Fisher, D. T. (2015). Fever and the thermal regulation of immunity: the immune system feels the heat. *Nat Rev Immunol* **15**, 335-349.

Fabian, M., Baumer, A. & Steinhagen, D. (2013). Do wild fish species contribute to the transmission of koi herpesvirus to carp in hatchery ponds? *J Fish Dis* **36**, 505-514.

FAO (2012a). Global Aquaculture Production 1950-2012.

FAO (2012b). Statistics and Information Service of the Fisheries and Aquaculture Department: Fishery and Aquaculture Statistics 2010. Rome: FAO.

FAO (2015). Common carp *Cyprinus carpio*. In *Aquaculture Feed and Fertilizer Resources Information System*.

Farmer, E. E. & Ryan, C. A. (1990). Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proc Natl Acad Sci U S A* **87**, 7713-7716.

Fawcett, D. W. (1956). Electron microscope observations on intracellular virus-like particles associated with the cells of the Lucke renal adenocarcinoma. *The Journal of biophysical and biochemical cytology* **2**, 725-741.

Fialho, R. F. & Schall, J. J. (1995). Thermal ecology of a malarial parasite and its insect vector: Consequences for the parasite's transmission success. *Journal of Animal Ecology* **64**, 553-562.

Fillatreau, S., Six, A., Magadan, S., Castro, R., Sunyer, J. O. & Boudinot, P. (2013). The astonishing diversity of Ig classes and B cell repertoires in teleost fish. *Front Immunol* **4**, 28.

Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M. & O'Garra, A. (1991). IL-10 inhibits cytokine production by activated macrophages. *J Immunol* **147**, 3815-3822.

Fisher, J. J. & Hajek, A. E. (2014). Thermoregulatory behavior and fungal infection of Anoplophora glabripennis (Coleoptera: Cerambycidae). *Environ Entomol* **43**, 384-392.

Florez-Duquet, M., Peloso, E. & Satinoff, E. (2001). Fever and behavioral thermoregulation in young and old rats. *Am J Physiol Regul Integr Comp Physiol* **280**, R1457-1461.

Fontenot, D. K. & Neiffer, D. L. (2004). Wound management in teleost fish: biology of the healing process, evaluation, and treatment. *Vet Clin North Am Exot Anim Pract* **7**, 57-86.

Forlenza, M., Magez, S., Scharsack, J. P., Westphal, A., Savelkoul, H. F. & Wiegertjes, G. F. (2009). Receptor-mediated and lectin-like activities of carp (*Cyprinus carpio*) TNF-alpha. *J Immunol* **183**, 5319-5332.

Fournier, G., Boutier, M., Stalin Raj, V., Mast, J., Parmentier, E., Vanderwalle, P., Peeters, D., Lieffrig, F., Farnir, F., Gillet, L. & Vanderplasschen, A. (2012). Feeding *Cyprinus carpio* with infectious materials mediates cyprinid herpesvirus 3 entry through infection of pharyngeal periodontal mucosa. *Vet Res* **43**, 6.

Fuchs, W., Fichtner, D., Bergmann, S. M. & Mettenleiter, T. C. (2011). Generation and characterization of koi herpesvirus recombinants lacking viral enzymes of nucleotide metabolism. *Arch Virol* **156**, 1059-1063.

Fuchs, W., Granzow, H., Dauber, M., Fichtner, D. & Mettenleiter, T. C. (2014). Identification of structural proteins of koi herpesvirus. *Arch Virol* **159**, 3257-3268.

Fujiki, K., Shin, D. H., Nakao, M. & Yano, T. (2000). Molecular cloning and expression analysis of carp (*Cyprinus carpio*) interleukin-1 beta, high affinity immunoglobulin E Fc receptor gamma subunit and serum amyloid A. *Fish Shellfish Immunol* **10**, 229-242.

Fujioka, H., Yamasaki, K., Furusawa, K., Tamura, K., Oguro, K., Kurihara, S., Seki, S., Oshima, S. & Imajoh, M. (2015). Prevalence and characteristics of Cyprinid herpesvirus 3 (CyHV-3) infection in common carp (*Cyprinus carpio* L.) inhabiting three rivers in Kochi Prefecture, Japan. *Vet Microbiol* **175**, 362-368.

Galic, M. A., Riazi, K. & Pittman, Q. J. (2012). Cytokines and brain excitability. *Front Neuroendocrinol* **33**, 116-125.

Garver, K. A., Al-Hussinee, L., Hawley, L. M., Schroeder, T., Edes, S., LePage, V., Contador, E., Russell, S., Lord, S., Stevenson, R. M., Souter, B., Wright, E. & Lumsden, J. S. (2010). Mass mortality associated with koi herpesvirus in wild common carp in Canada. *J Wildl Dis* **46**, 1242-1251.

Geiser, F., Drury, R. L., Körtner, G., Turbill, C., Pavey, C. R. & Brigham, R. (2004). Passive rewarming from torpor in mammals and birds: energetic, ecological and evolutionary implications. In *Life in the cold:evolution, adaptation and application*, pp. 51-62. Edited by B. M. Barnes & H. V. Carey. Institute of Artic Biology, University of Alaska, Fairbam: Biological paper of the University of Alaska.

Gilad, O., Yun, S., Adkison, M. A., Way, K., Willits, N. H., Bercovier, H. & Hedrick, R. P. (2003). Molecular comparison of isolates of an emerging fish pathogen, koi herpesvirus, and the effect of water temperature on mortality of experimentally infected koi. *J Gen Virol* **84**, 2661-2667.

Gilad, O., Yun, S., Andree, K. B., Adkison, M. A., Zlotkin, A., Bercovier, H., Eldar, A. & Hedrick, R. P. (2002). Initial characteristics of koi herpesvirus and development of a polymerase chain reaction assay to detect the virus in koi, *Cyprinus carpio koi*. *Dis Aquat Organ* **48**, 101-108.

Gilad, O., Yun, S., Zagmutt-Vergara, F. J., Leutenegger, C. M., Bercovier, H. & Hedrick, R. P. (2004). Concentrations of a Koi herpesvirus (KHV) in tissues of experimentally infected *Cyprinus carpio koi* as assessed by real-time TaqMan PCR. *Dis Aquat Organ* **60**, 179-187.

Gomez, D., Sunyer, J. O. & Salinas, I. (2013). The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. *Fish Shellfish Immunol* **35**, 1729-1739.

Gomez, D. K., Joh, S. J., Jang, H., Shin, S. P., Choresca, C. H., Han, J. E., Kim, J. H., Jun, J. W. & Park, S. C. (2011). Detection of koi herpesvirus (KHV) from koi (*Cyprinus carpio koi*) broodstock in South Korea. *Aquaculture* **311**, 42-47.

Gonzalez, S. F., Buchmann, K. & Nielsen, M. E. (2007a). Complement expression in common carp (*Cyprinus carpio* L.) during infection with Ichthyophthirius multifiliis. *Dev Comp Immunol* **31**, 576-586.

Gonzalez, S. F., Chatziandreou, N., Nielsen, M. E., Li, W., Rogers, J., Taylor, R., Santos, Y. & Cossins, A. (2007b). Cutaneous immune responses in the common carp detected using transcript analysis. *Mol Immunol* **44**, 1664-1679.

Goodwin, A. E., Sadler, J., Merry, G. E. & Marecaux, E. N. (2009). Herpesviral haematopoietic necrosis virus (CyHV-2) infection: case studies from commercial goldfish farms. *J Fish Dis* **32**, 271-278.

Gotesman, M., Kattlun, J., Bergmann, S. M. & El-Matbouli, M. (2013). CyHV-3: the third cyprinid herpesvirus. *Dis Aquat Organ* **105**, 163-174.

Gotesman, M., Soliman, H., Besch, R. & El-Matbouli, M. (2014). *In vitro* inhibition of Cyprinid herpesvirus-3 replication by RNAi. *J Virol Methods* **206**, 63-66.

Goulson, D. (1997). Wipfelkrankheit : modification of host behaviour during baculoviral infection. *Oecologia* **109**, 219-228.

Grans, A., Rosengren, M., Niklasson, L. & Axelsson, M. (2012). Behavioural fever boosts the inflammatory response in rainbow trout *Oncorhynchus mykiss*. *J Fish Biol* **81**, 1111-1117.

Gravell, M., Granoff, A. & Darlington, R. W. (1968). Viruses and renal carcinoma of *Rana pipiens*. VII. Propagation of a herpes-type frog virus. *Virology* **36**, 467-475.

Gray, W. L., Mullis, L., LaPatra, S. E., Groff, J. M. & Goodwin, A. (2002). Detection of koi herpesvirus DNA in tissues of infected fish. *J Fish Dis* **25**, 171-178.

Grimmett, S. G., Warg, J. V., Getchell, R. G., Johnson, D. J. & Bowser, P. R. (2006). An unusual koi herpesvirus associated with a mortality event of common carp *Cyprinus carpio* in New York State, USA. *J Wildl Dis* **42**, 658-662.

Gunimaladevi, I., Kono, T., Venugopal, M. N. & Sakai, M. (2004). Detection of koi herpesvirus in common carp, *Cyprinus carpio* L., by loop-mediated isothermal amplification. *J Fish Dis* **27**, 583-589.

Haenen, O. L. M., Dijkstra, S. G., Van Tulden, P. W., Davidse, A., Van Nieuwstadt, A. P., Wagenaar, F. & Wellenberg, G. J. (2002). Herpesvirus anguillae (HVA) isolations from disease outbreaks in cultured European eel, *Anguilla anguilla* in the Netherlands since 1996. *B Eur Assoc Fish Pat* **22**, 247-257.

Haenen, O. L. M., Way, K., Bergmann, S. M. & Ariel, E. (2004). The emergence of koi herpesvirus and its significance to European aquaculture. *B Eur Assoc Fish Pat* **24**, 293-307.

Han, J. E., Kim, J. H., Renault, T., Jr, C. C., Shin, S. P., Jun, J. W. & Park, S. C. (2013). Identifying the viral genes encoding envelope glycoproteins for differentiation of Cyprinid herpesvirus 3 isolates. *Viruses* **5**, 568-576.

Hansen, J. D., Landis, E. D. & Phillips, R. B. (2005). Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *Proc Natl Acad Sci U S A* **102**, 6919-6924.

Hanson, L., Dishon, A. & Kotler, M. (2011). Herpesviruses that infect fish. *Viruses* **3**, 2160-2191.

Hara, H., Aikawa, H., Usui, K. & Nakanishi, T. (2006). Outbreaks of Koi Herpesvirus Disease in Rivers of Kanagawa Prefecture. *Fish Pathol* **41**, 81-83.

Haramoto, E., Kitajima, M., Katayama, H., Ito, T. & Ohgaki, S. (2009). Development of virus concentration methods for detection of koi herpesvirus in water. *J Fish Dis* **32**, 297-300.

Haramoto, E., Kitajima, M., Katayama, H. & Ohgaki, S. (2007). Detection of koi herpesvirus DNA in river water in Japan. *J Fish Dis* **30**, 59-61.

Harmache, A., LeBerre, M., Droineau, S., Giovannini, M. & Bremont, M. (2006). Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for Novirhabdovirus. *J Virol* **80**, 3655-3659.

Hedrick, R. P., Gilad, O., Yun, S., Spangenberg, J., Marty, R., Nordhausen, M., Kebus, M., Bercovier, H. & Eldar, A. (2000). A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. *J Aquat Anim Health* **12**, 44-55.

Hedrick, R. P., Waltzek, T. B. & McDowell, T. S. (2006). Susceptibility of Koi Carp, Common Carp, Goldfish, and Goldfish × Common Carp Hybrids to Cyprinid Herpesvirus-2 and Herpesvirus-3. *J Aquat Anim Health* **18**, 26-34.

Hemachudha, T., Laothamatas, J. & Rupprecht, C. E. (2002). Human rabies: a disease of complex neuropathogenetic mechanisms and diagnostic challenges. *Lancet Neurol* **1**, 101-109.

Hong, S., Li, R., Xu, Q., Secombes, C. J. & Wang, T. (2013). Two types of TNF-alpha exist in teleost fish: phylogeny, expression, and bioactivity analysis of type-II TNF-alpha3 in rainbow trout *Oncorhynchus mykiss*. *J Immunol* **191**, 5959-5972.

Hong, S., Zou, J., Crampe, M., Peddie, S., Scapigliati, G., Bols, N., Cunningham, C. & Secombes, C. J. (2001). The production and bioactivity of rainbow trout (*Oncorhynchus mykiss*) recombinant IL-1 beta. *Vet Immunol Immunopathol* **81**, 1-14.

Honjo, M. N., Minamoto, T. & Kawabata, Z. (2012). Reservoirs of Cyprinid herpesvirus 3 (CyHV-3) DNA in sediments of natural lakes and ponds. *Vet Microbiol* **155**, 183-190.

Honjo, M. N., Minamoto, T., Matsui, K., Uchii, K., Yamanaka, H., Suzuki, A. A., Kohmatsu, Y., Iida, T. & Kawabata, Z. (2010). Quantification of cyprinid herpesvirus 3 in environmental water by using an external standard virus. *Appl Environ Microbiol* **76**, 161-168.

Hoover, K., Grove, M., Gardner, M., Hughes, D. P., McNeil, J. & Slavicek, J. (2011). A gene for an extended phenotype. *Science* **333**, 1401.

Horst, D., Ressing, M. E. & Wiertz, E. J. (2011). Exploiting human herpesvirus immune evasion for therapeutic gain: potential and pitfalls. *Immunol Cell Biol* **89**, 359-366.

Hoseinifar, S. H., Mirvaghefi, A., Amoozegar, M. A., Sharifian, M. & Esteban, M. A. (2015). Modulation of innate immune response, mucosal parameters and disease resistance in rainbow trout (*Oncorhynchus mykiss*) upon synbiotic feeding. *Fish Shellfish Immunol* **45**, 27-32.

Hunt, V. L. & Charnley, A. K. (2011). The inhibitory effect of the fungal toxin, destruxin A, on behavioural fever in the desert locust. *J Insect Physiol* **57**, 1341-1346.

Hutchison, V. H. & Erskine, D. J. (1981). Thermal Selection and Prostaglandin E1 Fever in the Salamander Necturus maculosus. *Herpetologica* **37**, 195-198.

Hutoran, M., Ronen, A., Perelberg, A., Ilouze, M., Dishon, A., Bejerano, I., Chen, N. & Kotler, M. (2005). Description of an as yet unclassified DNA virus from diseased *Cyprinus carpio* species. *J Virol* **79**, 1983-1991.

Huttenhuis, H. B. (2005). Ontogeny of the common carp (*Cyprinus carpio* L.) immune system, p. 173: Wageningen University.

Huttenhuis, H. B., Grou, C. P., Taverne-Thiele, A. J., Taverne, N. & Rombout, J. H. (2006a). Carp (*Cyprinus carpio* L.) innate immune factors are present before hatching. *Fish Shellfish Immunol* **20**, 586-596.

Huttenhuis, H. B., Romano, N., Van Oosterhoud, C. N., Taverne-Thiele, A. J., Mastrolia, L., Van Muiswinkel, W. B. & Rombout, J. H. (2006b). The ontogeny of mucosal immune cells in common carp (*Cyprinus carpio* L.). *Anat Embryol (Berl)* **211**, 19-29.

Ilouze, M., Davidovich, M., Diamant, A., Kotler, M. & Dishon, A. (2010). The outbreak of carp disease caused by CyHV-3 as a model for new emerging viral diseases in aquaculture: a review. *Ecological Research* **26**, 885-892.

Ilouze, M., Dishon, A., Kahan, T. & Kotler, M. (2006a). Cyprinid herpes virus-3 (CyHV-3) bears genes of genetically distant large DNA viruses. *FEBS Lett* **580**, 4473-4478.

Ilouze, M., Dishon, A. & Kotler, M. (2006b). Characterization of a novel virus causing a lethal disease in carp and koi. *Microbiol Mol Biol Rev* **70**, 147-156.

Ilouze, M., Dishon, A. & Kotler, M. (2012a). Coordinated and sequential transcription of the cyprinid herpesvirus-3 annotated genes. *Virus Res* **169**, 98-106.

Ilouze, M., Dishon, A. & Kotler, M. (2012b). Down-regulation of the cyprinid herpesvirus-3 annotated genes in cultured cells maintained at restrictive high temperature. *Virus Res* **169**, 289-295.

Imajoh, M., Fujioka, H., Furusawa, K., Tamura, K., Yamasaki, K., Kurihara, S., Yamane, J., Kawai, K. & Oshima, S. (2015). Establishment of a new cell line susceptible to Cyprinid herpesvirus 3 (CyHV-3) and possible latency of CyHV-3 by temperature shift in the cells. *J Fish Dis* **38**, 507-514.

Imeri, L. & Opp, M. R. (2009). How (and why) the immune system makes us sleep. *Nat Rev Neurosci* **10**, 199-210.

Irwin, M. R. & Cole, S. W. (2011). Reciprocal regulation of the neural and innate immune systems. *Nat Rev Immunol* **11**, 625-632.

Ishioka, T., Yoshizumi, M., Izumi, S., Suzuki, K., Suzuki, H., Kozawa, K., Arai, M., Nobusawa, K., Morita, Y., Kato, M., Hoshino, T., Iida, T., Kosuge, K. & Kimura, H. (2005). Detection and sequence analysis of DNA polymerase and major envelope protein genes in koi herpesviruses derived from *Cyprinus carpio* in Gunma prefecture, Japan. *Vet Microbiol* **110**, 27-33.

Ito, T., Kurita, J. & Yuasa, K. (2014). Differences in the susceptibility of Japanese indigenous and domesticated Eurasian common carp (*Cyprinus carpio*), identified by mitochondrial DNA typing, to cyprinid herpesvirus 3 (CyHV-3). *Vet Microbiol* **171**, 31-40.

Ito, T. & Maeno, Y. (2014). Effects of experimentally induced infections of goldfish *Carassius auratus* with cyprinid herpesvirus 2 (CyHV-2) at various water temperatures. *Dis Aquat Organ* **110**, 193-200.

Ito, T., Sano, M., Kurita, J., Yuasa, K. & Iida, T. (2007). Carp larvae are not susceptible to Koi Herpesvirus. *Fish Pathol* **42**, 107-109.

Janeway, C. A., Travers, P., Walport, M. & Shlomchik, M. J. (2001). Pathogens have evolved various means of evading or subverting normal host defenses. In *Immunobiology: The Immune System in Health and Disease 5th edition*. Edited by G. Science.

Jimenez-Clavero, M. A. (2012). Animal viral diseases and global change: bluetongue and West Nile fever as paradigms. *Frontiers in genetics* **3**, 105.

Jones, K. D., Aoki, Y., Chang, Y., Moore, P. S., Yarchoan, R. & Tosato, G. (1999). *Involvement of Interleukin-10 (IL-10) and Viral IL-6 in the Spontaneous Growth of Kaposi's Sarcoma Herpesvirus-Associated Infected Primary Effusion Lymphoma Cells*.

Kamita, S. G., Nagasaka, K., Chua, J. W., Shimada, T., Mita, K., Kobayashi, M., Maeda, S. & Hammock, B. D. (2005). A baculovirus-encoded protein tyrosine phosphatase gene induces enhanced locomotory activity in a lepidopteran host. *Proc Natl Acad Sci U S A* **102**, 2584-2589.

Katsuma, S., Koyano, Y., Kang, W., Kokusho, R., Kamita, S. G. & Shimada, T. (2012). The baculovirus uses a captured host phosphatase to induce enhanced locomotory activity in host caterpillars. *PLoS Pathog* **8**, e1002644.

Kattlun, J., Menanteau-Ledouble, S. & El-Matbouli, M. (2014). Non-structural protein pORF 12 of cyprinid herpesvirus 3 is recognized by the immune system of the common carp *Cyprinus carpio*. *Dis Aquat Organ* **111**, 269-273.

Kelley, G. O., Waltzek, T. B., McDowell, T. S., Yun, S. C., LaPatra, S. E. & Hedrick, R. P. (2005). Genetic Relationships among Herpes-Like Viruses Isolated from Sturgeon. *J Aquat Anim Health* **17**, 297-303.

Kelley, L. A. & Sternberg, M. J. E. (2009). Protein structure prediction on the web: a case study using the Phyre server. *Nat Protocols* **4**, 363-371.

Kempter, J. & Bergmann, S. M. (2007). Detection of koi herpesvirus (KHV) genome in wild and farmed fish from Northern Poland. *Aquaculture* **272**, S275-S275.

Kempter, J., Kielpinski, M., Panicz, R., Sadowski, J., Myslowski, B. & Bergmann, S. M. (2012). Horizontal transmission of koi herpes virus (KHV) from potential vector species to common carp. *B Eur Assoc Fish Pat* **32**, 212-219.

Kempter, J., Sadowski, J., Schütze, H., Fischer, U., Dauber, M., Fichtner, D., Panicz, R. & Bergmann, S. M. (2009). Koi Herpes Virus: Do Acipenserid Restitution Programs Pose a Threat to Carp Farms in the Disease-Free Zones? *Acta Ichthyologica Et Piscatoria* **39**, 119-126.

Kettelhut, I. C. & Goldberg, A. L. (1988). Tumor necrosis factor can induce fever in rats without activating protein breakdown in muscle or lipolysis in adipose tissue. *J Clin Invest* **81**, 1384-1389.

Kielpinski, M., Kempter, J., Panicz, R., Sadowski, J., Schütze, H., Ohlemeyer, S. & Bergmann, S. M. (2010). Detection of KHV in Freshwater Mussels and Crustaceans from Ponds with KHV History in Common Carp (*Cyprinus carpio*). *Isr J Aquacult-Bamid* **62**, 28-37.

Kim, H. J. & Kwon, S. R. (2013). Evidence for two koi herpesvirus (KHV) genotypes in South Korea. *Dis Aquat Organ* **104**, 197-202.

Kluger, M. J. (1977). Fever in the frog *Hyla Cinerea*. *J Therm Biol* **2**, 79-81.

Kluger, M. J. (1986). Is fever beneficial? *Yale J Biol Med* **59**, 89-95.

Kluger, M. J., Ringler, D. H. & Anver, M. R. (1975). Fever and survival. *Science* **188**, 166-168.

Kongchum, P., Sandel, E., Lutzky, S., Hallerman, E. M., Hulata, G., David, L. & Palti, Y. (2011). Association between IL-10a single nucleotide polymorphisms and resistance to cyprinid herpesvirus-3 infection in common carp (*Cyprinus carpio*). *Aquaculture* **315**, 417-421.

Kucuktas, H. & Brady, Y. J. (1999). Molecular biology of channel catfish virus. *Aquaculture* **172**, 147-161.

Kurita, J., Yuasa, K., Ito, T., Sano, M., Hedrick, R. P., Engelsma, M. Y., Haenen, O. L. M., Sunarto, A., Kholidin, E. B., Chou, H. Y., Tung, M. C., de la Pena, L., Lio-Po, G., Tu, C., Way, K. & Iida, T. (2009). Molecular Epidemiology of Koi Herpesvirus. *Fish Pathol* **44**, 59-66.

Kurobe, T., Kelley, G. O., Waltzek, T. B. & Hedrick, R. P. (2008). Revised phylogenetic relationships among herpesviruses isolated from sturgeons. *J Aquat Anim Health* **20**, 96-102.

Langevin, C., Aleksejeva, E., Passoni, G., Palha, N., Levraud, J. P. & Boudinot, P. (2013). The antiviral innate immune response in fish: evolution and conservation of the IFN system. *J Mol Biol* **425**, 4904-4920.

Laothamatas, J., Hemachudha, T., Mitrabhakdi, E., Wannakrairot, P. & Tulayadaechanont, S. (2003). MR imaging in human rabies. *AJNR Am J Neuroradiol* **24**, 1102-1109.

Laothamatas, J., Wacharapluesadee, S., Lumlertdacha, B., Ampawong, S., Tepsumethanon, V., Shuangshoti, S., Phumesin, P., Asavaphatiboon, S., Worapruekjaru, L., Avihingsanon, Y., Israsena, N., Lafon, M., Wilde, H. & Hemachudha, T. (2008). Furious and paralytic rabies of canine origin: neuroimaging with virological and cytokine studies. *J Neurovirol* **14**, 119-129.

Laurila, S. & Holopainen, I. J. (1990). Features of embryonic and larval development of crucian carp, *Carassius carassius* (L.) with a note on species identification. *Annales Zoologici Fennici* **27**, 361-367.

Le Morvan, C., Troutaud, D. & Deschaux, P. (1998). Differential effects of temperature on specific and nonspecific immune defences in fish. *J Exp Biol* **201**, 165-168.

Lee, N. S., Jung, S. H., Park, J. W. & Do, J. W. (2012). *In situ* Hybridization Detection of Koi Herpesvirus in Paraffin-embedded Tissues of Common Carp *Cyprinus carpio* Collected in 1998 in Korea. *Fish Pathol* **47**, 100-103.

Lefevre, T. & Thomas, F. (2008). Behind the scene, something else is pulling the strings: emphasizing parasitic manipulation in vector-borne diseases. *Infect Genet Evol* **8**, 504-519.

Li, J., Barreda, D. R., Zhang, Y. A., Boshra, H., Gelman, A. E., Lapatra, S., Tort, L. & Sunyer, J. O. (2006). B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nat Immunol* **7**, 1116-1124.

Li, M. & Leatherland, J. F. (2012). The interaction between maternal stress and the ontogeny of the innate immune system during teleost embryogenesis: implications for aquaculture practice. *J Fish Biol* **81**, 1793-1814.

Li, W., Lee, X., Weng, S., He, J. & Dong, C. (2015). Whole-genome sequence of a novel Chinese cyprinid herpesvirus 3 isolate reveals the existence of a distinct European genotype in East Asia. *Vet Microbiol* **175**, 185-194.

Lin, S. L., Cheng, Y. H., Wen, C. M. & Chen, S. N. (2013). Characterization of a novel cell line from the caudal fin of koi carp *Cyprinus carpio*. *J Fish Biol* **82**, 1888-1903.

Lio-Po, G. D. (2011). Recent developments in the study and surveillance of koi herpesvirus (KHV) in Asia. In *Diseases in Asian Aquaculture*, pp. 13-28. Edited by M. G. Bondad-Reantaso, J. B. Jones, F. Corsin & T. Aoki. Taipei, Taiwan: Fish Health Section, Asian Fisheries Society.

Liu, Z., Ke, H., Ma, Y., Hao, L., Feng, G., Ma, J., Liang, Z. & Li, Y. (2014). Oral passive immunization of carp *Cyprinus carpio* with anti-CyHV-3 chicken egg yolk immunoglobulin (IgY). *Fish Pathol* **49**, 113-120.

Lopez-Castejon, G. & Brough, D. (2011). Understanding the mechanism of IL-1beta secretion. *Cytokine Growth Factor Rev* **22**, 189-195.

Loret, S., Guay, G. & Lippe, R. (2008). Comprehensive characterization of extracellular herpes simplex virus type 1 virions. *J Virol* **82**, 8605-8618.

Lugo-Villarino, G., Balla, K. M., Stachura, D. L., Banuelos, K., Werneck, M. B. & Traver, D. (2010). Identification of dendritic antigen-presenting cells in the zebrafish. *Proc Natl Acad Sci U S A* **107**, 15850-15855.

Mabika, M. & Laburn, H. (1999). The role of tumour necrosis factor-alpha (TNF-alpha) in fever and the acute phase reaction in rabbits. *Pflugers Arch* **438**, 218-223.

Mabuchi, K., Senou, H., Suzuki, T. & Nishida, M. (2005). Discovery of an ancient lineage of *Cyprinus carpio* from Lake Biwa, central Japan, based on mtDNA sequence data, with reference to possible multiple origins of koi. *J Fish Biol* **66**, 1516-1528.

Magnadottir, B. (2006). Innate immunity of fish (overview). *Fish Shellfish Immunol* **20**, 137-151.

Magnadottir, B., Lange, S., Gudmundsdottir, S., Bogwald, J. & Dalmo, R. A. (2005). Ontogeny of humoral immune parameters in fish. *Fish Shellfish Immunol* **19**, 429-439.

Marcos-Lopez, M., Waltzek, T. B., Hedrick, R. P., Baxa, D. V., Garber, A. F., Liston, R., Johnsen, E., Forward, B. S., Backman, S. & Ferguson, H. W. (2012). Characterization of a novel alloherpesvirus from Atlantic cod (*Gadus morhua*). *J Vet Diagn Invest* **24**, 65-73.

Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M. C., Farrer, R. A., Schmidt, B. R., Tobler, U., Goka, K., Lips, K. R., Muletz, C., Zamudio, K. R., Bosch, J., Lotters, S., Wombwell, E., Garner, T. W., Cunningham, A. A., Spitzen-van der Sluijs, A., Salvidio, S., Ducatelle, R., Nishikawa, K., Nguyen, T. T., Kolby, J. E., Van Bocxlaer, I., Bossuyt, F. & Pasmans, F. (2014). Wildlife disease. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science* **346**, 630-631.

McCleary, S., Ruane, N. M., Cheslett, D., Hickey, C., Rodger, H. D., Geoghegan, F. & Henshilwood, K. (2011). Detection of koi herpesvirus (KHV) in koi carp (*Cyprinus carpio* L.) imported into Ireland. *B Eur Assoc Fish Pat* **31**, 124-128.

McColl, K. A., Cooke, B. D. & Sunarto, A. (2014). Viral biocontrol of invasive vertebrates: Lessons from the past applied to cyprinid herpesvirus-3 and carp (*Cyprinus carpio*) control in Australia. *Biol Control* **72**, 109-117.

McDermott, C. & Palmeiro, B. (2013). Selected emerging infectious diseases of ornamental fish. *Vet Clin North Am Exot Anim Pract* **16**, 261-282.

McGeoch, D. J., Dolan, A. & Ralph, A. C. (2000). Toward a comprehensive phylogeny for mammalian and avian herpesviruses. *J Virol* **74**, 10401-10406.

McGeoch, D. J. & Gatherer, D. (2005). Integrating reptilian herpesviruses into the family *Herpesviridae*. *J Virol* **79**, 725-731.

McGeoch, D. J., Rixon, F. J. & Davison, A. J. (2006). Topics in herpesvirus genomics and evolution. *Virus Res* **117**, 90-104.

McKinnell, R. G. & Tarin, D. (1984). Temperature-dependent metastasis of the Lucke renal carcinoma and its significance for studies on mechanisms of metastasis. *Cancer Metastasis Rev* **3**, 373-386.

Medici, M. A., Sciortino, M. T., Perri, D., Amici, C., Avitabile, E., Ciotti, M., Balestrieri, E., De Smaele, E., Franzoso, G. & Mastino, A. (2003). Protection by herpes simplex virus glycoprotein D against Fas-mediated apoptosis: role of nuclear factor kappaB. *J Biol Chem* **278**, 36059-36067.

Meeusen, E. N., Walker, J., Peters, A., Pastoret, P. P. & Jungersen, G. (2007). Current status of veterinary vaccines. *Clin Microbiol Rev* **20**, 489-510.

Mettenleiter, T. C. (2004). Budding events in herpesvirus morphogenesis. *Virus Res* **106**, 167-180.

Mettenleiter, T. C., Klupp, B. G. & Granzow, H. (2009). Herpesvirus assembly: an update. *Virus Res* **143**, 222-234.

Michel, B., Fournier, G., Lieffrig, F., Costes, B. & Vanderplasschen, A. (2010a). Cyprinid herpesvirus 3. *Emerg Infect Dis* **16**, 1835-1843.

Michel, B., Leroy, B., Stalin Raj, V., Lieffrig, F., Mast, J., Wattiez, R., Vanderplasschen, A. F. & Costes, B. (2010b). The genome of cyprinid herpesvirus 3 encodes 40 proteins incorporated in mature virions. *J Gen Virol* **91**, 452-462.

Midtlyng, P. J., Storset, A., Michel, C., Slierendrecht, W. J. & Okamoto, N. (2002). Breeding for disease resistance in fish. *B Eur Assoc Fish Pat* **22**, 166-172.

Miest, J. J., Adamek, M., Pionnier, N., Harris, S., Matras, M., Rakus, K. L., Irnazarow, I., Steinhagen, D. & Hoole, D. (2015). Differential effects of alloherpesvirus CyHV-3 and rhabdovirus SVCV on apoptosis in fish cells. *Vet Microbiol* **176**, 19-31.

Minamoto, T., Honjo, M. N. & Kawabata, Z. (2009a). Seasonal distribution of cyprinid herpesvirus 3 in Lake Biwa, Japan. *Appl Environ Microbiol* **75**, 6900-6904.

Minamoto, T., Honjo, M. N., Uchii, K., Yamanaka, H., Suzuki, A. A., Kohmatsu, Y., Iida, T. & Kawabata, Z. (2009b). Detection of cyprinid herpesvirus 3 DNA in river water during and after an outbreak. *Vet Microbiol* **135**, 261-266.

Minamoto, T., Honjo, M. N., Yamanaka, H., Tanaka, N., Itayama, T. & Kawabata, Z. (2011). Detection of cyprinid herpesvirus-3 DNA in lake plankton. *Res Vet Sci* **90**, 530-532.

Minamoto, T., Honjo, M. N., Yamanaka, H., Uchii, K. & Kawabata, Z. (2012). Nationwide Cyprinid herpesvirus 3 contamination in natural rivers of Japan. *Res Vet Sci* **93**, 508-514.

Miwa, S., Ito, T. & Sano, M. (2007). Morphogenesis of koi herpesvirus observed by electron microscopy. *J Fish Dis* **30**, 715-722.

Miwa, S., Kiryu, I., Yuasa, K., Ito, T. & Kaneko, T. (2014). Pathogenesis of acute and chronic diseases caused by cyprinid herpesvirus-3. *J Fish Dis*.

Miyazaki, T., Kuzuya, Y., Yasumoto, S., Yasuda, M. & Kobayashi, T. (2008). Histopathological and ultrastructural features of Koi herpesvirus (KHV)-infected carp *Cyprinus carpio*, and the morphology and morphogenesis of KHV. *Dis Aquat Organ* **80**, 1-11.

Miyazaki, T., Yasumoto, S., Kuzuya, Y. & Yoshimura, T. (2005). A Primary Study on Oral Vaccination with Liposomes Entrapping Koi Herpesvirus (KHV) Antigens Against KHV Infection in Carp. In *Diseases in Asian Aquaculture*, pp. 99-104. Colombo, Sri Lanka.

Monaghan, S. J., Thompson, K. D., Adams, A. & Bergmann, S. M. (2015a). Sensitivity of seven PCRs for early detection of koi herpesvirus in experimentally infected carp, *Cyprinus carpio* L., by lethal and non-lethal sampling methods. *J Fish Dis* **38**, 303-319.

Monaghan, S. J., Thompson, K. D., Adams, A., Kempter, J. & Bergmann, S. M. (2015b). Examination of the early infection stages of koi herpesvirus (KHV) in experimentally infected carp, *Cyprinus carpio* L. using *in situ* hybridization. *J Fish Dis* **38**, 477-489.

Morrison, T. E., Mauser, A., Klingelhutz, A. & Kenney, S. C. (2004). Epstein-Barr virus immediate-early protein BZLF1 inhibits tumor necrosis factor alpha-induced signaling and apoptosis by downregulating tumor necrosis factor receptor 1. *J Virol* **78**, 544-549.

Mulero, I., García-Ayala, A., Meseguer, J. & Mulero, V. (2007). Maternal transfer of immunity and ontogeny of autologous immunocompetence of fish: A minireview. *Aquaculture* **268**, 244-250.

Musa, N., Leong, N. K. & Sunarto, A. (2005). Koi herpesvirus (KHV) – an emerging pathogen in koi. In *Colloquium on Viruses of Veterinary and Public Health Importance*, pp. 146–147. Bangi, Malaysia.

Myhre, K., Cabanac, M. & Myhre, G. (1977). Fever and behavioural temperature regulation in the frog Rana esculenta. *Acta Physiol Scand* **101**, 219-229.

Nakamura, H., Seto, Y., Motoyoshi, S., Kadokawa, T. & Sunahara, N. (1988). Recombinant human tumor necrosis factor causes long-lasting and prostaglandin-mediated fever, with little tolerance, in rabbits. *J Pharmacol Exp Ther* **245**, 336-341.

Nelson, D. O. & Prosser, C. L. (1979). Effect of preoptic lesions on behavioral thermoregulation of green sunfish,*Lepomis cyanellus*, and of goldfish,*Carassius auratus*. *J Comp Physiol* **129**, 193-197.

Netea, M. G., Kullberg, B. J. & Van der Meer, J. W. M. (2000). Circulating cytokines as mediators of fever. *Clin Infect Dis* **31**, 178-184.

Neukirch, M., Böttcher, K. & Bunnajrakul, S. (1999). Isolation of a virus from koi with altered gills. *B Eur Assoc Fish Pat* **19**, 221-224.

Neukirch, M. & Kunz, U. (2001). Isolation and preliminary characterization of several viruses from koi (*Cyprinus carpio*) suffering gill necrosis and mortality. *B Eur Assoc Fish Pat* **21**, 125-135.

O'Connor, M. R., Farver, T. B., Malm, K. V., Yun, S. C., Marty, G. D., Salonius, K., Dishon, A. & Weber, E. P., 3rd (2014). Protective immunity of a modified-live cyprinid herpesvirus 3 vaccine in koi (*Cyprinus carpio koi*) 13 months after vaccination. *Am J Vet Res* **75**, 905-911.

Ødegård, J., Olesena, I., Dixonb, P., Jeneyc, Z., Nielsena, H.-M., Wayb, K., Joinerb, C., Jeneyc, G., Ardóc, L., Rónyaic, A. & Gjerdea, B. (2010). Genetic analysis of common carp (*Cyprinus carpio*) strains. II: Resistance to koi herpesvirus and *Aeromonas hydrophila* and their relationship with pond survival *Aquaculture* **304**, 7-13.

OIE (2012). Chapter 2.3.6. Koi Herpesvirus Disease. In *Manual of Diagnostic Tests for Aquatic Animals 2012*, pp. 328-344.

OIE (2015). Infectious salmon anaemia virus (HPR-deleted or HPR0 genotypes) (Infection with), Norway. In *WAHID interface: Weekly Disease information*.

Ouyang, P., Rakus, K., Boutier, M., Reschner, A., Leroy, B., Ronsmans, M., Fournier, G., Scohy, S., Costes, B., Wattiez, R. & Vanderplasschen, A. (2013). The IL-10 homologue encoded by cyprinid herpesvirus 3 is essential neither for viral replication *in vitro* nor for virulence *in vivo*. *Vet Res* **44**, 53.

Ouyang, P., Rakus, K., van Beurden, S. J., Westphal, A. H., Davison, A. J., Gatherer, D. & Vanderplasschen, A. F. (2014). IL-10 encoded by viruses: a remarkable example of independent acquisition of a cellular gene by viruses and its subsequent evolution in the viral genome. *J Gen Virol* **95**, 245-262.

Palaksha, K. J., Shin, G. W., Kim, Y. R. & Jung, T. S. (2008). Evaluation of non-specific immune components from the skin mucus of olive flounder (Paralichthys olivaceus). *Fish Shellfish Immunol* **24**, 479-488.

Pellett, P. E., Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., Lacoste, V., Minson, A. C., Nicholas, J., Roizman, B., Studdert, M. J. & Wang, F. (2011a). *Alloherpesviridae*. In *Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses* pp. 108-110. Edited by A. M. Q. King, E. Lefkowitz, M. J. Adams & E. B. Carstens: Elsevier Academic Press, London, United Kingdom.

Pellett, P. E., Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., Lacoste, V., Minson, A. C., Nicholas, J., Roizman, B., Studdert, M. J. & Wang, F. (2011b). *Herpesvirales*. In *Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses* pp. 99-107. Edited by A. M. Q. King, E. Lefkowitz, M. J. Adams & E. B. Carstens: Elsevier Academic Press, London, United Kingdom.

Pellett, P. E., Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., Lacoste, V., Minson, A. C., Nicholas, J., Roizman, B., Studdert, M. J. & Wang, F. (2011c). *Herpesviridae*. In *Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses* pp. 111-122. Edited by A. M. Q. King, E. Lefkowitz, M. J. Adams & E. B. Carstens: Elsevier Academic Press, London, United Kingdom.

Pellett, P. E., Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., Lacoste, V., Minson, A. C., Nicholas, J., Roizman, B., Studdert, M. J. & Wang, F. (2011d). *Malacoherpesviridae*. In *Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses* p. 123. Edited by A. M. Q. King, E. Lefkowitz, M. J. Adams & E. B. Carstens: Elsevier Academic Press, London, United Kingdom.

Perelberg, A., Ilouze, M., Kotler, M. & Steinitz, M. (2008). Antibody response and resistance of *Cyprinus carpio* immunized with cyprinid herpes virus 3 (CyHV-3). *Vaccine* **26**, 3750-3756.

Perelberg, A., Ronen, A., Hutoran, M., Smith, Y. & Kotler, M. (2005). Protection of cultured *Cyprinus carpio* against a lethal viral disease by an attenuated virus vaccine. *Vaccine* **23**, 3396-3403.

Perelberg, A., Smirnov, M., Hutoran, M., Diamant, A., Bejerano, Y. & Kotler, M. (2003). Epidemiological description of a new viral disease afflicting cultured *Cyprinus carpio* in Israel. *Isr J Aquacult-Bamid* **55**, 5-12.

Piackova, V., Flajshans, M., Pokorova, D., Reschova, S., Gela, D., Cizek, A. & Vesely, T. (2013). Sensitivity of common carp, *Cyprinus carpio* L., strains and crossbreeds reared in the Czech Republic to infection by cyprinid herpesvirus 3 (CyHV-3; KHV). *J Fish Dis* **36**, 75-80.

Piazzon, M. C., Savelkoul, H. S., Pietretti, D., Wiegertjes, G. F. & Forlenza, M. (2015). Carp Il10 Has Anti-Inflammatory Activities on Phagocytes, Promotes Proliferation of Memory T Cells, and Regulates B Cell Differentiation and Antibody Secretion. *J Immunol* **194**, 187-199.

Pietretti, D. & Wiegertjes, G. F. (2014). Ligand specificities of Toll-like receptors in fish: indications from infection studies. *Dev Comp Immunol* **43**, 205-222.

Pikarsky, E., Ronen, A., Abramowitz, J., Levavi-Sivan, B., Hutoran, M., Shapira, Y., Steinitz, M., Perelberg, A., Soffer, D. & Kotler, M. (2004). Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. *J Virol* **78**, 9544-9551.

Pikulkaew, S., Meeyam, T. & Banlunara, W. (2009). The outbreak of koi herpesvirus (KHV) in koi (*Cyprinus carpio koi*) from Chiang Mai province, Thailand. *Thai J Vet Med* **39**, 53-58.

Pionnier, N., Adamek, M., Miest, J. J., Harris, S. J., Matras, M., Rakus, K. L., Irnazarow, I. & Hoole, D. (2014). C-reactive protein and complement as acute phase reactants in common carp *Cyprinus carpio* during CyHV-3 infection. *Dis Aquat Organ* **109**, 187-199.

Plant, K. P. & Lapatra, S. E. (2011). Advances in fish vaccine delivery. *Dev Comp Immunol* **35**, 1256-1262.

Pokorova, D., Reschova, S., Hulova, J., Vicenova, M., Vesely, T. & Piackova, V. (2010). Detection of Cyprinid Herpesvirus-3 in Field Samples of Common and Koi Carp by Various Single-Round and Nested PCR Methods. *Journal of the World Aquaculture Society* **41**, 773-779.

Pokorova, D., Vesely, T., Piackova, V., Reschova, S. & Hulova, J. (2005). Current knowledge on koi herpesvirus (KHV): a review. *Vet Med (Praha)* **5**, 139-147.

Poulin, R. (1995). "Adaptive" changes in the behaviour of parasitized animals: a critical review. *Int J Parasitol* **25**, 1371-1383.

Pretto, T., Manfrin, A., Ceolin, C., Dalla Pozza, M., Zelco, S., Quartesan, R., Abbadi, M., Panzarin, V. & Toffan, A. (2013). First isolation of koi herpes virus (KHV) in Italy from imported koi (*Cyprinus carpio koi*). *B Eur Assoc Fish Pat* **33**, 126-133.

Prosser, C. L. & Nelson, D. O. (1981). The role of nervous systems in temperature adaptation of poikilotherms. *Annu Rev Physiol* **43**, 281-300.

Radosavljevic, V., Jeremic, S., Cirkovic, M., Lako, B., Milicevic, V., Potkonjak, A. & Nikolin, V. (2012). Common fish species in polyculture with carp as cyprinid herpes virus 3 carriers. *Acta Vet Brno* **62**, 675-681.

Rahman, M. M. & McFadden, G. (2006). Modulation of tumor necrosis factor by microbial pathogens. *PLoS Pathog* **2**, e4.

Raj, V. S., Fournier, G., Rakus, K., Ronsmans, M., Ouyang, P., Michel, B., Delforges, C., Costes, B., Farnir, F., Leroy, B., Wattiez, R., Melard, C., Mast, J., Lieffrig, F. & Vanderplasschen, A. (2011). Skin mucus of *Cyprinus carpio* inhibits cyprinid herpesvirus 3 binding to epidermal cells. *Vet Res* **42**, 92.

Rakus, K. L., Irnazarow, I., Adamek, M., Palmeira, L., Kawana, Y., Hirono, I., Kondo, H., Matras, M., Steinhagen, D., Flasz, B., Brogden, G., Vanderplasschen, A. & Aoki, T. (2012). Gene expression analysis of common carp (*Cyprinus carpio* L.) lines during Cyprinid herpesvirus 3 infection yields insights into differential immune responses. *Dev Comp Immunol* **37**, 65-76.

Rakus, K. L., Ouyang, P., Boutier, M., Ronsmans, M., Reschner, A., Vancsok, C., Jazowiecka-Rakus, J. & Vanderplasschen, A. (2013). Cyprinid herpesvirus 3: an interesting virus for applied and fundamental research. *Vet Res* **44**, 85.

Rakus, K. L., Wiegertjes, G. F., Adamek, M., Siwicki, A. K., Lepa, A. & Irnazarow, I. (2009a). Resistance of common carp (*Cyprinus carpio* L.) to Cyprinid herpesvirus-3 is influenced by major histocompatibility (MH) class II *B* gene polymorphism. *Fish Shellfish Immunol* **26**, 737-743.

Rakus, K. L., Wiegertjes, G. F., Jurecka, P., Walker, P. D., Pilarczyk, A. & Irnazarow, I. (2009b). Major histocompatibility (MH) class II *B* gene polymorphism influences disease resistance of common carp (*Cyprinus carpio* L.). *Aquaculture* **288**, 44-50.

Reed, A. N., Izume, S., Dolan, B. P., LaPatra, S., Kent, M., Dong, J. & Jin, L. (2014). Identification of B cells as a major site for cyprinid herpesvirus 3 latency. *J Virol* **88**, 9297-9309.

Reyes-Cerpa, S., Maisey, K., Reyes-López, F., Toro-Ascuy, D., Sandino, A. M. & Imarai, M. (2012). *Fish Cytokines and Immune Response*.

Reynolds, W. W., Casterlin, M. E. & Covert, J. B. (1976). Behavioural fever in teleost fishes. *Nature* **259**, 41-42.

Reynolds, W. W., Covert, J. B. & Casterlin, M. E. (1978). Febrile responses of goldfish *Carassius auratus* (L.)to *Aeromonas hydrophila* and to Escherichia coli endotoxin. *J Fish Dis* **1**, 271-273.

Riazi, K., Galic, M. A., Kuzmiski, J. B., Ho, W., Sharkey, K. A. & Pittman, Q. J. (2008). Microglial activation and TNFalpha production mediate altered CNS excitability following peripheral inflammation. *Proc Natl Acad Sci U S A* **105**, 17151-17156.

Rixon, F. J. & Schmid, M. F. (2014). Structural similarities in DNA packaging and delivery apparatuses in Herpesvirus and dsDNA bacteriophages. *Curr Opin Virol* **5**, 105-110.

Roberts, R. J. & Ellis, A. E. (2012). The Anatomy and Physiology of Teleosts. In *Fish Pathol*, pp. 17-61: Wiley-Blackwell.

Rombout, J. H., Yang, G. & Kiron, V. (2014). Adaptive immune responses at mucosal surfaces of teleost fish. *Fish Shellfish Immunol* **40**, 634-643.

Ronen, A., Perelberg, A., Abramowitz, J., Hutoran, M., Tinman, S., Bejerano, I., Steinitz, M. & Kotler, M. (2003). Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*. *Vaccine* **21**, 4677-4684.

Ronsmans, M., Boutier, M., Rakus, K., Farnir, F., Desmecht, D., Ectors, F., Vandecan, M., Lieffrig, F., Melard, C. & Vanderplasschen, A. (2014). Sensitivity and permissivity of *Cyprinus carpio* to cyprinid herpesvirus 3 during the early stages of its development: importance of the epidermal mucus as an innate immune barrier. *Vet Res* **45**, 100.

Rosenkranz, D., Klupp, B. G., Teifke, J. P., Granzow, H., Fichtner, D., Mettenleiter, T. C. & Fuchs, W. (2008). Identification of envelope protein pORF81 of koi herpesvirus. *J Gen Virol* **89**, 896-900.

Roth, J. & Blatteis, C. M. (2014). Mechanisms of fever production and lysis: lessons from experimental LPS fever. *Compr Physiol* **4**, 1563-1604.

Rothenburg, S., Deigendesch, N., Dittmar, K., Koch-Nolte, F., Haag, F., Lowenhaupt, K. & Rich, A. (2005). A PKR-like eukaryotic initiation factor 2alpha kinase from zebrafish contains Z-DNA binding domains instead of dsRNA binding domains. *Proc Natl Acad Sci U S A* **102**, 1602-1607.

Rueckert, C. & Guzman, C. A. (2012). Vaccines: from empirical development to rational design. *PLoS Pathog* **8**, e1003001.

Ryo, S., Wijdeven, R. H., Tyagi, A., Hermsen, T., Kono, T., Karunasagar, I., Rombout, J. H., Sakai, M., Verburg-van Kemenade, B. M. & Savan, R. (2010). Common carp have two subclasses of bonyfish specific antibody IgZ showing differential expression in response to infection. *Dev Comp Immunol* **34**, 1183-1190.

Sadler, J., Marecaux, E. & Goodwin, A. E. (2008). Detection of koi herpes virus (CyHV-3) in goldfish, *Carassius auratus* (L.), exposed to infected koi. *J Fish Dis* **31**, 71-72.

Salinas, I. & Miller, R. D. (2015). Comparative phylogeny of the mucosa-associated lymphoid tissue. In *Mucosal Immunol*. Edited by M. J., S. W., R. M.W., K. B.L., C. H. & L. B.N.: Elsevier

Sano, M., Fukuda, H. & Sano, T. (1990). Isolation and characterization of a new herpesvirus from eel. In *Pathology in Marine Sciences*, pp. 15-31. Edited by P. O. Perkins & T. C. Cheng: Academic Press, San Diego.

Sano, M., Ito, T., Kurita, J., Yanai, T., Watanabe, N., Satoshi, M. & Iida, T. (2004). First detection of koi herpes virus in cultured common carp *Cyprinus carpio* in Japan. *Fish Pathol* **39**, 165-168.

Sano, T., Fukuda, H. & Furukawa, M. (1985). Herpesvirus cyprini: Biological and oncogenic properties. *Fish Pathol* **20**, 381-388.

Santini, F., Harmon, L., Carnevale, G. & Alfaro, M. (2009). Did genome duplication drive the origin of teleosts? A comparative study of diversification in ray-finned fishes. *BMC Evol Biol* **9**, 194.

Saper, C. B., Romanovsky, A. A. & Scammell, T. E. (2012). Neural circuitry engaged by prostaglandins during the sickness syndrome. *Nat Neurosci* **15**, 1088-1095.

Sarid, R., Sato, T., Bohenzky, R. A., Russo, J. J. & Chang, Y. (1997). Kaposi's sarcoma-associated herpesvirus encodes a functional bcl-2 homologue. *Nat Med* **3**, 293-298.

Satinoff, E., McEwen, G. N., Jr. & Williams, B. A. (1976). Behavioral fever in newborn rabbits. *Science* **193**, 1139-1140.

Scheller, J., Chalaris, A., Schmidt-Arras, D. & Rose-John, S. (2011). The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta* **1813**, 878-888.

Schubert, G. H. (1966). The infective agent in carp pox. *Bull Off Int Epizoot* **65**, 1011-1022.

Sedy, J. R., Spear, P. G. & Ware, C. F. (2008). Cross-regulation between herpesviruses and the TNF superfamily members. *Nature review* **8**, 361-373.

Seet, B. T., Johnston, J. B., Brunetti, C. R., Barrett, J. W., Everett, H., Cameron, C., Sypula, J., Nazarian, S. H., Lucas, A. & McFadden, G. (2003). Poxviruses and immune evasion. *Annu Rev Immunol* **21**, 377-423.

Shapira, Y., Magen, Y., Zak, T., Kotler, M., Hulata, G. & Levavi-Sivan, B. (2005). Differential resistance to koi herpes virus (KHV)/carp interstitial nephritis and gill necrosis virus (CNGV) among common carp (*Cyprinus carpio* L.) strains and crossbreds. *Aquaculture* **245**, 1-11.

Shephard, K. L. (1994). Functions for fish mucus. *Rev Fish Biol Fish* **4**, 401-429.

Shimizu, T., Yoshida, N., Kasai, H. & Yoshimizu, M. (2006). Survival of koi herpesvirus (KHV) in environmental water. *Fish Pathol* **41**, 153-157.

Sievers, F. & Higgins, D. G. (2014). Clustal Omega. *Current Protocols in Bioinformatics* **48**, 3.13.11-13.13.16.

Siwicki, A., Kazuń, K., Kazuń, B. & Majewicz-Zbikowska, E. (2012). Impact of cyprinid herpesvirus-3, which causes interstitial nephritis and gill necrosis, on the activity of carp (*Cyprinus carpio* L.) macrophages and lymphocytes. *Archives of Polish Fisheries* **20**, 123-128.

Soliman, H. & El-Matbouli, M. (2010). Loop mediated isothermal amplification combined with nucleic acid lateral flow strip for diagnosis of cyprinid herpes virus-3. *Mol Cell Probes* **24**, 38-43.

Sommerset, I., Krossoy, B., Biering, E. & Frost, P. (2005). Vaccines for fish in aquaculture. *Expert Rev Vaccines* **4**, 89-101.

Spear, P. G. & Longnecker, R. (2003). Herpesvirus entry: an update. *J Virol* **77**, 10179-10185.

St-Hilaire, S., Beevers, N., Joiner, C., Hedrick, R. P. & Way, K. (2009). Antibody response of two populations of common carp, *Cyprinus carpio* L., exposed to koi herpesvirus. *J Fish Dis* **32**, 311-320.

St-Hilaire, S., Beevers, N., Way, K., Le Deuff, R. M., Martin, P. & Joiner, C. (2005). Reactivation of koi herpesvirus infections in common carp *Cyprinus carpio*. *Dis Aquat Organ* **67**, 15-23.

Stahlschmidt, Z. R. & Adamo, S. A. (2013). Context dependency and generality of fever in insects. *Naturwissenschaften* **100**, 691-696.

Stingley, R. L. & Gray, W. L. (2000). Transcriptional regulation of the channel catfish virus genome direct repeat region. *J Gen Virol* **81**, 2005-2010.

Subramanian, S., MacKinnon, S. L. & Ross, N. W. (2007). A comparative study on innate immune parameters in the epidermal mucus of various fish species. *Comp Biochem Physiol B Biochem Mol Biol* **148**, 256-263.

Subramanian, S., Ross, N. W. & MacKinnon, S. L. (2008). Comparison of antimicrobial activity in the epidermal mucus extracts of fish. *Comp Biochem Physiol B Biochem Mol Biol* **150**, 85-92.

Sunarto, A., Liongue, C., McColl, K. A., Adams, M. M., Bulach, D., Crane, M. S., Schat, K. A., Slobedman, B., Barnes, A. C., Ward, A. C. & Walker, P. J. (2012). Koi herpesvirus encodes and expresses a functional interleukin-10. *J Virol* **86**, 11512-11520.

Sunarto, A., McColl, K. A., Crane, M. S., Schat, K. A., Slobedman, B., Barnes, A. C. & Walker, P. J. (2014). Characteristics of cyprinid herpesvirus 3 in different phases of infection: implications for disease transmission and control. *Virus Res* **188**, 45-53.

Sunarto, A., McColl, K. A., Crane, M. S., Sumiati, T., Hyatt, A. D., Barnes, A. C. & Walker, P. J. (2011). Isolation and characterization of koi herpesvirus (KHV) from Indonesia: identification of a new genetic lineage. *J Fish Dis* **34**, 87-101.

Sunarto, A., Rukyani, A. & Itami, T. (2005). Indonesian Experience on the Outbreak of Koi Herpesvirus in Koi and Carp (*Cyprinus carpio*). *Bulletin of Fisheries Research Agency* **Supplement 2**, 15-21.

Swain, P. & Nayak, S. K. (2009). Role of maternally derived immunity in fish. *Fish Shellfish Immunol* **27**, 89-99.

Syakuri, H., Adamek, M., Brogden, G., Rakus, K. L., Matras, M., Irnazarow, I. & Steinhagen, D. (2013). Intestinal barrier of carp (*Cyprinus carpio* L.) during a cyprinid herpesvirus 3-infection: molecular identification and regulation of the mRNA expression of claudin encoding genes. *Fish Shellfish Immunol* **34**, 305-314.

Takahara, T., Honjo, M. N., Uchii, K., Minamoto, T., Doi, H., Ito, T. & Kawabata, Z. (2014). Effects of daily temperature fluctuation on the survival of carp infected with Cyprinid herpesvirus 3. *Aquaculture* **433**, 208-213.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* **30**, 2725-2729.

Taylor, N. G., Dixon, P. F., Jeffery, K. R., Peeler, E. J., Denham, K. L. & Way, K. (2010a). Koi herpesvirus: distribution and prospects for control in England and Wales. *J Fish Dis* **33**, 221-230.

Taylor, N. G., Norman, R. A., Way, K. & Peeler, E. J. (2011). Modelling the koi herpesvirus (KHV) epidemic highlights the importance of active surveillance within a national control policy. *J Appl Ecol* **48**, 348-355.

Taylor, N. G., Way, K., Jeffery, K. R. & Peeler, E. J. (2010b). The role of live fish movements in spreading koi herpesvirus throughout England and Wales. *J Fish Dis* **33**, 1005-1007.

Terhune, J. S., Grizzle, J. M., Hayden, K., McClenahan, S. D., Lamprecht, S. D. & White, M. G. (2004). First report of koi herpesvirus in wild common carp in the Western Hemisphere. *Fish Health Newsletter* **32**, 8-9.

Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meinl, E., Neipel, F., Mattmann, C., Burns, K., Bodmer, J. L., Schroter, M., Scaffidi, C., Krammer, P. H., Peter, M. E. & Tschopp, J. (1997). Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* **386**, 517-521.

Tischer, B. K. & Kaufer, B. B. (2012). Viral bacterial artificial chromosomes: generation, mutagenesis, and removal of mini-F sequences. *Journal of biomedicine & biotechnology* **2012**, 472537.

Tome, A. R., Kus, K., Correia, S., Paulo, L. M., Zacarias, S., de Rosa, M., Figueiredo, D., Parkhouse, R. M. & Athanasiadis, A. (2013). Crystal structure of a poxvirus-like zalpha domain from cyprinid herpesvirus 3. *J Virol* **87**, 3998-4004.

Tu, C., Lu, Y. P., Hsieh, C. Y., Huang, S. M., Chang, S. K. & Chen, M. M. (2014). Production of monoclonal antibody against ORF72 of koi herpesvirus isolated in Taiwan. *Folia Microbiol (Praha)* **59**, 159-165.

Tu, C., Weng, M. C., Shiau, J. R. & Lin, S. Y. (2004). Detection of koi herpesvirus in koi *Cyprinus carpio* in Taiwan. *Fish Pathol* **39**, 109-110.

Tyring, S., Gottlieb, A., Papp, K., Gordon, K., Leonardi, C., Wang, A., Lalla, D., Woolley, M., Jahreis, A., Zitnik, R., Cella, D. & Krishnan, R. (2006). Etanercept and clinical outcomes, fatigue, and depression in psoriasis: double-blind placebo-controlled randomised phase III trial. *Lancet* **367**, 29-35.

Uchii, K., Matsui, K., Iida, T. & Kawabata, Z. (2009). Distribution of the introduced cyprinid herpesvirus 3 in a wild population of common carp, *Cyprinus carpio* L. *J Fish Dis* **32**, 857-864.

Uchii, K., Minamoto, T., Honjo, M. N. & Kawabata, Z. (2014). Seasonal reactivation enables Cyprinid herpesvirus 3 to persist in a wild host population. *FEMS microbiology ecology* **87**, 536-542.

Uchii, K., Okuda, N., Minamoto, T. & Kawabata, Z. (2013). An emerging infectious pathogen endangers an ancient lineage of common carp by acting synergistically with conspecific exotic strains. *Anim Conserv* **16**, 324-330.

Uchii, K., Telschow, A., Minamoto, T., Yamanaka, H., Honjo, M. N., Matsui, K. & Kawabata, Z. (2011). Transmission dynamics of an emerging infectious disease in wildlife through host reproductive cycles. *ISME Journal* **5**, 244-251.

Vadstein, O., Bergh, Ø., Gatesoupe, F.-J., Galindo-Villegas, J., Mulero, V., Picchietti, S., Scapigliati, G., Makridis, P., Olsen, Y., Dierckens, K., Defoirdt, T., Boon, N., De Schryver, P. & Bossier, P. (2013). Microbiology and immunology of fish larvae. *Rev Aquac* **5**, S1-S25.

van Beurden, S. J., Bossers, A., Voorbergen-Laarman, M. H., Haenen, O. L., Peters, S., Abma-Henkens, M. H., Peeters, B. P., Rottier, P. J. & Engelsma, M. Y. (2010). Complete genome sequence and taxonomic position of anguillid herpesvirus 1. *J Gen Virol* **91**, 880-887.

van Beurden, S. J. & Engelsma, M. Y. (2012). Herpesviruses of fish, amphibians and invertebrates. In *Herpesviridae - a look into this unique family of viruses*, pp. 217-242. Edited by G. D. Magel & S. Tyring: InTech, Rijeka.

van Beurden, S. J., Engelsma, M. Y., Roozenburg, I., Voorbergen-Laarman, M. A., van Tulden, P. W., Kerkhoff, S., van Nieuwstadt, A. P., Davidse, A. & Haenen, O. L. (2012a). Viral diseases of wild and farmed European eel Anguilla anguilla with particular reference to the Netherlands. *Dis Aquat Organ* **101**, 69-86.

van Beurden, S. J., Forlenza, M., Westphal, A. H., Wiegertjes, G. F., Haenen, O. L. & Engelsma, M. Y. (2011a). The alloherpesviral counterparts of interleukin 10 in European eel and common carp. *Fish Shellfish Immunol* **31**, 1211-1217.

van Beurden, S. J., Gatherer, D., Kerr, K., Galbraith, J., Herzyk, P., Peeters, B. P., Rottier, P. J., Engelsma, M. Y. & Davison, A. J. (2012b). Anguillid herpesvirus 1 transcriptome. *J Virol* **86**, 10150-10161.

van Beurden, S. J., Leroy, B., Wattiez, R., Haenen, O. L., Boeren, S., Vervoort, J. J., Peeters, B. P., Rottier, P. J., Engelsma, M. Y. & Vanderplasschen, A. F. (2011b). Identification and localization of the structural proteins of anguillid herpesvirus 1. *Vet Res* **42**, 105.

van Beurden, S. J., Peeters, B. P., Rottier, P. J., Davison, A. J. & Engelsma, M. Y. (2013). Genome-wide gene expression analysis of anguillid herpesvirus 1. *BMC Genomics* **14**, 83.

van der Marel, M., Adamek, M., Gonzalez, S. F., Frost, P., Rombout, J. H., Wiegertjes, G. F., Savelkoul, H. F. & Steinhagen, D. (2012). Molecular cloning and expression of two beta-defensin and two mucin genes in common carp (*Cyprinus carpio* L.) and their up-regulation after beta-glucan feeding. *Fish Shellfish Immunol* **32**, 494-501.

van Houte, S., Ros, V. I. & van Oers, M. M. (2013). Walking with insects: molecular mechanisms behind parasitic manipulation of host behaviour. *Mol Ecol* **22**, 3458-3475.

Vaughn, L. K., Bernheim, H. A. & Kluger, M. J. (1974). Fever in the lizard *Dipsosaurus dorsalis*. *Nature* **252**, 473-474.

Vilizzi, L. & Walker, K. F. (1999). The Onset of the Juvenile Period in Carp, *Cyprinus Carpio*: a Literature Survey. *Environ Biol Fishes* **56**, 93-102.

Vrancken, R., Boutier, M., Ronsmans, M., Reschner, A., Leclipteux, T., Lieffrig, F., Collard, A., Melard, C., Wera, S., Neyts, J., Goris, N. & Vanderplasschen, A. (2013). Laboratory validation of a lateral flow device for the detection of CyHV-3 antigens in gill swabs. *J Virol Methods* **193**, 679-682.

Wajant, H., Pfizenmaier, K. & Scheurich, P. (2003). Tumor necrosis factor signaling. *Cell Death Differ* **10**, 45-65.

Walczak, H. (2011). TNF and ubiquitin at the crossroads of gene activation, cell death, inflammation, and cancer. *Immunol Rev* **244**, 9-28.

Walster, C. I. (1999). Clinical observations of severe mortalities in koi carp, *Cyprinus carpio*, with gill disease. *Fish Vet J* **3**, 54-58.

Waltzek, T. B., Kelley, G. O., Alfaro, M. E., Kurobe, T., Davison, A. J. & Hedrick, R. P. (2009). Phylogenetic relationships in the family *Alloherpesviridae*. *Dis Aquat Organ* **84**, 179-194.

Waltzek, T. B., Kelley, G. O., Stone, D. M., Way, K., Hanson, L., Fukuda, H., Hirono, I., Aoki, T., Davison, A. J. & Hedrick, R. P. (2005). Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family *Herpesviridae*. *J Gen Virol* **86**, 1659-1667.

Wang, Y., Zeng, W., Li, Y., Liang, H., Liu, C., Pan, H., Lee, P., Wu, S., Bergmann, S. M. & Wang, Q. (2015). Development and characterization of a cell line from the snout of koi (*Cyprinus carpio* L.) for detection of koi herpesvirus. *Aquaculture* **435**, 310-317.

Weber, E. P., 3rd, Malm, K. V., Yun, S. C., Campbell, L. A., Kass, P. H., Marty, G. D., Salonius, K. & Dishon, A. (2014). Efficacy and safety of a modified-live cyprinid herpesvirus 3 vaccine in koi (*Cyprinus carpio koi*) for prevention of koi herpesvirus disease. *Am J Vet Res* **75**, 899-904.

Wegner, N. C., Snodgrass, O. E., Dewar, H. & Hyde, J. R. (2015). Whole-body endothermy in a mesopelagic fish, the opah, Lampris guttatus. *Science* **348**, 786-789.

Whittington, R. J., Crockford, M., Jordan, D. & Jones, B. (2008). Herpesvirus that caused epizootic mortality in 1995 and 1998 in pilchard, *Sardinops sagax neopilchardus* (Steindachner), in Australia is now endemic. *J Fish Dis* **31**, 97-105.

Whyte, S. K. (2007). The innate immune response of finfish--a review of current knowledge. *Fish Shellfish Immunol* **23**, 1127-1151.

Wiens, G. D. & Glenney, G. W. (2011). Origin and evolution of TNF and TNF receptor superfamilies. *Dev Comp Immunol* **35**, 1324-1335.

Winton, J., Batts, W., deKinkelin, P., LeBerre, M., Bremont, M. & Fijan, N. (2010). Current lineages of the *epithelioma papulosum cyprini* (EPC) cell line are contaminated with fathead minnow, *Pimephales promelas*, cells. *J Fish Dis* **33**, 701-704.

Wolf, B. B. & Green, D. R. (1999). Suicidal tendencies: apoptotic cell death by caspase family proteinases. *J Biol Chem* **274**, 20049-20052.

Wolf, K. (1988). Channel catfish virus disease. In *Fish viruses and fish viral diseases*, pp. 21-42. Edited by K. Wolf: Cornell University Press, Ithaca, New York.

Wolf, K. & Darlington, R. W. (1971). Channel catfish virus: a new herpesvirus of ictalurid fish. *J Virol* **8**, 525-533.

Woolhouse, M. E., Haydon, D. T. & Antia, R. (2005). Emerging pathogens: the epidemiology and evolution of species jumps. *Trends in ecology & evolution* **20**, 238-244.

Workenhe, S. T., Rise, M. L., Kibenge, M. J. & Kibenge, F. S. (2010). The fight between the teleost fish immune response and aquatic viruses. *Mol Immunol* **47**, 2525-2536.

Xu, J. R., Bently, J., Beck, L., Reed, A., Miller-Morgan, T., Heidel, J. R., Kent, M. L., Rockey, D. D. & Jin, L. (2013a). Analysis of koi herpesvirus latency in wild common carp and ornamental koi in Oregon, USA. *J Virol Methods* **187**, 372-379.

Xu, Z., Parra, D., Gomez, D., Salinas, I., Zhang, Y. A., von Gersdorff Jorgensen, L., Heinecke, R. D., Buchmann, K., LaPatra, S. & Sunyer, J. O. (2013b). Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. *Proc Natl Acad Sci U S A* **110**, 13097-13102.

Yasumoto, S., Kuzuya, Y., Yasuda, M., Yoshimura, T. & Miyazaki, T. (2006). Oral Immunization of Common Carp with a Liposome Vaccine Fusing Koi Herpesvirus Antigen. *Fish Pathol* **41**, 141-145.

Yi, Y., Zhang, H., Lee, X., Weng, S., He, J. & Dong, C. (2014). Extracellular virion proteins of two Chinese CyHV-3/KHV isolates, and identification of two novel envelope proteins. *Virus Res* **191**, 108-116.

Yoshida, N., Sasaki, R. K., Kasai, H. & Yoshimizu, M. (2013). Inactivation of koi-herpesvirus in water using bacteria isolated from carp intestines and carp habitats. *J Fish Dis* **36**, 997-1005.

Yoshino, M., Watari, H., Kojima, T. & Ikedo, M. (2006). Sensitive and Rapid Detection of Koi Herpesvirus by LAMP Method. *Fish Pathol* **41**, 19-27.

Yoshino, M., Watari, H., Kojima, T., Ikedo, M. & Kurita, J. (2009). Rapid, sensitive and simple detection method for koi herpesvirus using loop-mediated isothermal amplification. *Microbiol Immunol* **53**, 375-383.

Yuasa, K., Ito, T. & Sano, M. (2008). Effect of Water Temperature on Mortality and Virus Shedding in Carp Experimentally Infected with Koi Herpesvirus. *Fish Pathol* **43**, 83-85.

Yuasa, K., Kurita, J., Kawana, M., Kiryu, I., Oseko, N. & Sano, M. (2012a). Development of mRNA-specific RT-PCR for the detection of koi herpesvirus (KHV) replication stage. *Dis Aquat Organ* **100**, 11-18.

Yuasa, K. & Sano, M. (2009). Koi herpesvirus: Status of outbreaks, diagnosis, surveillance, and research. *Isr J Aquacult-Bamid* **61**, 169-179.

Yuasa, K., Sano, M., Kurita, J., Ito, T. & Iida, T. (2005). Improvement of a PCR method with the Sph I-5 primer set for the detection of koi herpesvirus (KHV). *Fish Pathol* **40**, 37-39.

Yuasa, K., Sano, M. & Oseko, N. (2012b). Effective procedures for culture isolation of koi herpesvirus (KHV). *Fish Pathol* **47**, 97-99.

Yuasa, K., Sano, M. & Oseko, N. (2013). Goldfish is Not a Susceptible Host of Koi Herpesvirus (KHV) Disease. *Fish Pathol* **48**, 52-55.

Zak, T., Perelberg, A., Magen, I., Milstein, A. & Joseph, D. (2007). Heterosis in the Growth Rate of Hungarian-Israeli Common Carp Crossbreeds and Evaluation of their Sensitivity to Koi Herpes Virus (KHV) Disease. *Isr J Aquacult-Bamid* **59**, 63-72.

Zapata, A., Diez, B., Cejalvo, T., Gutierrez-de Frias, C. & Cortes, A. (2006). Ontogeny of the immune system of fish. *Fish Shellfish Immunol* **20**, 126-136.

Żbikowska, E., Cichy, A. & Papierkiewicz, D. (2013). Viral pyrogen affects thermoregulatory behavior of wintering Planorbarius corneus (L.) snails (Mollusca: Gastropoda). *J Therm Biol* **38**, 543-547.

Zhang, S., Wang, Z. & Wang, H. (2013). Maternal immunity in fish. *Dev Comp Immunol* **39**, 72-78.

Zhang, Y. A., Salinas, I., Li, J., Parra, D., Bjork, S., Xu, Z., LaPatra, S. E., Bartholomew, J. & Sunyer, J. O. (2010). IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nat Immunol* **11**, 827-835.

Zhou, J., Wang, H., Li, X. W., Zhu, X., Lu, W. & Zhang, D. (2014a). Construction of KHV-CJ ORF25 DNA vaccine and immune challenge test. *J Fish Dis* **37**, 319-325.

Zhou, J., Wang, H., Zhu, X., Li, X., Lv, W. & Zhang, D. (2013). The primary culture of mirror carp snout and caudal fin tissues and the isolation of Koi herpesvirus. *In Vitro Cell Dev Biol Anim* **49**, 734-742.

Zhou, J., Xue, J., Wang, Q., Zhu, X., Li, X., Lv, W. & Zhang, D. (2014b). Vaccination of plasmid DNA encoding ORF81 gene of CJ strains of KHV provides protection to immunized carp. *In Vitro Cell Dev Biol Anim* **50**, 489-495.

Annex

Annex

Rational development of an attenuated recombinant cyprinid herpesvirus 3 vaccine using prokaryotic mutagenesis and *in vivo* bioluminescent imaging

Plos Pathogens (2015), 11(2):e1004690

Maxime Boutier, Maygane Ronsmans, Ping Ouyang, Guillaume Fournier, Anca Reschner, Krzysztof Rakus, Gavin S. Wilkie, Frédéric Farnir, Calixte Bayrou, François Lieffrig, Hong Li, Daniel Desmecht, Andrew J. Davison and Alain Vanderplasschen

Preamble

This thesis focused on fundamental aspects of two innate immune mechanisms developed by carp against CyHV-3. However, it was also an opportunity to contribute to an applied project aiming to develop a safe and efficacious attenuated recombinant vaccine against CyHV-3. This study has been published in *Plos Pathogens* and is provided as an annex hereafter.

My contribution to this applied study consisted to participate to the production of a recombinant form of the vaccine candidate expressing luciferase as a reporter gene. I also contributed to the IVIS experiments performed with this recombinant to investigate the effect of the ORF56-57 deletion on the tropism of the produced CyHV-3 vaccine and its capability to spread from vaccinated fish to naïve cohabitant subjects.

PLOS | PATHOGENS

RESEARCH ARTICLE

Rational Development of an Attenuated Recombinant Cyprinid Herpesvirus 3 Vaccine Using Prokaryotic Mutagenesis and In Vivo Bioluminescent Imaging

Maxime Boutier[1], Maygane Ronsmans[1], Ping Ouyang[1¤], Guillaume Fournier[1], Anca Reschner[1], Krzysztof Rakus[1], Gavin S. Wilkie[2], Frédéric Farnir[3], Calixte Bayrou[4], François Lieffrig[5], Hong Li[6], Daniel Desmecht[4], Andrew J. Davison[2], Alain Vanderplasschen[1]*

1 Immunology-Vaccinology, Department of Infectious and Parasitic Diseases, Fundamental and Applied Research for Animals & Health (FARAH), Faculty of Veterinary Medicine, University of Liège, Liège, Belgium, **2** MRC—University of Glasgow Centre for Virus Research, Glasgow, United Kingdom, **3** Biostatistics and Bioinformatics, Fundamental and Applied Research for Animals & Health (FARAH), Faculty of Veterinary Medicine, University of Liège, Liège, Belgium, **4** Pathology, Department of Morphology and Pathology, Fundamental and Applied Research for Animals & Health (FARAH), Faculty of Veterinary Medicine, University of Liège, Liège, Belgium, **5** Fish Pathology Lab, Department of Biotechnology, CER Groupe, Marloie, Belgium, **6** USDA-ARS-ADRU, Washington State University, Pullman, Pullman, Washington, United States of America

¤ Current address: Key Laboratory of Animal Disease, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan, China
* A.vdplasschen@ulg.ac.be

Abstract

Cyprinid herpesvirus 3 (CyHV-3) is causing severe economic losses worldwide in common and koi carp industries, and a safe and efficacious attenuated vaccine compatible with mass vaccination is needed. We produced single deleted recombinants using prokaryotic mutagenesis. When producing a recombinant lacking open reading frame 134 (ORF134), we unexpectedly obtained a clone with additional deletion of ORF56 and ORF57. This triple deleted recombinant replicated efficiently in vitro and expressed an in vivo safety/efficacy profile compatible with use as an attenuated vaccine. To determine the role of the double ORF56-57 deletion in the phenotype and to improve further the quality of the vaccine candidate, a series of deleted recombinants was produced and tested in vivo. These experiments led to the selection of a double deleted recombinant lacking ORF56 and ORF57 as a vaccine candidate. The safety and efficacy of this strain were studied using an in vivo bioluminescent imaging system (IVIS), qPCR, and histopathological examination, which demonstrated that it enters fish via skin infection similar to the wild type strain. However, compared to the parental wild type strain, the vaccine candidate replicated at lower levels and spread less efficiently to secondary sites of infection. Transmission experiments allowing water contamination with or without additional physical contact between fish demonstrated that the vaccine candidate has a reduced ability to spread from vaccinated fish to naïve sentinel cohabitants. Finally, IVIS analyses demonstrated that the vaccine candidate

OPEN ACCESS

Citation: Boutier M, Ronsmans M, Ouyang P, Fournier G, Reschner A, Rakus K, et al. (2015) Rational Development of an Attenuated Recombinant Cyprinid Herpesvirus 3 Vaccine Using Prokaryotic Mutagenesis and In Vivo Bioluminescent Imaging. PLoS Pathog 11(2): e1004690. doi:10.1371/journal.ppat.1004690

Editor: Lindsey Hutt-Fletcher, Louisiana State University Health Sciences Center, United States

Received: November 29, 2014

Accepted: January 20, 2015

Published: February 20, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by MSD Animal Health grant KHV2013-2014 to AV, by Belgian Science Policy grant R.SSTC.0454-BELVIR IAP7/45 to AV, by FNRS grants R.FNRS.2165 2.4622.10 and R.FNRS.2597-1.A290.12 to AV and by UK Medical Research Council grant MC_UU_12014/3 to AD. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The present study was financially supported at least in part by MSD animal health. This does not alter our adherence to all PLOS policies on sharing data and materials.

induces a protective mucosal immune response at the portal of entry. Thus, the present study is the first to report the rational development of a recombinant attenuated vaccine against CyHV-3 for mass vaccination of carp. We also demonstrated the relevance of the CyHV-3 carp model for studying alloherpesvirus transmission and mucosal immunity in teleost skin.

Author Summary

Common carp, and its colorful ornamental variety koi, is one of the most economically valuable species in aquaculture. Since the late 1990s, the common and koi carp culture industries have suffered devastating worldwide losses due to cyprinid herpesvirus 3 (CyHV-3). In the present study, we report the development of an attenuated recombinant vaccine against CyHV-3. Two genes were deleted from the viral genome, leading to a recombinant virus that is no longer capable of causing the disease but can be propagated in cell culture (for vaccine production) and infect fish when added to the water, thereby immunizing the fish. This attenuated recombinant vaccine also had a drastic defect in spreading from vaccinated to non-vaccinated cohabitant fish. The vaccine induced a protective mucosal immune response capable of preventing the entry of virulent CyHV-3 and is compatible with the simultaneous vaccination of a large number of carp by simply immersing the fish in water containing the vaccine. This vaccine represents a promising tool for controlling the most dreadful disease ever encountered by the carp culture industries. In addition, the present study highlights the importance of the CyHV-3 - carp model for studying alloherpesvirus transmission and mucosal immunity in teleost skin.

Introduction

Aquaculture is currently one of the world's fastest growing food production sectors, with an annual growth rate of 6.2% between 2000 and 2012 [1]. Global aquaculture currently provides half of the fish consumed worldwide. However, aquaculture is suffering important economic losses due to outbreaks of infectious and parasitic diseases [2–4], which are promoted by high rearing densities under artificial conditions [5], the efficient abiotic vector properties of water [5], and the international trade of genitors and fingerlings [6].

Inland aquaculture of freshwater finfishes dominates global aquaculture, representing 57.9% (38.6 million tons) of global production of aquaculture, far greater than mollusks (22.8%), crustaceans (9.7%), and mariculture of finfishes (8.3%) and other aquatic animals (1.3%) [1]. The common carp (*Cyprinus carpio*) is one of the oldest cultivated freshwater fish species. In China, carp cultivation dates back to at least the 5th century BC, and in Europe carp farming began during the Roman Empire [7]. Common carp is currently one of the most economically valuable species in aquaculture; it is one of the main fish species cultivated for human consumption, with worldwide production of 3.8 million tons (the third most important species based on the number of tons produced) in 2012, representing 9.8% of all freshwater fish production and US$5.2 billion [8]. Common carp is also produced and stocked in fishing areas for angling purposes. In addition, its colorful ornamental varieties (koi carp) grown for personal pleasure and competitive exhibitions represent one the most expensive markets for individual freshwater fish, with some prize-winners being sold for US$10,000-1,000,000 [9].

Koi herpesvirus (KHV), also known as cyprinid herpesvirus 3 (CyHV-3; species *Cyprinid herpesvirus 3*, genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales*), is the etiological agent of an emerging and mortal disease in common and koi carp [10]. Since its emergence in the late 1990s, this highly contagious disease has caused severe economic losses worldwide in both common and koi carp culture industries [9,11,12]. Outbreaks of CyHV-3 disease are associated with a mortality rate of 80–100% [9]. As an example, outbreaks of CyHV-3 in Indonesia in 2002 and 2003 caused an estimated economic loss of US$15 million [13,14].

The economic losses caused by infectious and parasitic diseases in aquaculture have motivated the development of efficient prophylactic vaccines [2,3]. In addition to the safety/efficacy issues that apply to all vaccines independent of the target species (humans or animals), vaccines for fish and production animals in general are under additional constraints. First, the vaccine must be compatible with mass vaccination and administered via a single dose as early as possible in life. Second, the cost-benefit ratio should be as low as possible, implying the lowest cost for vaccine production and administration [5]. Ideally, cost-effective mass vaccination of young fish is performed by bath vaccination, meaning that the fish are immersed in water containing the vaccine [15]. This procedure allows vaccination of a large number of subjects when their individual value is still low and their susceptibility to the disease highest [15]. The use of injectable vaccines for mass vaccination of fish is restricted to limited circumstances, i.e. when the value of an individual subject is relatively high and when vaccination can be delayed until an age when the size of the fish is compatible with their manipulation [16,17].

Various anti-CyHV-3 vaccine candidates have been developed [18–27]. Injectable DNA vaccines are efficacious under experimental conditions [21,22] but incompatible with most of the field constraints described above (i.e., the value of an individual common carp is very low and they should be vaccinated when only a few grams). In contrast, attenuated vaccines could meet these constraints but raise safety concerns, such as residual virulence, reversion to virulence, and spreading from vaccinated to naïve subjects. A conventional anti-CyHV-3 attenuated vaccine has been developed by serial passages in cell culture and UV irradiation [18–20,26,27]. This vaccine is commercialized in Israel by the KoVax Company for the vaccination of koi and common carp by immersion in water containing the attenuated strain and was recently launched in the US market under the name Cavoy but was withdrawn from sale after just a year. This vaccine has two major disadvantages. First, the attenuated strain has residual virulence for fish weighing less than 50 g [18,27]. Second, the determinism of the attenuation is unknown, and consequently, reversions to a pathogenic phenotype cannot be excluded [28].

Due to scientific advances in molecular biology and molecular virology, the development of attenuated vaccines is evolving from empirical to rational design [28–30]. A viral genome can be edited to delete genes encoding virulence factors in such a way that reversion to virulence can be excluded. This approach has been tested for CyHV-3 by targeting different genes thought to encode virulence factors, such as open reading frame (ORF) 16, ORF55, ORF123, and ORF134, which encode a G protein-coupled receptor, thymidine kinase (TK), deoxyuridine triphosphatase, and an Interleukine-10 (IL-10) homolog, respectively. Unfortunately, none of the recombinants lacking these genes express a safety/efficacy profile compatible with use as an attenuated recombinant vaccine [23,24,31].

In the present study, we took advantage of a recombinant strain that was obtained by accident. This strain was deleted of three genes and expressed a safety/efficacy profile compatible with use as an attenuated vaccine. Using prokaryotic recombination technologies, a series of recombinants was produced to identify the determinism of the attenuation and to improve further some properties of the vaccine candidate. Based on the results, a double deleted strain was selected as a vaccine candidate. The safety and efficacy of this strain as a recombinant

attenuated vaccine against CyHV-3 were investigated using various approaches, including an in vivo bioluminescent imaging system (IVIS). Taken together, the results of the present study demonstrate that this vaccine candidate is appropriate for safe and efficacious mass vaccination of carp against CyHV-3, inducing a protective mucosal immune response at the portal of entry. In addition to its importance for applied research, the present study is also important for fundamental research by demonstrating the potential of the CyHV-3 - carp model for studying the transmission of members of the family *Alloherpesviridae* and mucosal immunity in teleost skin.

Results

Determinism of the attenuated phenotype observed for a triple deleted CyHV-3 strain

In an earlier study, we produced a CyHV-3 recombinant strain lacking ORF134, which encodes a viral IL-10 [31]. This recombinant was produced using a bacterial artificial chromosome (BAC) clone of the CyHV-3 FL strain and prokaryotic recombination technologies. To reconstitute infectious viral particles encoding a wild-type ORF55 locus (encoding TK) in which the BAC cassette was inserted, the FL BAC ORF134 Del galactokinase (*galK)* plasmid (deleted for ORF134) was co-transfected with the pGEMT-TK plasmid in permissive *Cyprinus carpio* brain (CCB) cells. For one of the viral clones we obtained, this procedure led to unexpected non-homologous recombination between the pGEMT vector and the beginning of ORF57, leading to reversion to a wild-type ORF55 locus and deletion of CyHV-3 genome from coordinates 97001 to 99726. This deleted region encodes most of ORF56 and the beginning of ORF57 (Fig. 1A). Despite this triple deletion (ORF56-57 and ORF134), this strain replicated efficiently in vitro, reaching a titer of 10^6 plaque forming units (pfu)/ml. Moreover, it expressed in vivo a safety/efficacy profile compatible with use as an attenuated vaccine for vaccination of carp against CyHV-3 by immersion in water containing the virus.

Here, we produced and tested a series of recombinant strains to establish the contribution of the ORF134 and the ORF56-57 deletions to the observed safety/efficacy profile of the triple deleted recombinant described above. Based on our recent study demonstrating that ORF134 is not essential for virulence in vivo [31], we hypothesized that the attenuated phenotype of the triple deleted strain was mainly, if not exclusively, determined by the ORF56-57 deletion. However, deletion of ORF134, which encodes an IL-10 homolog, could potentially contribute to the safety observed for the triple recombinant and/or the immune response it induced. To test these hypotheses, two groups of recombinants were produced independently using BAC cloning technologies according to the strategy described in Fig. 1B. Both groups encoded the ORF56-57 deletion (Fig. 1A) encompassing coordinates 97001 to 99750. This deletion was slightly longer on the 3' end than the deletion observed in the triple deleted strain described above in order to remove two potential alternative ATG start codons present in the beginning of ORF57. In addition to the ORF56-57 deletion, one of the groups also encoded an ORF134 deletion. When reconstituting infectious virions from recombinant BAC plasmids, the BAC cassette was removed by homologous recombination, leading to a wild-type ORF55 locus, or by cre-loxP-mediated excision, leading to a truncated ORF55 locus (TKtrunc genotype). The molecular structures of all recombinant strains were controlled by SacI restriction fragment length polymorphism (RFLP), Southern blot analysis (S1 Fig.), and sequencing of the genome region encoding ORF55 to ORF57. Strains encoding wild-type loci had a sequence identical to the reference sequence available in the GenBank (Accession number NC_009127.1) and all recombinant strains had the expected modified sequences. All recombinants were tested for their virulence (i.e., safety) and their ability to induce immune protection against a lethal challenge

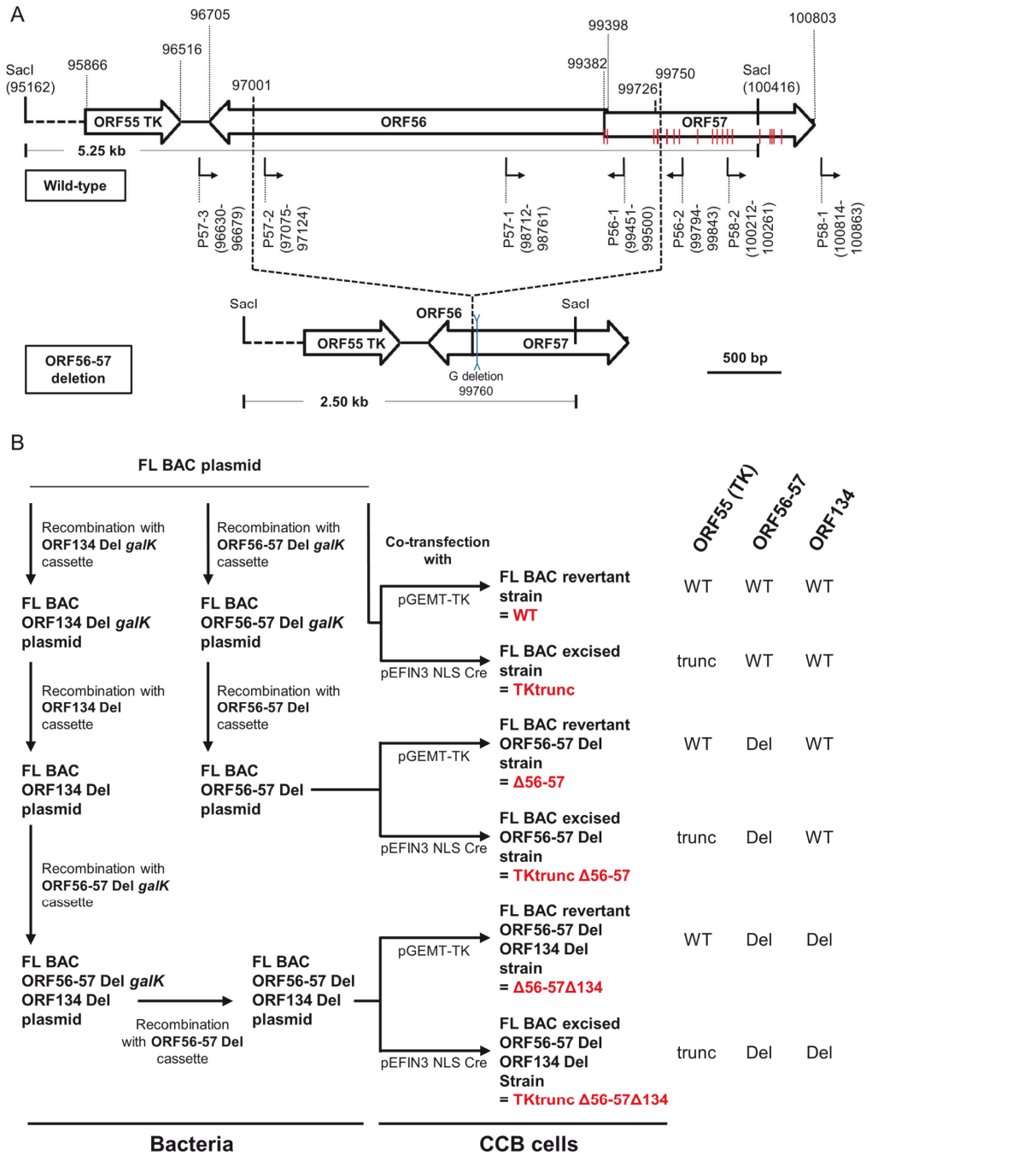


Fig 1. Production of CyHV-3 recombinants. (A) Schematic representation of wild-type ORF56-57 and ORF56-57 deleted genotypes. CyHV-3 ORFs are represented by white arrows. Predicted promoters in the ORF56-ORF57 region are represented by angular black arrows below the wild-type ORF56-57

genotype. In-frame ATGs of ORF57 are indicated by red lines. SacI restriction sites and predicted restriction fragments (in kb) are shown. Coordinates are those of the CyHV-3 reference strain available in GenBank (Accession number NC_009127.1). (B) Flowchart of the production of double (Δ56-57) and triple (Δ56-57Δ134) deleted recombinants for CyHV-3 ORF134 and/or ORF56-57. To simplify the reading of the manuscript, recombinants were given a nickname (in red) that will be used hereafter. The right side of the figure summarizes the genotype of the strains for ORF55 (TK), ORF56-57, and ORF134. Del, deleted; trunc, truncated.

doi:10.1371/journal.ppat.1004690.g001

(i.e., efficacy) (Figs. 2 and S2). Independent of the ORF55 genotype (wild-type versus truncated (TKtrunc)) and ORF134 genotype (wild-type versus deleted (Δ134)), all recombinants encoding the double ORF56-57 deletion (Δ56-57) expressed comparable safety/efficacy profiles. Fish infected with the WT (Fig. 2) or the TKtrunc (S2 Fig.) strains exhibited all clinical signs

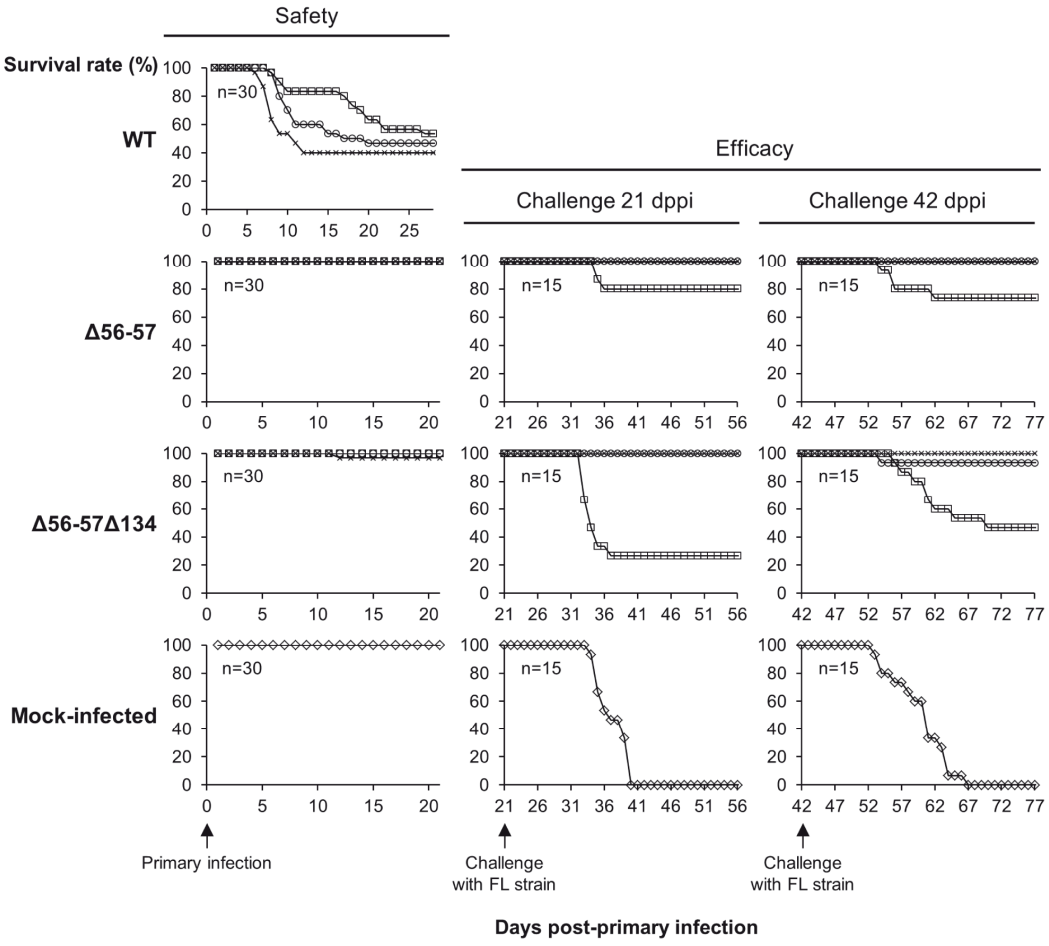


Fig 2. Safety-efficacy profile of double Δ56-57 and triple Δ56-57Δ134 deleted recombinants encoding a wild-type TK locus. The safety and efficacy of the indicated recombinant strains was tested in common carp (average weight 3.77 g ± 1.95 g, 7 months old). On day 0, fish were infected for 2 h by immersion in water containing 4 (□), 40 (○), or 400 (x) pfu/ml. Safety was investigated by measuring the survival rate for 21 days in a group of 30 carp. Efficacy was tested at 21 and 42 dppi. Mock-infected fish and fish that survived the primary infection were distributed in tanks (n = 15) and challenged by cohabitation with fish infected with the FL strain.

doi:10.1371/journal.ppat.1004690.g002

associated with CyHV-3 disease, including apathy, folding of the dorsal fin, hyperemia, increased mucus secretion, skin lesions, suffocation, erratic swimming, and loss of equilibrium. Independent of the dose tested, mortality ranged from 47 to 87%. In contrast, all double ORF56-57 deleted (Δ56-57) strains expressed an attenuated phenotype. No clinical signs were observed in fish inoculated at 4 or 40 pfu/ml, and only a few fish expressed transient mild hyperemia and folding of the dorsal fin at the higher dose (400 pfu/ml). Importantly, all fish exhibited unaltered swimming and feeding behavior. These observations were confirmed by the survival rate, which was very high in all groups (a single fish died in the group inoculated with the highest dose of the Δ56-57Δ134 strain). To investigate if the fish initially inoculated with strains encoding the ORF56-57 deletion developed a protective anti-CyHV-3 immune response, they were challenged at 21 and 42 days post-primary infection (dppi) by cohabitation with fish that were freshly inoculated with the parental FL strain (Fig. 2 and S2 Fig.). Though mock-infected fish were very sensitive to this challenge (reaching 100% mortality rate in nearly all cases), fish previously infected with strains encoding the ORF56-57 deletion at 400 pfu/ml did not express the disease and had a survival rate of 100%. Fish initially inoculated at 40 pfu/ml exhibited also very high survival rates (100% or 93%). Fish initially inoculated at 4 pfu/ml exhibited partial protection ranging from 27% to 80% survival rate. Interestingly, the challenges performed 21 dppi led to comparable results 42 dppi, indicating early onset of protective immunity. The results presented above demonstrate that the double ORF56-57 deletion correlated with a safety/efficacy profile compatible with use of the encoding strain as an attenuated vaccine. Additional truncation of ORF55 and/or deletion of ORF134 did not improve the safety/efficacy profile. The latter result is consistent with our recent study demonstrating that ORF134 does not affect CyHV-3 virulence [31] as observed for other virally encoded IL-10 homologs [32].

Taking into account the results for safety and efficacy, as well as the stability of the ORF56-57 deletion observed when the virus was passed extensively in cell culture (demonstrated by sequencing of both ORFs after passage of the virus under industrial conditions corresponding to vaccine production, Master Seed Virus +5; sequences observed were identical to initially edited sequences), the Δ56-57 strain was selected as an attenuated recombinant vaccine candidate against CyHV-3 disease.

Safety of the Δ56-57 strain studied by the IVIS

Recently, we demonstrated the usefulness of the IVIS to study the portal of entry and spreading of CyHV-3 into its host [33–36]. In the present study, we exploited this technology to investigate the safety of the Δ56-57 strain. To achieve this goal, a recombinant strain encoding both the ORF56-57 deletion and a luciferase (Luc) expression cassette inserted in a previously studied insertion site [33], hereafter called the Δ56-57 Luc strain, was produced by homologous recombination in eukaryotic cells (S3 Fig.). The molecular structure of this strain was confirmed by RFLP, Southern blot analysis (S4 Fig.), and full-length genome sequencing. Full length genome sequence of WT Luc and Δ56-57 Luc strains have been deposited in the GenBank (Accession numbers KP343683 and KP343684, respectively). The two strains share a number of defects in genes other than those noted in other strains. This includes one point deletion in ORF129 (GGGG>GGG), one inversion/deletion in ORF122, one deletion affecting the ends of ORF27 (already mutated in some other CyHV-3 strains) and ORF28. However, since these mutations are present in both WT Luc and Δ56-57 Luc strains, they are not relevant to the attenuated observed phenotype.

Before its use in vivo, the Δ56-57 Luc strain was tested in vitro. First, even if the insertion of the Luc expression cassette in a wild type strain was previously shown to have no effect on viral

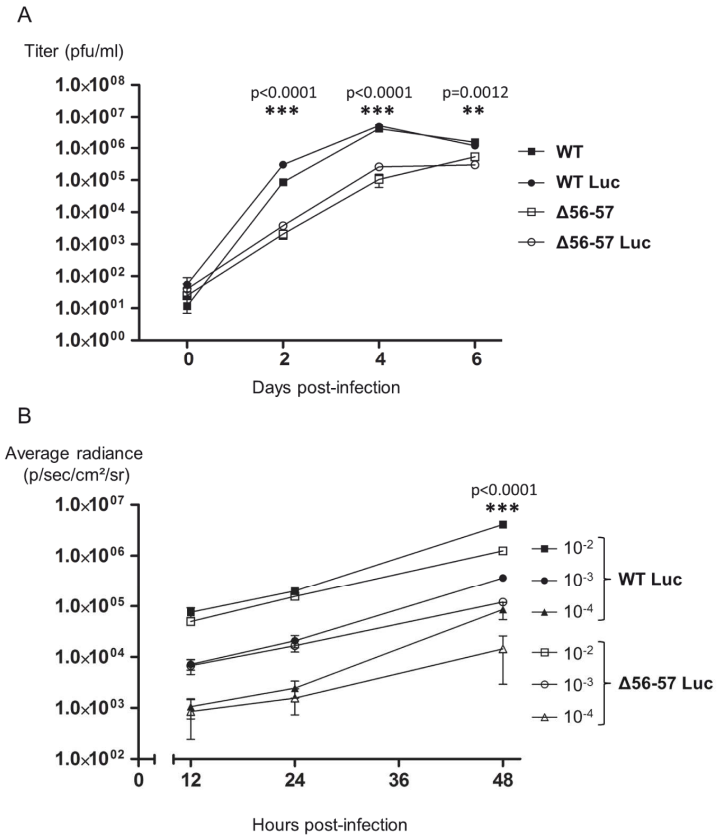


Fig 3. Effect of the double ORF56-57 deletion on CyHV-3 replication in vitro. (A) Multi-step growth curves. CCB cells were infected with the indicated strains and the viral titer (pfu/ml) in the cell supernatant determined at the indicated time points post-infection. Data presented are the mean ± SD of triplicate measurements. Significant differences between the two ORF56-57 wild-type strains (WT and WT Luc) and the two double ORF56–57 deleted strains (Δ56-57 and Δ56-57 Luc) were tested using three-way ANOVA taking ORF56–57 genotype (wild-type vs. deleted), presence or absence of Luc, and time post-infection as variables. The p-values demonstrated a significant effect of ORF56-57 genotype independent of the Luc genotype. (B) Luc activity. CCB cells were infected with the indicated strains at the indicated MOI. Luc expression was analyzed by the IVIS at different times post-infection. Data presented are the radiance ± SD of quadruplicate analyses. Significant differences were tested using three-way ANOVA taking viral strain, MOI, and time post-infection as variables. The p-values demonstrated a significant effect of the viral strain independent of the MOI. The raw data of these experiments (panels A and B) are provided online (S1 Dataset).

doi:10.1371/journal.ppat.1004690.g003

growth in cell culture and virulence in vivo [33], we tested whether this insertion affects viral growth for the Δ56-57 genotype (Fig. 3A). Replication of the Δ56-57 Luc strain was comparable to that of the Δ56-57 strain. Consistent with an earlier report [33], replication of the WT Luc strain was comparable to that of the WT strain. These data demonstrate that insertion of the Luc cassette between ORF136 and ORF137 had no effect on viral growth. However, this experiment revealed that both strains encoding the ORF56-57 deletion replicated at a significantly lower level than the strains encoding the ORF56-57 wild-type genotype ($p<0.0001$ at 2 and 4

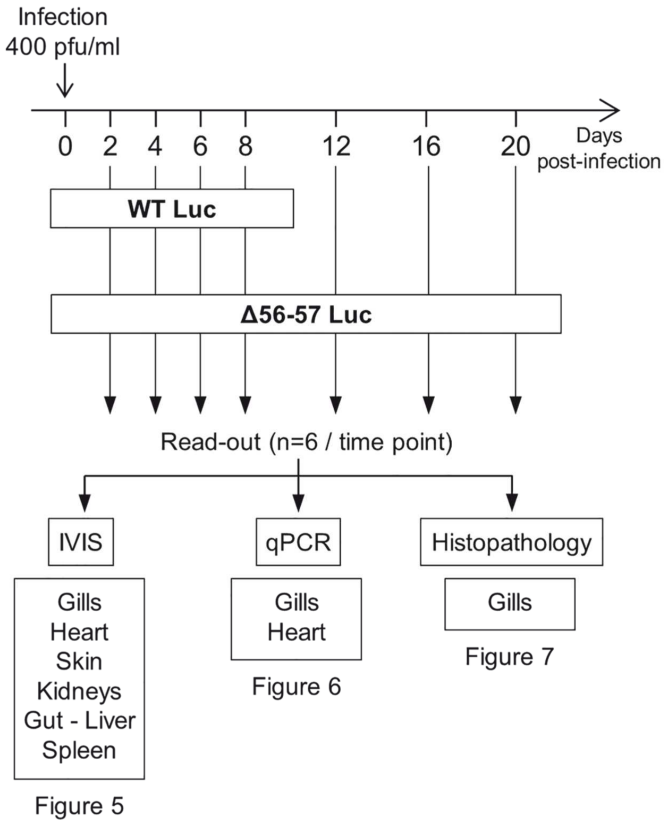


Fig 4. Flow chart of experiments performed to study the effect of the double ORF56-57 deletion on CyHV-3 pathogenesis. At time 0, carp (mean weight ± SD 30.40 g ± 9.12 g, 9 months old) were infected for 2 h by immersion in water containing WT Luc strain or the Δ56-57 Luc strain at 400 pfu/ml, then returned to larger tanks. At the indicated time points (restricted to maximum 8 dpi for the WT Luc strain to precede the peak of mortality), fish were sampled and submitted to the read-outs listed in the lower part of the figure. The read-out data are presented in Figs. 5–7. Throughout these figures, the data obtained for each fish (according to viral strain and time post-infection) are represented by the same symbol to allow correlation of the data obtained for the different organs and with the different read-outs.

doi:10.1371/journal.ppat.1004690.g004

days post-infection (dpi), and p = 0.0012 at 6 dpi). Second, to investigate whether the ORF56-57 deletion affects the expression of the Luc expression cassette, CCB cells were infected at different multiplicity of infection (MOI) with the WT Luc strain and Δ56-57 Luc strain (Fig. 3B). IVIS analyses performed 12 and 24 hours post-infection (hpi) demonstrated that the transduced Luc expression was comparable between the two strains. In contrast, analyses performed 48 hpi revealed significantly faster replication of the WT Luc strain compared to the Δ56-57 Luc strain ($p<0.0001$). Taken together, the results demonstrate that Δ56-57 Luc strain replication in vitro is comparable to that of the Δ56-57 strain, and it transduces Luc expression comparably to the WT Luc strain. These data validate the use of these two Luc recombinant strains to investigate the effect of the double ORF56-57 deletion on the pathogenesis of CyHV-3. This question was investigated by performing the experiment described in Fig. 4.

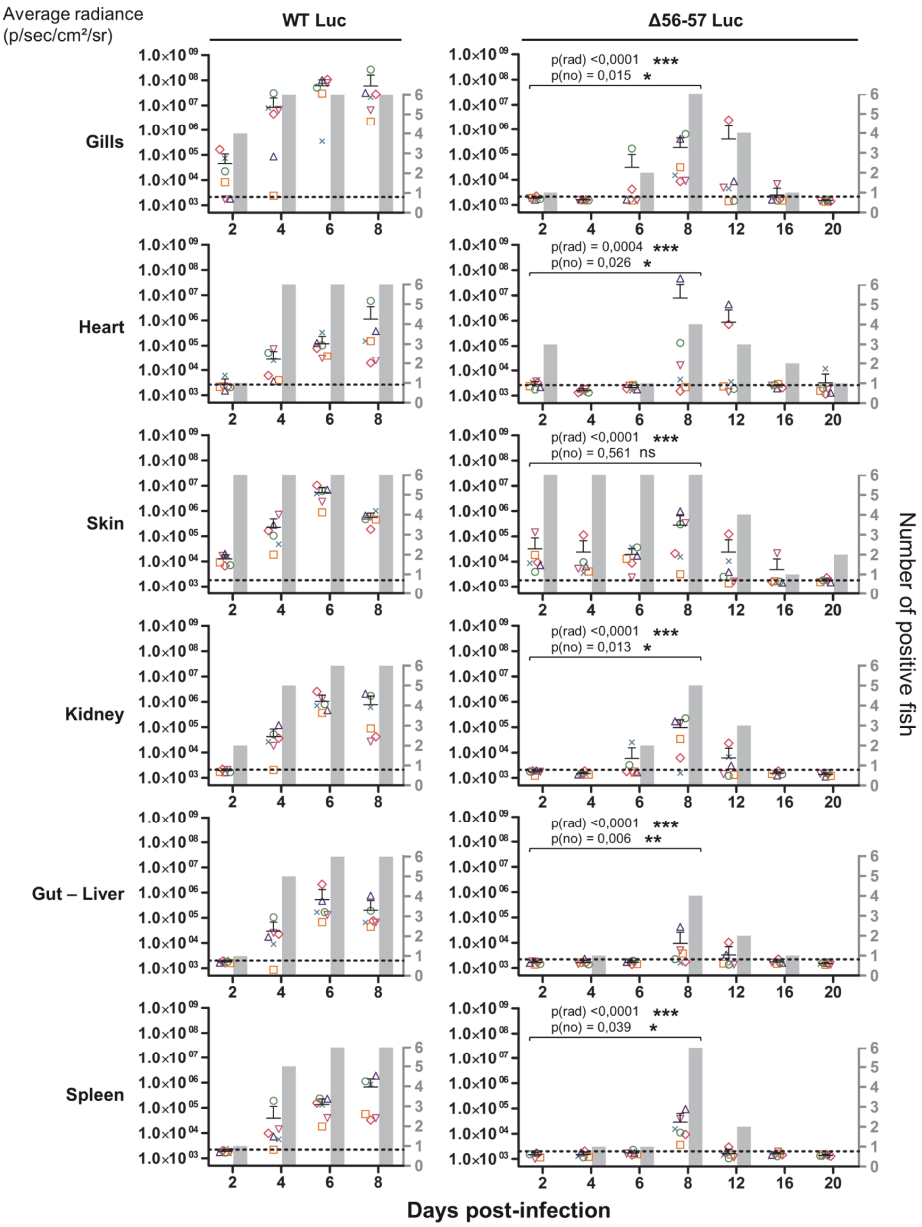


Fig 5. Effect of the double ORF56-57 deletion on viral tropism according to the IVIS. The graphs present the average radiance emitted (p/sec/cm^2/sr) per fish and per indicated organ according to time post-infection (mean + SD is represented for each time point). The average radiance was measured over a region of interest manually drawn following the outline of dissected organs. For the skin, the average radiance (individual values, mean + SD) was measured on both sides of the body, and the results for individual fish are expressed as the mean of both sides. The discontinuous line represents the cut-off for positivity and represents the mean + 3 SD ($p < 0.00135$) of the values obtained (not represented) for mock-infected fish (negative control). The number of positive fish among six analyzed fish is represented by grey bars. Statistical analyses compared the first four time points (2, 4, 6, and 8 dpi) available for both strains. The p-value and level of significance are indicated. The average emitted radiances were compared using a two-way ANOVA taking viral strain and time post-infection as variables (p(rad)), whereas the number of positive fish per group was compared using a permutation test (p(no)).

doi:10.1371/journal.ppat.1004690.g005

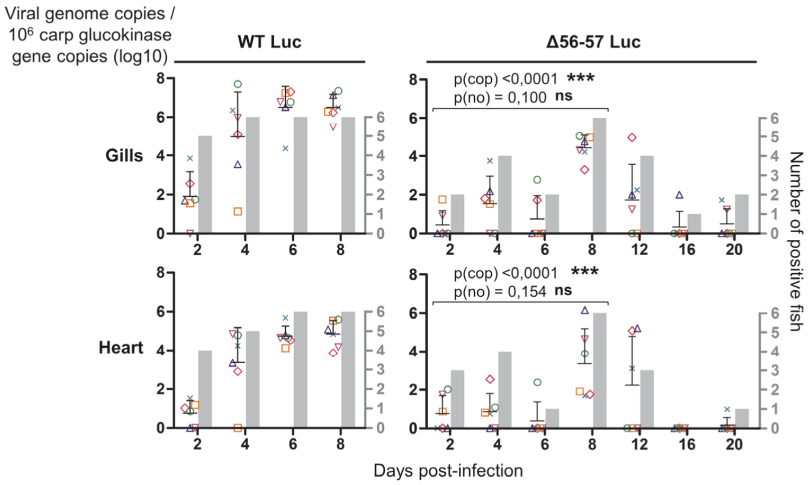


Fig 6. Effect of the double ORF56-57 deletion on viral tropism according to qPCR analysis. The number of viral genome copies is expressed as log_{10} per 10^6 carp glucokinase gene copies. Individual values represent the mean of duplicate measurements. Mock-infected fish were used as a negative control and no viral genome copies were detected in these fish. The number of positive fish among six analyzed fish is represented by grey bars. Statistical analyses compared the first four time points (2, 4, 6, and 8 dpi) available for both strains. Viral charge (Viral genome copies/10^6 carp glucokinase gene copies (log_{10})) was compared using two-way ANOVA taking viral strain and time post-infection as variables (p(cop)), whereas the number of positive fish per group was compared using a permutation test (p(no)).

doi:10.1371/journal.ppat.1004690.g006

Fish were inoculated by immersion in water containing the WT Luc strain or Δ56-57 Luc strain. At different times post-infection (restricted to 8 dpi for the WT Luc strain to precede the peak of mortality), fish were collected and each fish analyzed by the IVIS (Fig. 5), qPCR (Fig. 6), and histopathological examination (Fig. 7). The results of these read-outs were presented throughout the figures using a distinctive constant symbol for each analyzed fish according to the viral strain inoculated and the time post-infection at which the fish was collected. This mode of presentation allows identification of all results obtained for a particular fish in the figures. The skin has been shown to be the major portal of entry of CyHV-3 after inoculation by immersion in water containing the virus [33]. Consistent with this earlier report, we observed that, independent of the inoculated virus, all tested fish expressed light foci on their skin as early as 2 dpi (Fig. 5). Though the signal increased in intensity from 2 to 6 dpi on fish inoculated with the WT Luc strain, it remained stable for 12 dpi on fish inoculated with the Δ56-57 Luc strain. Global statistical analysis of the data obtained during the first 8 dpi demonstrated no significant difference in the number of positive fish between the two virus strains, as all fish were positive, but significantly less light emission was observed for fish infected with the double ORF56-57 deleted recombinant. After initial replication in the skin, the WT Luc strain spread and replicated rapidly in all tested organs, and all fish were positive in all organs at 6 dpi (Fig. 5). These results are consistent with earlier reports [33,34]. Spreading of the Δ56-57 Luc strain within infected fish was significantly delayed, reduced both quantitatively (i.e., number of positive fish) and qualitatively (i.e., intensity of the signal), and transient (Fig. 5, compare left and right columns). The reduced ability of the Δ56-57 Luc strain to spread into infected fish was further supported by qPCR analysis performed on the gills and heart (Fig. 6).

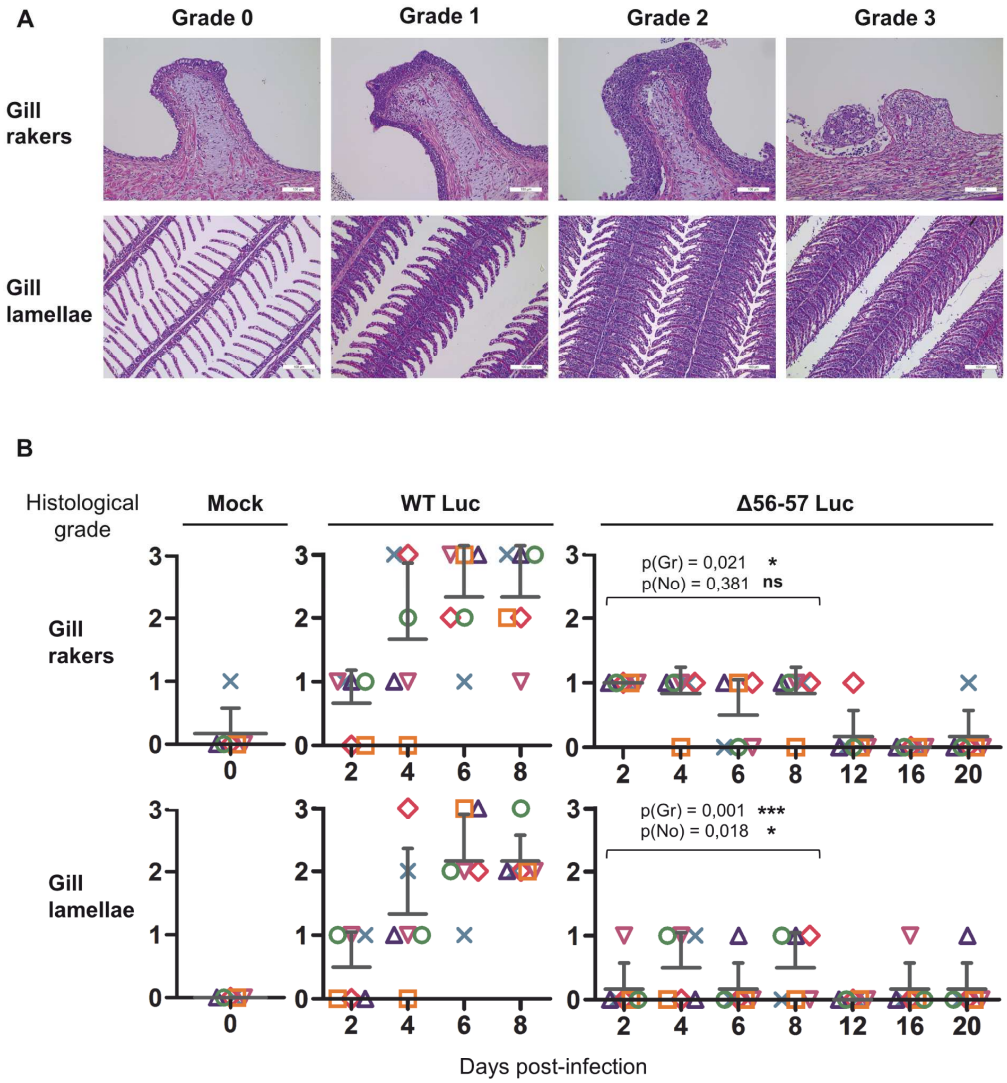


Fig 7. Effect of the double ORF56-57 deletion on viral pathogenesis based on histopathological analysis of gills. (A) Grading used to characterize the lesions observed on gill rakers and gill lamellae. Scale bars = 100 μm. (B) Histological preparations were observed by three independent observers. The grade selected by at least two of the three observers was retained as the final score. The data represent the mean grade + SD for each group at each time point. Statistical analyses compared the first four time points (2, 4, 6, and 8 dpi) available for both strains. The p-value and level of significance are indicated. Two types of statistical analyses were performed using permutation tests: comparison of the severity of the lesions (p(gr)) and comparison of the number of positive fish (p(no)) according to the viral strain.

doi:10.1371/journal.ppat.1004690.g007

Compared to fish infected with the WT Luc strain, fish infected with the double ORF56-57 deleted variant expressed significantly lower viral loads. Taken together, the results in Fig. 5 and 6 demonstrate that the Δ56-57 Luc strain was as capable of entering fish as the wild-type control

strain, but it had a reduced ability to spread in the infected animal, thereby explaining its attenuated phenotype. Finally, as the gills have been shown to support drastic anatomopathological modifications during CyHV-3 disease, they were examined at different times post-infection with the two viral strains (Fig. 7). Examination of both gill rakers and gill lamellae led to the conclusion that the Δ56-57 Luc strain induced much less severe lesions than the WT Luc strain, as demonstrated by the significantly fewer positive fish (gill lamellae but not gill rakers) and significantly lower lesion scores (gill lamellae and gill rakers). These results support the attenuation conferred by the double ORF56-57 deletion and justify the selection of the Δ56-57 strain as a recombinant attenuated vaccine candidate for mass vaccination of carp by immersion in water containing the vaccine.

A key safety aspect of an attenuated recombinant vaccine is the possible spread from vaccinated to unvaccinated naïve cohabitant subjects. This aspect is particularly important for vaccination in aquaculture, where water can act as an efficient abiotic vector. To address this issue, we used the two recombinant Luc strains described above to investigate the effect of the double ORF56-57 deletion on the ability of CyHV-3 to spread from newly infected fish to naïve sentinel fish (Fig. 8). The spread of CyHV-3 was studied using two experimental settings designed to allow transmission of the virus through infectious water (water sharing) or through infectious water and physical contact between infected and naïve sentinel fish (tank sharing). Fish were infected with either the WT Luc strain or the Δ56-57 Luc strain for 2 h in water containing the virus. After rinsing in fresh water, infected fish were distributed in tanks (Fig. 8) for water sharing and tank sharing experiments. The IVIS analysis of initially infected fish 2 dpi (n = 4 for each virus strain) demonstrated that they all expressed luciferase activity on their skin as described in Fig. 5 (2 days post-infection, skin analysis), thereby demonstrating successful infection. The IVIS analysis of sentinel fish was performed after different periods of cohabitation to investigate potential spreading; the experiment was stopped at day 14 for the WT Luc strain because the vast majority of fish were dying from the infection. Under water sharing conditions, no transmission was detected for the Δ56-57 Luc strain over 18 days. In contrast, for the WT Luc strain, positive fish (2 out of 5) were detected as early as 6 days of cohabitation. Both the number and radiance of positive fish increased with time. Under tank sharing conditions, erratic and rare transmission was observed for the Δ56-57 Luc strain. Only two of the 25 analyzed fish were positive respectively after 10 and 18 days of cohabitation. In contrast, naïve sentinel fish cohabiting with fish infected with the WT Luc strain became infected as early as 6 days of cohabitation. Taken together, the results demonstrate that the double ORF56-57 deletion drastically impaired the ability of CyHV-3 to spread from freshly infected fish to naïve sentinel fish.

Efficacy of the Δ56-57 strain studied by the IVIS

The experiments presented above demonstrate the usefulness of the IVIS for studying the safety of an attenuated recombinant vaccine candidate. In the last section of this study, we also used the IVIS to characterize the immune protection conferred by the Δ56-57 strain (Fig. 9). Fish were vaccinated by immersion in water containing two different doses of the Δ56-57 strain. Six weeks post-primary infection, fish were challenged with the WT Luc strain and analyzed by the IVIS at 2, 4, and 8 days post-challenge. Analyses performed on day 2 post-challenge revealed few vaccinated fish (2 fish vaccinated at 40 pfu/ml and 1 fish vaccinated at 400 pfu/ml) expressing a low luciferase signal close to the threshold determined for the mock-infected/mock-challenged group. None of the fish analyzed on day 2 post-challenge expressed a positive signal in internal organs. No positive signal was observed on the skin or internal organs of vaccinated fish at later time points, independent of the dose used for vaccination. In contrast, mock-infected fish

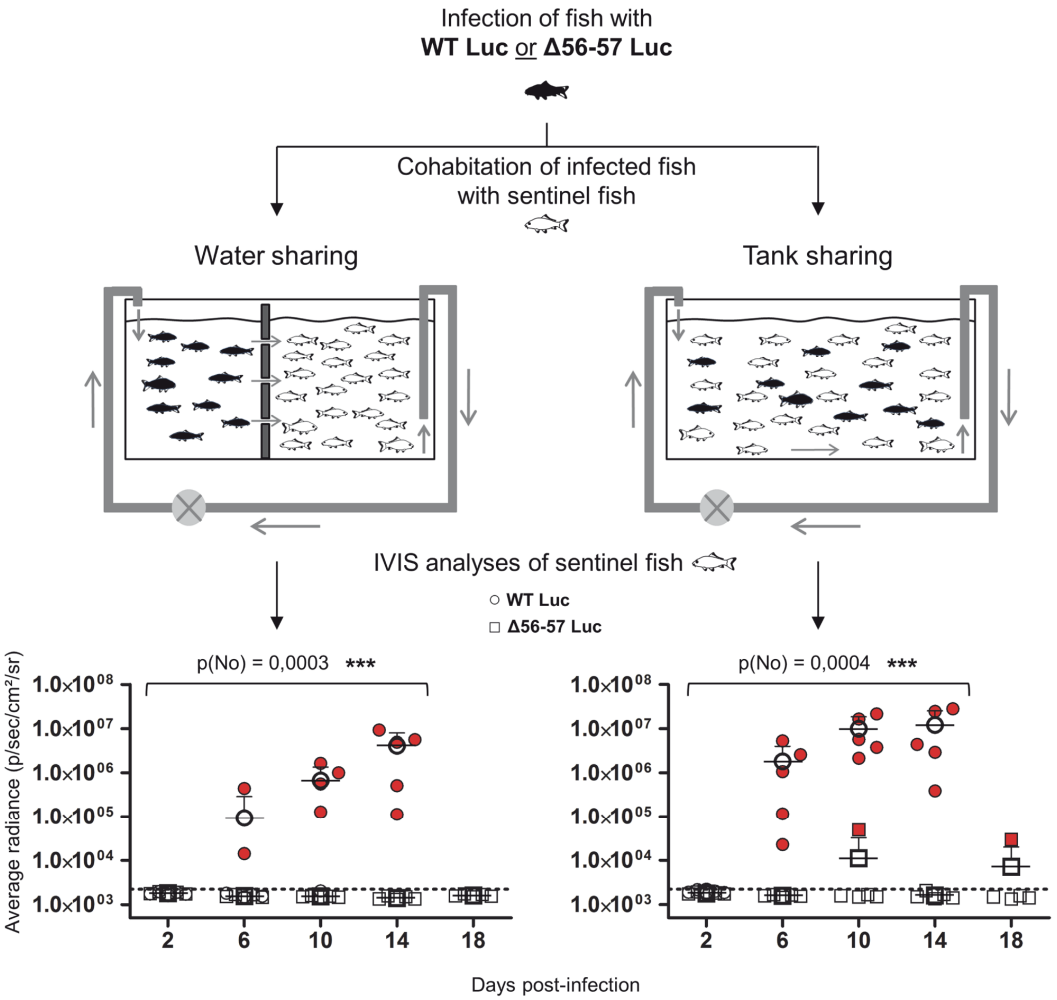


Fig 8. Effect of the double ORF56-57 deletion on CyHV-3 spread from infected fish to naïve fish. This experiment was performed using a group of common carp (mean weight ± SD 7.09 g ± 2.38 g, 9 months old). At time 0, some fish were infected (represented by black fish) by immersion for 2 h in water containing the WT Luc strain or the Δ56-57 Luc strain at 400 pfu/ml. At the end of the inoculation period, infected fish were collected, marked by cutting a fragment of the superior lobe of the caudal fin, and rinsed twice in water before distribution into two types of 60 L tanks (n = 12 per tank). The two types of tanks were designed to study virus transmission through only the water (left, water sharing) or through the water and direct fish-to-fish contact (right, tank sharing). Naive fish (represented by white fish; n = 30 per tank) were distributed to the tanks as illustrated. At the indicated time points post-infection, five naïve fish were collected from each of the four tanks (2 viral strains × 2 tank conditions) and analyzed by IVIS for Luc expression on the skin. The discontinuous line represents the cut-off for positivity ($p < 0.00135$) determined based on the analysis of mock-infected fish. Data represent the analysis of individual fish. Positive scores are presented in red. The mean radiance + SD is presented for each time point. Statistical analyses compared the number of positive fish at the first four time points (2, 4, 6, and 8 dpi) available for both strains using a permutation test. The p-value and level of significance are indicated.

doi:10.1371/journal.ppat.1004690.g008

challenged with the WT Luc strain expressed increasing light intensity according to time post-infection. The data demonstrate that vaccination with the Δ56-57 strain induces a protective mucosal immune response at the portal of entry.

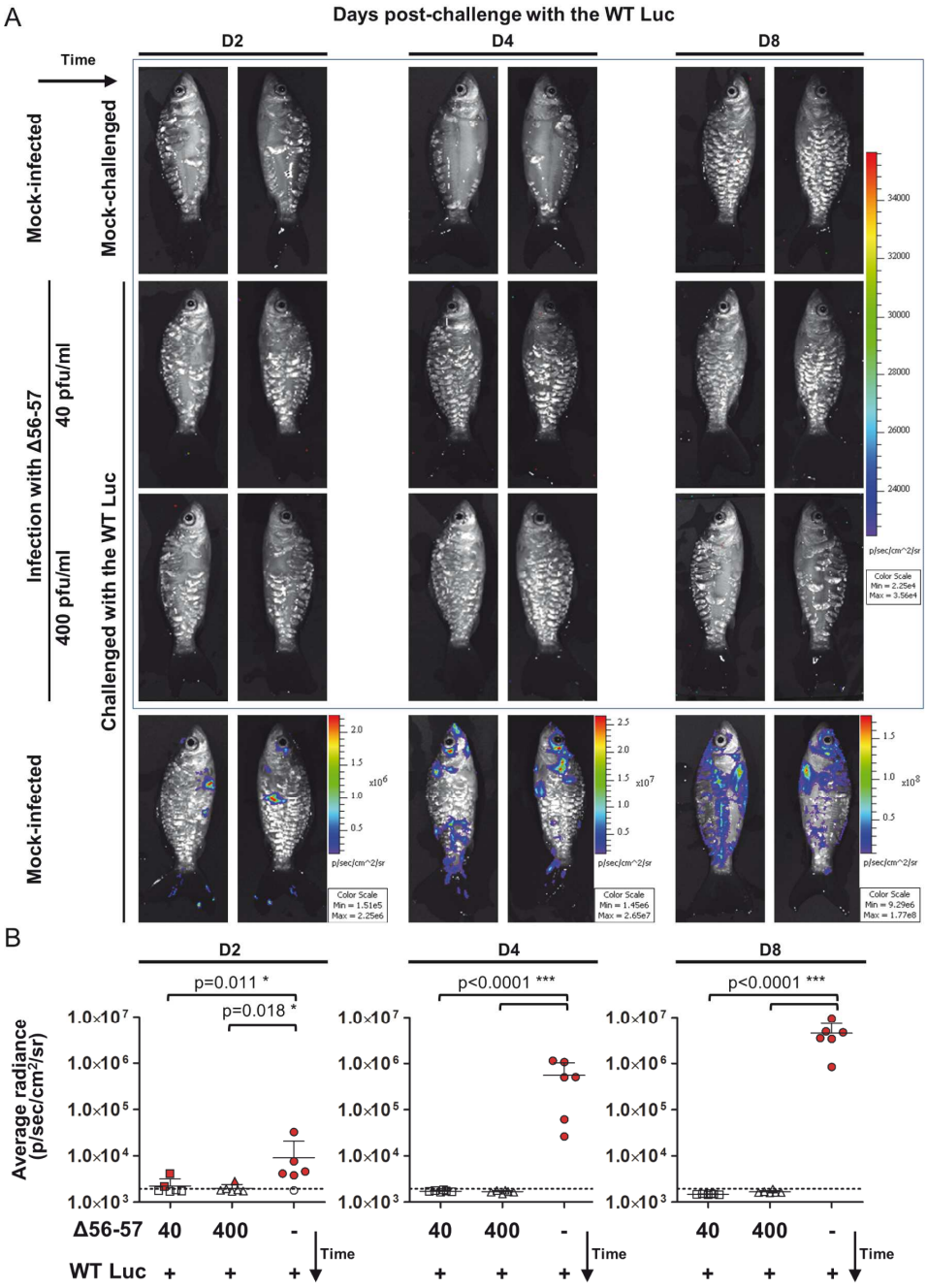


Fig 9. Immune protection conferred by the Δ56-57 strain revealed by the IVIS. Common carp (mean ± SD weight 13.82 ± 5.00 g, 9 months old) were infected for 2 h by immersion in water containing 40 or 400 pfu/ml of the Δ56–57 strain or mock-infected. None of the fish died from primary infection. Forty-two dppi, fish were challenged by immersion for 2 h in water containing 200 pfu/ml of the WT Luc strain. At the indicated time post-challenge, fish (n = 6) were

analyzed using the IVIS. (A) Representative images. Images within the blue frame were normalized using the same scale. (B) Average radiance (individual values, mean + SD) measured on the entire body surface of the fish (individual values represent the mean of the left and right sides obtained for each fish). The discontinuous line represents the cut-off for positivity, which is the mean + 3 SD ($p < 0.00135$) of the values obtained (not presented) for mock-infected and mock–challenged fish (negative control). Positive fish are represented by red filled dots. Significant differences in the mean of the average radiance were identified by post-hoc t-test after two-way ANOVA analysis taking the treatment and the time post-challenge as variables.

doi:10.1371/journal.ppat.1004690.g009

Discussion

In the present study, we took advantage of an accidentally obtained recombinant CyHV-3 strain that exhibited a safety/efficacy profile compatible with use as an attenuated vaccine. This recombinant strain had deletions at three loci (ORF56-57, and ORF134). To determine the role of the double ORF56–57 deletion in the phenotype and to improve further the quality of the vaccine candidate, a series of deleted recombinants was produced and tested in vivo. The Δ56-57 strain with a deletion encompassing ORF56 and ORF57 was selected and characterized as an attenuated recombinant vaccine candidate against CyHV-3. This strain exhibited properties compatible with use as an attenuated recombinant vaccine for mass vaccination of carp by immersion in water containing the virus. It replicated efficiently in vitro, though at a lower level than the parental wild-type strain, expressed a safe attenuated phenotype, and induced a protective mucosal immune response against a lethal challenge by blocking viral infection at the portal of entry.

Deletion of ORF56 and/or ORF57 has not been reported previously for CyHV-3. Sequencing of the Cavoy attenuated anti-CyHV-3 vaccine demonstrated that it encodes a wild-type ORF56 and ORF57 sequence identical to the reference sequence available in the GenBank (Accession number NC_009127.1, sequencing from coordinates 96370 to 101248), whereas immunofluorescent staining of infected cells demonstrated the expression of both proteins (S5 Fig.). The proteins pORF56 and pORF57 have unknown functions. They both lack a signal peptide and transmembrane domain. Though pORF57 is one of the most abundant proteins of the CyHV-3 virion, pORF56 is a non-structural protein [37,38]. Extensive bioinformatic analyses did not generate a hypothesis concerning their putative functions. Homologs of CyHV-3 ORF56 and ORF57 are found in orthologous positions in the two other cyprinid herpesviruses (CyHV-1 and CyHV-2) [39], among which CyHV-2 (also known as goldfish hematopoietic necrosis virus) is responsible for a severe disease initially reported in goldfish (*Carassius auratus auratus*) that recently emerged in gibel carp (*Carassius auratus gibelio*) [40]. Based on the positional orthology of ORF56 and ORF57 in cyprinid herpesviruses, the ORF56-57 deletion reported here for CyHV-3 can likely be exploited to produce attenuated CyHV-1 and CyHV-2 recombinant vaccine candidates. Homologs of CyHV-3 ORF57 are also present in the cyprinivirus anguillid herpesvirus 1 (AngHV-1) [41] and a more distantly related virus, crocodilepox virus (CRV) [39,42], which suggests an ancestral origin for the gene. AngHV-1 infects European and Japanese eel (*Anguilla anguilla* and *Anguilla japonica*) and is responsible for mortalities of up to 30% in cultured and wild eel populations [43]. As part of a follow-up project of the present study, ORF56 and ORF57 single deleted recombinants were produced and were tested in vivo. The data obtained demonstrate that most of the attenuation observed for the double deletion ORF56-57 relied on the deletion of ORF57. These data identify the ORF57 homologue encoded by AngHV-1 (ORF35) as an obvious locus for production of an attenuated recombinant vaccine candidate.

Our earlier finding based on a wild-type CyHV-3 strain suggested that the skin is the major portal of entry after inoculation by immersion in water containing the virus [33]. After initial replication in the skin, wild-type CyHV-3 spread rapidly to virtually all organs (Fig. 5, left column). Soon after the skin, the gills, followed by other organs, support viral infection [34,44,45].

Even if the skin was always the first organ to express luciferase, the early positivity of the gills led to the hypothesis that they could represent an alternative and possibly parallel portal of entry for the virus [45–47]. The present study of the tropism of the Δ56-57 strain demonstrated that it also spreads from the skin to all tested organs. However, compared to the wild-type strain, its systemic spread to the other organs was much slower, and its replication was reduced in intensity and duration (Figs. 5 and 6). The slower spread of the Δ56-57 vaccine strain within infected fish allowed better discrimination of the portal(s) of entry from secondary sites of infection. Though the skin of all fish was positive as early as 2 dpi, all of the other tested organs (including gills and gut) were positive in the majority of fish after 6 dpi. These data further demonstrate that the skin is the major portal of entry of CyHV-3 after infection by immersion and suggest that the other organs (including gills and gut) represent secondary sites of replication.

The double ORF56-57 deletion reduced the ability of the virus to spread within infected fish and impaired virus transmission from infected fish to naïve sentinel fish. In the present study, we designed two tank systems to test the ability of CyHV-3 strains to spread from infected to naïve sentinel subjects through indirect or indirect-and-direct contact (Fig. 8). The absence of detectable transmission under water sharing conditions and the very low level of transmission detected under tank sharing conditions suggested that fish inoculated with the vaccine candidate strain excrete very low amounts of infectious particles during the 18 days following vaccination. Combined with the observation that all vaccinated fish supported skin infection during the first 8 dpi, these data demonstrate that the ORF56-57 deletion drastically impaired viral excretion by infected subjects. Notably, the spread of the vaccine candidate strain was tested in the present study by cohabitation of fish immediately after vaccination. This is in contrast to previous studies that tested spreading by starting cohabitation weeks after vaccination, when replication of the attenuated vaccine strain was ending [27].

In addition to its relevance for applied research, the CyHV-3 - carp model has numerous qualities as a subject of fundamental research [9,11,12]. First, it is phylogenetically distant from the vast majority of herpesviruses studied so far, thereby providing an original field of research [10,39,48]. Second, it can be studied in laboratories by infection of its natural host (homologous virus-host model). Third, it allows the study of the complete infectious cycle of an alloherpesvirus, including transmission from infected to naïve animals. Transmission is an essential step in the biological cycle of pathogens and acts as a main motor of their evolution. However, very few laboratory models currently studied among members of the *Herpesvirales* allow the study of transmission [49]. Here, we report two systems allowing the study of CyHV-3 transmission by indirect or direct contact between infected and naïve sentinel fish. They will be useful for understanding the biology of an alloherpesvirus and for evaluating the ability of prophylactic strategies to inhibit the spread of wild-type strains in a fish population. The difference in transmission kinetic observed between the two systems demonstrated that direct contact between subjects promotes transmission of CyHV-3. Early replication of the virus at the portal of entry should contribute not only to the spread of the virus in infected fish, but also to the spread of the virus in the fish population. As early as 2 to 3 dpi, infected fish rubbed themselves against each other or objects. This behavior could contribute to skin-to-skin transmission [35]. Later during infection, transmission could also occur when uninfected fish peck macroscopic skin herpetic lesions developed by infected fish, thereby being infected by both skin to skin contact and infection of the pharyngeal periodontal mucosa [34]. These data highlight the importance of inducing a mucosal immune response through vaccination against CyHV-3.

Immunization of carp by immersion in water containing the vaccine candidate strain induced protective mucosal immunity, preventing replication of a challenging strain. Vaccination by immersion has several advantages. Firstly, it is compatible with mass vaccination.

Secondly, in contrast to injection based vaccination, it is not restricted by a minimum fish size. Though the experiments reported in the present study were performed with fish older than 6 months, the safety and the efficacy of the Δ56-57 strain has been demonstrated in younger fish (4 months old fish, mean weight of 1.3 g). These data demonstrated that the Δ56-57 strain is compatible with vaccination of carp soon after they acquired a competent adaptive immune system (at the end of the second month of life [50,51]). Thirdly, in contrast to the oral route, which favors the greediest fish, vaccination by immersion delivers a comparable dose to each fish. Finally, it induces antigen exposition and, hopefully, an adaptive immune response at the portal of entry used by the pathogen. The data presented in Fig. 9 demonstrate that the adaptive immune response induced by the vaccine candidate prevented infection of a challenging strain at the portal of entry. The mucosal immunity induced against CyHV-3 at the portal of entry could contribute not only to protecting vaccinated fish from CyHV-3 disease (clinical protection), but also to preventing them from transmitting virulent circulating strains (sterile immunity), thereby inducing herd immunity. This hypothesis is currently being tested using the two tank systems described above. The skin of teleost fish is a pluristratified epithelium composed exclusively of living cells covered by a mucus layer [35]. The large surface area of this mucosa, combined with its easy access, promoted the study of its innate and adaptive immune components. Interest in teleost skin as a model for studying comparative mucosal immunity recently increased with the discovery of a new immunoglobulin isotype, IgT (or IgZ) [52–54], specialized in mucosal immunity [55–58]. This specific mucosal adaptive immune response further supports the importance of antigen presentation at the pathogen's portal of entry to induce topologically adequate immune protection capable of blocking pathogen entry into the host [59,60]. Future studies are required to unravel the mechanisms underlying the mucosal immune protection conferred by the anti-CyHV-3 vaccine candidate developed in the present study. However, this study provides an original model for studying epidermal mucosal immunity against an infectious agent in teleosts.

Since it was first described in the late 1990s, CyHV-3 rapidly spread to different continents, causing severe financial losses in the common carp and koi culture industries worldwide [9]. In addition to its negative economical and societal impacts, CyHV-3 also has a negative environmental impact by affecting wild carp populations [61,62]. Thus, CyHV-3 rapidly became the subject of applied research aiming to develop diagnostic methods and a safe/efficacious vaccine. In the present study, we used BAC cloning mutagenesis and IVIS technology to develop and characterize the first rationally designed attenuated recombinant CyHV-3 vaccine compatible with mass vaccination. In addition, the present study demonstrated the importance of the CyHV-3 - carp model as an interesting and original fundamental subject of research. This model allows the study of the complete infectious cycle (including transmission from infected to naïve animals) of an alloherpesvirus by infection of its natural host. Furthermore, infection of carp by CyHV-3 represents a unique model for studying skin mucosal immunity in teleosts in response to a natural infection.

Materials and Methods

Cells and viruses

CCB cells [63] were cultured in minimum essential medium (Sigma) containing 4.5 g/L glucose (D-glucose monohydrate; Merck) and 10% fetal calf serum (FCS) as described previously [24]. The CyHV-3 FL strain was isolated from the kidney of a fish that died from CyHV-3 infection and previously used to produce the FL BAC plasmid [24]. The FL BAC revertant ORF136 Luc strain (called WT Luc in the present study) of CyHV-3 was derived from the FL BAC plasmid

by prokaryotic mutagenesis [33]. The Cavoy strain was cultured from the Cavoy vaccine (Novartis).

Fish

Common carp (*Cyprinus carpio carpio*) were kept in 60 L tanks at 24°C. Water parameters were checked twice per week. Microbiological, parasitic, and clinical examinations of the fish just before the experiments demonstrated that they were healthy. All experiments were preceded by an acclimation period of at least 2 weeks.

Inoculation of fish with CyHV-3

Two modes of inoculation were used: inoculation by immersion in infectious water and inoculation by cohabitation with newly infected fish. Fish were inoculated by immersion in water (volume adapted based on fish size and fish number to use a biomass around 10%) containing the virus for 2 h under constant aeration. At the end of the incubation period, the fish were returned to 60 L tanks. For inoculation by cohabitation, newly infected fish were produced by immersion of naïve fish for 2 h in water containing 200 pfu/ml of the FL strain. At the end of the incubation period, newly infected fish were released into the tank of fish to be contaminated at a ratio of 2 newly infected fish per 15 fish to be contaminated.

Ethics statement

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

Production of CyHV-3 recombinant strains using BAC cloning and prokaryotic recombination technologies

Different recombinant BAC plasmids were produced using the FL BAC [24] and FL BAC ORF134 Del plasmids [31] as parental plasmids (Fig. 1B). Recombinant plasmids were produced based on the strategy described in Fig. 1B using *galK* positive/negative selection in bacteria as described previously [64]. Recombination cassettes encoding *galK* were produced by PCR using the primers listed in Table 1 and the p*galK* vector as the template. The ORF56-57 Del cassette consisted of 250 bp upstream (coordinates 96751-97000) and 249 bp downstream (coordinates 99751-100000 with deletion of base 99760) of the ORF56-57 deletion (Fig. 1A, ORF56-57 deletion). To reconstitute the infectious virus, the recombinant BAC plasmids were co-transfected in CCB cells using polyethylenimine (3 μg polyethylenimine per 1 μg DNA) with either the pGEMT-TK plasmid or pEFIN3 NLS Cre (molecular ratio 1:75) [24]. Transfection with pGEMT-TK plasmid induced recombination upstream and downstream of the BAC cassette, leading to its complete removal and consequent reversion to a wild-type TK locus (FL BAC revertant strains). Transfection with pEFIN3 NLS Cre induced expression of a nuclear Cre recombinase and cre-loxP-mediated excision of the BAC cassette. Viruses reconstituted (FL BAC excised strains) by this procedure express a truncated form of TK due to a 172 bp foreign sequence of the BAC cassette left in the ORF55 locus. Independent of the method, EGFP-negative plaques (the BAC cassette encodes an EGFP expression cassette) were picked and amplified.

Table 1. Primers used in this study.

	Primer name	Sequence (5'- 3')	Coordinates*/ Accession number
		Synthesis of probes for Southern blot analysis	
Probe name			
CyHV-3 ORF55	ORF55InF	AGCGCTACACCGAAGAGTCC	95990–96009
	ORF55stopR	TCACAGGATAGATATGTTACAAG	96516–96494
CyHV-3 ORF 56–57 Del	ORF56–57Pr5F	GGTACAAGACGGCCTGCTG	97247–97265
	ORF56–57Pr9R	GCCAGCACGTAGAGCTTGTG	99686–99667
CyHV-3 ORF134 Del	ORF134InF	GGTTTCTCTTTGTAGTTTTCCG	229362–229383
	ORF134InR	CACCCCAACTTTTGAGACAAC	229795–229765
CyHV-3 ORF136–137	ORF136F2	ATGAAGGCCTCTAAACTGCTG	231339–231359
	ORF137R2	ATGGACAGCACAAACGTTAC	233804–233823
Luc	LUCF3	TTGTGGATCTGGATACCGGG	
	LUCR3	GACACCTGCGTCGAAGATGT	
		qPCR analysis	
Gene amplified			
CyHV-3 ORF89	KHV-86F	GACGCCGGAGACCTTGTG	AF411803
	KHV-163R	CGGGTTCTTATTTTTGTCCTTGTT	
	KHV-109P	(6FAM) CTTCCTCTGCTCGGCGAGCACG (BHQ1)	
Carp glucokinase	CgGluc-162F	ACTGCGAGTGGAGACACATGAT	AF053332
	CgGluc-230R	TCAGGTGTGGAGCGGACAT	
	CgGluc-185P	(6FAM) AAGCCAGTGTCAAAATGCTGCCCACT (BHQ1)	
		Synthesis of recombination cassettes	
Cassette name			
ORF 134 Del *galK*	ORF134 *galK* F	ATGTTCCTTGCAGTGCTACTAACCGCGACCATCTTCTTCGAGGCTCGGGG*CCTGTTGACAATTAATCATCGGCA*	229791–229840
	ORF134 *galK* R	TCAATGTTTGCGCTTGGTTTTCATGTTCTTGACGTCTTTTGCGACCAGGA*TCAGCACTGTCCTGCTCCTT*	229217–229266
ORF 56–57 Del *galK*	ORF56–57 *galK* F	GTCCCTCGACAGCCCCAGCCCGCACAGCAGTCGCCACTCTTCCCTGTTGA*TCAGCACTGTCCTGCTCCTT*	96951–97000
	ORF56–57 *galK* R	AACCCGTACACGACGCGCTCAAGCAGCTTGATCTTGACGACGTCGTGCAC*CCTGTTGACAATTAATCATCGGCA*	99800–99751

*Coordinates based on the reference CyHV-3 genome (GenBank accession number: NC_009127.1)

Underlined: 50 bp corresponding to the CyHV-3 sequence

Italic: sequence corresponding to *galK*

doi:10.1371/journal.ppat.1004690.t001

Production of the Δ56-57 Luc strain by recombination in eukaryotic cells

The Δ56-57 Luc strain was produced by co-infection of CCB cells with two parental strains (S3 Fig.). CCB cells were superinfected at the MOI of 10 pfu/cell with the WT Luc strain and Δ56-57 strain (ratio 1:1). When all cells expressed a cytopathic effect, the supernatant containing progeny virions was collected and submitted to limiting dilution of clone virions. The recombinant viral clone expressing both Luc and ORF56-57 deletion was selected by successive screening with the IVIS and PCR genotyping, respectively.

Production of antibodies raised against pORF56 and pORF57

Mouse polyclonal antibodies (pAb) directed against the unstructured domain (IUPred, http://iupred.enzim.hu) of pORF56 encoded by coordinates 98049-99398 (GenBank accession number NC_009127.1) were produced by DNA immunization using a customized commercial service (DelphiGenetics). Mouse monoclonal antibodies (mAb) directed against pORF57 were selected from a bank of mAbs raised against CyHV-3 structural proteins; mAb 6B2 recognizes an epitope expressed in the last 165 amino acid residues of pORF57 (genomic coordinates 100309-100803, GenBank accession number NC_009127.1).

Indirect immunofluorescence staining

Cells were fixed in PBS containing 4% (w/v) paraformaldehyde at 4°C for 15 min and then 20°C for 30 min. After washing with PBS, samples were permeabilized in PBS containing 0.1% (v/v) NP-40 at 37°C for 15 min. Immunofluorescent staining (incubation and washes) was performed in PBS containing 10% FCS (v/v). Mouse pAb raised against pORF56 (diluted 1:500), mAb 6B2 raised against pORF57 (diluted 1:2500), and rabbit pAb raised against CyHV-3 structural proteins (diluted 1:1500) were used as the primary antibodies. The primary antibody was incubated at 37°C for 1 h. Alexa Fluor 488 goat anti-mouse immunoglobulin G (H+L) and Alexa Fluor 568 goat anti-rabbit immunoglobulin G (H+L) (Invitrogen) were used as the secondary antibodies. The secondary antibody was incubated at 37°C for 30 min. After washing, cells were mounted using Prolong Gold Antifade Reagent with DAPI (Invitrogen).

Genetic characterization of CyHV-3 recombinants

CyHV-3 recombinants were characterized by RFLP using SacI digestion, Southern blot analyses [31,33], sequencing of regions of interest (ROIs), and for some recombinants, full-length genome sequencing. For full-length genome sequencing, DNA (500 ng) was sheared by sonication to an average size of 400 bp and prepared for sequencing using a KAPA library preparation kit (KAPA Biosystems, Woburn, MA, USA). The fragments were A-tailed, ligated to the NEBnext Illumina adaptor (New England Biolabs, Ipswich, MA, USA), and amplified by PCR. Index tags were added by six cycles of PCR using KAPA HiFi HotStart and NEBnext indexing primers. The samples were analyzed using a MiSeq DNA sequencer running v2 chemistry (Illumina, San Diego, CA, USA). Approximately 1 million 250-nucleotide paired-end reads were obtained per sample. The reads were prepared for assembly using Trim Galore v. 0.2.2 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore). A scaffold was assembled de novo for the WT Luc strain using AbySS [65] and finished by referencing GenBank accession number NC_009127.1. Sequence accuracy was checked by assembling the reads against this sequence using BWA v. 0.6.2-r126 [66] and visualizing the alignment using Tablet v. 1.13.08.05 [67]. The other sequences were obtained by assembling the relevant reads against conceptual modifications of the sequence of the WT Luc strain using BWA and checking it using Tablet. In all viral genomes, two regions were of undetermined length: an A repeat and a GA repeat

located at 32540–32465 and 177568-177730 nt, respectively, in NC_009127.1. The full length genome sequence of the WT Luc and Δ56-57 Luc strains were deposited in the GenBank (Accession numbers KP343683 and KP343684, respectively).

Multi-step growth curves

Triplicate cultures of CCB cells were infected at a MOI of 0.1 pfu/cell. After an incubation period of 2 h, cells were washed with PBS and overlaid with Dulbecco's modified essential medium (DMEM, Sigma) containing 4.5 g of glucose/L and 10% FCS. Supernatant was removed from the infected cultures at successive intervals (0, 2, 4, and 6 dpi) and stored at-80°C. The titration of infectious viral particles was determined by duplicate plaque assays in CCB cells as described previously [31,33].

In vivo bioluminescent imaging

Firefly (*Photinus pyralis*) luciferase was imaged using an IVIS (IVIS spectrum, Caliper LifeSciences) as described previously [33–35]. For cell culture analysis, the culture medium was replaced with fresh medium containing D-luciferin (150 μg/ml) (Caliper LifeSciences). Analyses were performed after an incubation period of 5 min at room temperature. For in vivo analyses, fish were anesthetized with benzocaine (25 mg/L of water). Fifteen minutes before bioluminescence analysis, D-luciferin (150 mg/kg of body weight) was injected into the peritoneal cavity. After 15 minutes, the fish were analyzed in vivo lying on their right and left sides and ex vivo after euthanasia. Dissected organs were analyzed independent from the body. All images presented in this study were acquired using a field view of C or D, a maximum auto-exposure time of 1 minute, a binning factor of 4, and a f/stop of 1. The relative intensities of transmitted light from bioluminescence and scales were determined automatically and represented as a pseudocolor image ranging from violet (least intense) to red (most intense) using Living Image 3.2 software. ROIs were drawn manually by surrounding the organs or body outline, and the average radiance (p/sec/cm^2/sr) was taken as the final measure of the bioluminescence emitted over the ROI.

Quantification of virus genome copies in organs by real-time TaqMan PCR

The virus genome was quantified by real-time TaqMan PCR as described previously [31], by amplifying fragments of the CyHV-3 ORF89 and carp glucokinase genes. The primers and probes are listed in Table 1.

Histopathological analysis of gills

Gills were dissected immediately after euthanasia and fixed in 4% buffered formalin before embedding in paraffin [31]. Five-micrometer sections were stained with hematoxylin and eosin, mounted, and examined by microscopy. Three independent observers scored the lesions in a blind test mode using a 4-step scale. For each sample, a score was attributed for the gill rakers and gill lamellae. When at least two observers agreed on a grade, the corresponding grade was attributed. In a few exceptional cases, when all three examiners scored differently, additional analyses were performed until achieving a majority. The grading system evaluated the degree of epithelial hyperplasia, the presence of intra-nuclear viral inclusions, and cell degeneration (Fig. 7A). Briefly, grade 0 = physiological state; grade 1 = mild hyperplasia without evidence of degenerated cells and viral inclusions; grade 2 = severe hyperplasia and presence of few degenerated cells and viral inclusions; and grade 3 = the presence of abundant degenerated

cells and viral inclusions (gill lamellae and gill rakers), massive epithelial hyperplasia filling the entire secondary lamellae inter-space (gill lamellae), and ulcerative erosion of the epithelium (gill rakers).

Statistical analysis

The number of positive fish or positive organs (qualitative data) according to the two viral strains was compared by a permutation test (Figs. 5–8). Briefly, all occurrences were recorded in a dataset (observed dataset). A series of 10,000 random repetitions of the same procedure was performed to allocate positive events to the two groups, creating the shuffled datasets. For each dataset (observed dataset and shuffled datasets), the global difference between the two groups was calculated by summing the daily observed differences. The proportion of shuffled datasets with a global difference greater or equal to the global difference in the observed dataset was then taken as the p-value. Histopathological grading results were compared by a permutation test after calculating the value per group and per day corresponding to the sum of all individual grades obtained in each group (Fig. 7). Viral growth (Fig. 3A), IVIS (Figs. 3B, 5, and 9B), and qPCR results (Fig. 6) were compared as quantitative data (level of positivity) using two- or three-way ANOVA with interactions followed by post-hoc t-test. The variables used and comparisons retained for statistical illustrations are described in their respective figure legends. Presence or absence of statistical significance is represented as follows, ns: not significant, $*$ $p<0.05$, $**$ $p<0.01$, $***$ $p<0.001$.

Supporting Information

S1 Fig. Structural analysis of double Δ56-57 and triple Δ56-57Δ134 deleted recombinants. The indicated strains were analyzed by SacI restriction (left) and Southern blotting using ORF55, ORF56-57 Del, and ORF134 Del probes. Black and white arrowheads indicate fragments containing ORF134 and ORF56-57, respectively. Markers sizes (MS) are indicated on the left.
(TIF)

S2 Fig. Safety-efficacy profile of double Δ56-57 and triple Δ56-57Δ134 deleted recombinants encoding a truncated TK locus. The safety and efficacy of the indicated recombinant strains was tested in common carp (average weight 4.41 g ± 1.78 g, 7 months old). On day 0, fish were infected for 2 h by immersion in water containing 4 (□), 40 (○), or 400 (x) pfu/ml. Safety was investigated by measuring the survival rate for 21 days in a group of 30 carp. Efficacy was tested at 21 and 42 dppi. Mock-infected fish and fish that survived the primary infection were distributed in tanks (n = 15) and challenged by cohabitation with fish infected with the FL strain.
(TIF)

S3 Fig. Production of a recombinant Δ56-57 strain expressing Luciferase. (A) Molecular structure of the Δ56-57 strain and WT Luc strain used as parental strains for production of the Δ56-57 Luc strain by co-infection in eukaryotic cells. ORFs are represented by white or grey arrows, the Luc cassette by a grey rectangle, and CyHV-3 terminal repeats by white rectangles. The positions of SacI restriction sites and restriction fragment lengths are shown below each genotype. (B) Schematic representation of the method used to produce recombinant strains by co-infection of two parental strains. The molecular structures of the four possible recombinant strains resulting from unique or multiple cross-overs are illustrated.
(TIF)

S4 Fig. Structure analysis of the Δ56-57 Luc strain. The indicated strains were analyzed by SacI restriction (left) and Southern blotting using ORF55, ORF56-57 Del, ORF136-137, and Luc probes. White and white-outlined black arrowheads indicate fragments containing ORF56-57 loci and ORF136-137 loci, respectively. Black arrowheads indicate the restriction fragment containing most of the Luc cassette sequence. Marker sizes (MS) are indicated on the left.
(TIF)

S5 Fig. pORF56 and pORF57 expression by the Cavoy strain of CyHV-3. CCB cells were infected with the indicated strains. One day post-infection, cells were treated for indirect immunofluorescent staining and confocal microscopic analysis of pORF56 or pORF57 (green), CyHV-3 structural proteins (red), and cell nuclei (blue). The overlay represents the superposition of the three channels. White scale bars = 20 μm.
(TIF)

S1 Dataset. Raw data of the experiments presented in Fig. 3.
(XLSX)

Acknowledgments

The authors are grateful to Cédric Delforge, Emeline Deglaire, Justine Javaux, and Lorène Dams for their excellent technical assistance.

Author Contributions

Conceived and designed the experiments: MB MR AV. Performed the experiments: MB MR PO GF KR GSW FL HL AV. Analyzed the data: MB MR FF CB DD AJD AV. Contributed reagents/materials/analysis tools: AR KR GSW FL HL. Wrote the paper: MB MR FF CB AJD AV.

References

1. F.A.O. (2014) The State of World Fisheries and Aquaculture 2014. Rome. 223 p.
2. Muir J (2005) Managing to harvest? Perspectives on the potential of aquaculture. Philos Trans R Soc Lond B Biol Sci 360: 191–218. PMID: 15713597
3. Bostock J, McAndrew B, Richards R, Jauncey K, Telfer T, et al. (2010) Aquaculture: global status and trends. Philos Trans R Soc Lond B Biol Sci 365: 2897–2912. doi: 10.1098/rstb.2010.0170 PMID: 20713392
4. Salgado-Miranda C, Loza-Rubio E, Rojas-Anaya E, Garcia-Espinosa G (2013) Viral vaccines for bony fish: past, present and future. Expert Rev Vaccines 12: 567–578. doi: 10.1586/erv.13.38 PMID: 23659303
5. Sommerset I, Krossoy B, Biering E, Frost P (2005) Vaccines for fish in aquaculture. Expert Rev Vaccines 4: 89–101. PMID: 15757476
6. Rodgers CJ, Mohan CV, Peeler EJ (2011) The spread of pathogens through trade in aquatic animals and their products. Rev Sci Tech 30: 241–256. PMID: 21809767
7. Balon EK (1995) Origin and domestication of the wild carp, *Cyprinus carpio*: from Roman gourmets to the swimming flowers. Aquaculture 129: 3–48.
8. F.A.O. Global Aquaculture Production 1950–2012. Available http://www.fao.org/fishery/statistics/global-aquaculture-production/query/fr/en/. Accessed 22 May 2014.
9. Rakus K, Ouyang P, Boutier M, Ronsmans M, Reschner A, et al. (2013) Cyprinid herpesvirus 3: an interesting virus for applied and fundamental research. Vet Res 44: 85. doi: 10.1186/1297-9716-44-85 PMID: 24073814
10. Waltzek TB, Kelley GO, Stone DM, Way K, Hanson L, et al. (2005) Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family Herpesviridae. J Gen Virol 86: 1659–1667. PMID: 15914843

11. Michel B, Fournier G, Lieffrig F, Costes B, Vanderplasschen A (2010) Cyprinid herpesvirus 3. Emerg Infect Dis 16: 1835–1843. doi: 10.3201/eid1612.100593 PMID: 21122210
12. Adamek M, Steinhagen D, Irnazarow I, Hikima J, Jung TS, et al. (2014) Biology and host response to Cyprinid herpesvirus 3 infection in common carp. Dev Comp Immunol 43: 151–159. doi: 10.1016/j.dci.2013.08.015 PMID: 23981329
13. Bondad-Reantaso MG, Subasinghe RP, Arthur JR, Ogawa K, Chinabut S, et al. (2005) Disease and health management in Asian aquaculture. Vet Parasitol 132: 249–272. PMID: 16099592
14. Sunarto A, Rukyani A, Itami T (2005) Indonesian Experience on the Outbreak of Koi Herpesvirus in Koi and Carp (*Cyprinus carpio*). Bull Fish Res Agen Supplement 2: 15–21.
15. Brudeseth BE, Wiulsrod R, Fredriksen BN, Lindmo K, Lokling KE, et al. (2013) Status and future perspectives of vaccines for industrialised fin-fish farming. Fish Shellfish Immunol 35: 1759–1768. doi: 10.1016/j.fsi.2013.05.029 PMID: 23769873
16. Plant KP, Lapatra SE (2011) Advances in fish vaccine delivery. Dev Comp Immunol 35: 1256–1262. doi: 10.1016/j.dci.2011.03.007 PMID: 21414351
17. Lorenzen N, LaPatra SE (2005) DNA vaccines for aquacultured fish. Rev Sci Tech 24: 201–213. PMID: 16110889
18. Perelberg A, Ilouze M, Kotler M, Steinitz M (2008) Antibody response and resistance of *Cyprinus carpio* immunized with cyprinid herpes virus 3 (CyHV-3). Vaccine 26: 3750–3756. doi: 10.1016/j.vaccine.2008.04.057 PMID: 18534721
19. Perelberg A, Ronen A, Hutoran M, Smith Y, Kotler M (2005) Protection of cultured *Cyprinus carpio* against a lethal viral disease by an attenuated virus vaccine. Vaccine 23: 3396–3403. PMID: 15837363
20. Ronen A, Perelberg A, Abramowitz J, Hutoran M, Tinman S, et al. (2003) Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*. Vaccine 21: 4677–4684. PMID: 14585675
21. Zhou J, Xue J, Wang Q, Zhu X, Li X, et al. (2014) Vaccination of plasmid DNA encoding ORF81 gene of CJ strains of KHV provides protection to immunized carp. In Vitro Cell Dev Biol Anim 50: 489–495. doi: 10.1007/s11626-014-9737-2 PMID: 24619212
22. Zhou JX, Wang H, Li XW, Zhu X, Lu WL, et al. (2014) Construction of KHV-CJ ORF25 DNA vaccine and immune challenge test. J Fish Dis 37: 319–325. doi: 10.1111/jfd.12105 PMID: 23962282
23. Fuchs W, Fichtner D, Bergmann SM, Mettenleiter TC (2011) Generation and characterization of koi herpesvirus recombinants lacking viral enzymes of nucleotide metabolism. Arch Virol 156: 1059–1063. doi: 10.1007/s00705-011-0953-8 PMID: 21387205
24. Costes B, Fournier G, Michel B, Delforge C, Raj VS, et al. (2008) Cloning of the koi herpesvirus genome as an infectious bacterial artificial chromosome demonstrates that disruption of the thymidine kinase locus induces partial attenuation in *Cyprinus carpio koi*. J Virol 82: 4955–4964. doi: 10.1128/JVI.00211-08 PMID: 18337580
25. Yasumoto S, Kuzuya Y, Yasuda M, Yoshimura T, Miyazaki T (2006) Oral Immunization of Common Carp with a Liposome Vaccine Fusing Koi Herpesvirus Antigen. Fish Pathol 41: 141–145.
26. O'Connor MR, Farver TB, Malm KV, Yun SC, Marty GD, et al. (2014) Protective immunity of a modified-live cyprinid herpesvirus 3 vaccine in koi (*Cyprinus carpio koi*) 13 months after vaccination. Am J Vet Res 75: 905–911. doi: 10.2460/ajvr.75.10.905 PMID: 25255180
27. Weber EP 3rd, Malm KV, Yun SC, Campbell LA, Kass PH, et al. (2014) Efficacy and safety of a modified-live cyprinid herpesvirus 3 vaccine in koi (*Cyprinus carpio koi*) for prevention of koi herpesvirus disease. Am J Vet Res 75: 899–904. doi: 10.2460/ajvr.75.10.899 PMID: 25255179
28. Meeusen EN, Walker J, Peters A, Pastoret PP, Jungersen G (2007) Current status of veterinary vaccines. Clin Microbiol Rev 20: 489–510. PMID: 17630337
29. Lauring AS, Jones JO, Andino R (2010) Rationalizing the development of live attenuated virus vaccines. Nat Biotechnol 28: 573–579. doi: 10.1038/nbt.1635 PMID: 20531338
30. Rueckert C, Guzman CA (2012) Vaccines: from empirical development to rational design. PLoS Pathog 8: e1003001. doi: 10.1371/journal.ppat.1003001 PMID: 23144616
31. Ouyang P, Rakus K, Boutier M, Reschner A, Leroy B, et al. (2013) The IL-10 homologue encoded by cyprinid herpesvirus 3 is essential neither for viral replication in vitro nor for virulence in vivo. Vet Res 44: 53. doi: 10.1186/1297-9716-44-53 PMID: 23865540
32. Ouyang P, Rakus K, van Beurden SJ, Westphal AH, Davison AJ, et al. (2014) IL-10 encoded by viruses: a remarkable example of independent acquisition of a cellular gene by viruses and its subsequent evolution in the viral genome. J Gen Virol 95: 245–262. doi: 10.1099/vir.0.058966-0 PMID: 24225498

33. Costes B, Raj VS, Michel B, Fournier G, Thirion M, et al. (2009) The major portal of entry of koi herpesvirus in *Cyprinus carpio* is the skin. J Virol 83: 2819–2830. doi: 10.1128/JVI.02305-08 PMID: 19153228

34. Fournier G, Boutier M, Stalin Raj V, Mast J, Parmentier E, et al. (2012) Feeding *Cyprinus carpio* with infectious materials mediates cyprinid herpesvirus 3 entry through infection of pharyngeal periodontal mucosa. Vet Res 43: 6. doi: 10.1186/1297-9716-43-6 PMID: 22276598

35. Raj VS, Fournier G, Rakus K, Ronsmans M, Ouyang P, et al. (2011) Skin mucus of *Cyprinus carpio* inhibits cyprinid herpesvirus 3 binding to epidermal cells. Vet Res 42: 92. doi: 10.1186/1297-9716-42-92 PMID: 21816061

36. Ronsmans M, Boutier M, Rakus K, Farnir F, Desmecht D, et al. (2014) Sensitivity and permissivity of *Cyprinus carpio* to cyprinid herpesvirus 3 during the early stages of its development: importance of the epidermal mucus as an innate immune barrier. Vet Res 45: 100. PMID: 25281322

37. Michel B, Leroy B, Stalin Raj V, Lieffrig F, Mast J, et al. (2010) The genome of cyprinid herpesvirus 3 encodes 40 proteins incorporated in mature virions. J Gen Virol 91: 452–462. doi: 10.1099/vir.0.015198-0 PMID: 19846671

38. Yi Y, Zhang H, Lee X, Weng S, He J, et al. (2014) Extracellular virion proteins of two Chinese CyHV-3/KHV isolates, and identification of two novel envelope proteins. Virus Res doi: 10.1016/j.virusres.2014.07.034

39. Davison AJ, Kurobe T, Gatherer D, Cunningham C, Korf I, et al. (2013) Comparative genomics of carp herpesviruses. J Virol 87: 2908–2922. doi: 10.1128/JVI.03206-12 PMID: 23269803

40. Xu J, Zeng L, Zhang H, Zhou Y, Ma J, et al. (2013) Cyprinid herpesvirus 2 infection emerged in cultured gibel carp, *Carassius auratus gibelio* in China. Vet Microbiol 166: 138–144. doi: 10.1016/j.vetmic.2013.05.025 PMID: 23850442

41. van Beurden SJ, Bossers A, Voorbergen-Laarman MH, Haenen OL, Peters S, et al. (2010) Complete genome sequence and taxonomic position of anguillid herpesvirus 1. J Gen Virol 91: 880–887. doi: 10.1099/vir.0.016261-0 PMID: 20016040

42. Afonso CL, Tulman ER, Delhon G, Lu Z, Viljoen GJ, et al. (2006) Genome of crocodilepox virus. J Virol 80: 4978–4991. PMID: 16641289

43. van Beurden SJ, Engelsma MY, Roozenburg I, Voorbergen-Laarman MA, van Tulden PW, et al. (2012) Viral diseases of wild and farmed European eel *Anguilla anguilla* with particular reference to the Netherlands. Dis Aquat Organ 101: 69–86. doi: 10.3354/dao02501 PMID: 23047193

44. Gilad O, Yun S, Zagmutt-Vergara FJ, Leutenegger CM, Bercovier H, et al. (2004) Concentrations of a Koi herpesvirus (KHV) in tissues of experimentally infected *Cyprinus carpio koi* as assessed by real-time TaqMan PCR. Dis Aquat Organ 60: 179–187. PMID: 15521316

45. Pikarsky E, Ronen A, Abramowitz J, Levavi-Sivan B, Hutoran M, et al. (2004) Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. J Virol 78: 9544–9551. PMID: 15308746

46. Miyazaki T, Kuzuya Y, Yasumoto S, Yasuda M, Kobayashi T (2008) Histopathological and ultrastructural features of Koi herpesvirus (KHV)-infected carp *Cyprinus carpio*, and the morphology and morphogenesis of KHV. Dis Aquat Organ 80: 1–11. doi: 10.3354/dao01929 PMID: 18714678

47. Monaghan SJ, Thompson KD, Adams A, Kempter J, Bergmann SM (2014) Examination of the early infection stages of koi herpesvirus (KHV) in experimentally infected carp, *Cyprinus carpio* L. using in situ hybridization. J Fish Dis doi: 10.1111/jfd.12260

48. Waltzek TB, Kelley GO, Alfaro ME, Kurobe T, Davison AJ, et al. (2009) Phylogenetic relationships in the family Alloherpesviridae. Dis Aquat Organ 84: 179–194. doi: 10.3354/dao02023 PMID: 19565695

49. Francois S, Vidick S, Sarlet M, Desmecht D, Drion P, et al. (2013) Illumination of murine gammaherpesvirus-68 cycle reveals a sexual transmission route from females to males in laboratory mice. PLoS Pathog 9: e1003292. doi: 10.1371/journal.ppat.1003292 PMID: 23593002

50. Zapata A, Diez B, Cejalvo T, Gutiérrez-de Frías C, Cortés A (2006) Ontogeny of the immune system of fish. Fish Shellfish Immunol 20: 126–136. PMID: 15939627

51. Huttenhuis HBT, Grou CPO, Taverne-Thiele AJ, Taverne N, Rombout JHWM (2006) Carp (*Cyprinus carpio* L.) innate immune factors are present before hatching. Fish Shellfish Immunol 20: 586–596. PMID: 16169251

52. Danilova N, Bussmann J, Jekosch K, Steiner LA (2005) The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. Nat Immunol 6: 295–302. PMID: 15685175

53. Hansen JD, Landis ED, Phillips RB (2005) Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. Proc Natl Acad Sci U S A 102: 6919–6924. PMID: 15863615

54. Ryo S, Wijdeven RH, Tyagi A, Hermsen T, Kono T, et al. (2010) Common carp have two subclasses of bonyfish specific antibody IgZ showing differential expression in response to infection. Dev Comp Immunol 34: 1183–1190. doi: 10.1016/j.dci.2010.06.012 PMID: 20600275

55. Xu Z, Parra D, Gomez D, Salinas I, Zhang YA, et al. (2013) Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. Proc Natl Acad Sci U S A 110: 13097–13102. doi: 10.1073/pnas.1304319110 PMID: 23884653

56. Zhang YA, Salinas I, Li J, Parra D, Bjork S, et al. (2010) IgT, a primitive immunoglobulin class specialized in mucosal immunity. Nat Immunol 11: 827–835. doi: 10.1038/ni.1913 PMID: 20676094

57. Sunyer JO (2013) Fishing for mammalian paradigms in the teleost immune system. Nat Immunol 14: 320–326. doi: 10.1038/ni.2549 PMID: 23507645

58. Tacchi L, Musharrafieh R, Larragoite ET, Crossey K, Erhardt EB, et al. (2014) Nasal immunity is an ancient arm of the mucosal immune system of vertebrates. Nature communications 5: 5205. doi: 10.1038/ncomms6205 PMID: 25335508

59. Gomez D, Sunyer JO, Salinas I (2013) The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. Fish Shellfish Immunol 35: 1729–1739. doi: 10.1016/j.fsi.2013.09.032 PMID: 24099804

60. Rombout JH, Yang G, Kiron V (2014) Adaptive immune responses at mucosal surfaces of teleost fish. Fish Shellfish Immunol 40: 634–643. doi: 10.1016/j.fsi.2014.08.020 PMID: 25150451

61. Uchii K, Okuda N, Minamoto T, Kawabata Z (2013) An emerging infectious pathogen endangers an ancient lineage of common carp by acting synergistically with conspecific exotic strains. Anim Conserv 16: 324–330.

62. Minamoto T, Honjo MN, Yamanaka H, Uchii K, Kawabata Z (2012) Nationwide Cyprinid herpesvirus 3 contamination in natural rivers of Japan. Res Vet Sci 93: 508–514. doi: 10.1016/j.rvsc.2011.06.004 PMID: 21723573

63. Neukirch M, Böttcher K, Bunnajrakul S (1999) Isolation of a virus from koi with altered gills. B Eur Assoc Fish Pat 19: 221–224.

64. Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG (2005) Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res 33: e36. PMID: 15731329

65. Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJ, et al. (2009) ABySS: a parallel assembler for short read sequence data. Genome Res 19: 1117–1123. doi: 10.1101/gr.089532.108 PMID: 19251739

66. Li H, Durbin R (2010) Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 26: 589–595. doi: 10.1093/bioinformatics/btp698 PMID: 20080505

67. Milne I, Stephen G, Bayer M, Cock PJ, Pritchard L, et al. (2013) Using Tablet for visual exploration of second-generation sequencing data. Brief bioinform 14: 193–202. doi: 10.1093/bib/bbs012 PMID: 22445902

Presses de la Faculté de Médecine vétérinaire de l'Université de Liège

4000 Liège (Belgique)

D/2015/0480/15

ISBN 978-2-87543-065-6