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Interaction between type I interferon and cyprinid herpesvirus 3 in two genetic lines of common carp *Cyprinus carpio*

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ABSTRACT: Cyprinid herpesvirus 3 (CyHV-3) infection in common carp *Cyprinus carpio* L. and its ornamental koi varieties can induce the severe systemic disease known as koi herpesvirus disease. This disease is characterised by a rapid replication and spreading of the virus through multiple organs and results in a fast onset of mortality (starting on Day 6 post infection) in up to 100% of infected fish. During the first phase of viral infections, type I interferons (IFNs) have generally been proven to be essential in inducing an innate immune response; however, very little is known about the type I IFN response to herpesviruses in fish. The aim of this work was to study the type I IFN responses during CyHV-3 infection in 2 genetically divergent lines of common carp which presented differing survival rates. Our results show that CyHV-3 induced a systemic type I IFN response in carp, and the magnitude of type I IFN expression is correlated with the virus load found in skin and head kidney. In this *in vivo* experimental setup, the level of type I IFN response cannot be linked with higher survival of carp during CyHV-3 infection.

KEY WORDS: CyHV-3 · Koi · Infection · Innate immune response · Interferon stimulated genes

INTRODUCTION

Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), is considered to be among the most dangerous fish pathogens. CyHV-3 infection in common carp *Cyprinus carpio* L. and its ornamental koi varieties induces a severe systemic disease, known as koi herpesvirus disease (KHVD), which is regarded as a serious threat to modern carp aquaculture (Hanson et al. 2011, Rakus et al. 2013). KHVD is characterised by rapid viral replication and spreading of the virus to different organs, resulting in a fast

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onset of mortality, which starts as early as 6 d post infection and may affect up to 100% of infected carp (Rakus et al. 2009, Piačková et al. 2013). Accordingly, the time period to raise a host reaction against the virus is very limited.

In all vertebrates including fish, type I interferons (IFNs) are essential for the development of the innate immune response during the first phase of a viral infection (Robertsen 2006). Production and release of type I IFNs by virus-infected cells leads to activation and transcription of IFN-stimulated genes (ISGs), which encode for different antiviral proteins that interfere with viral replication via antiviral, antiproliferative and immunomodulatory activity (Samuel 2001, Zou & Secombes 2011).

Very little is known about the type I IFN response to alloherpesviruses in fish (Adamek et al. 2014). Recent *in vitro* studies have shown that CyHV-3 is capable of inhibiting a type I IFN response in fibroblastic cells derived from the brain of common carp (CCB) but not in head kidney leukocytes (HKL). Interestingly, a pre-stimulation of CCB cells with polyinosinic-polycytidylic acid (poly I:C) activates the type I IFN response, which subsequently results in a slower spreading of CyHV-3 through the cell monolayer of a cell culture (Adamek et al. 2012). Furthermore, *in vivo* studies showed that the transcription of IFN-encoding genes is up-regulated in skin and intestine during a CyHV-3 infection (Adamek et al. 2013, Syakuri et al. 2013).

Type I IFNs are essential for the control of rhabdovirus infections in fish. Recombinant type I IFNs were capable of protecting zebrafish *Danio rerio* against a fatal infection with spring viraemia of carp virus (López-Muñoz et al. 2009), and during an experimental infection of rainbow trout *Oncorhynchus mykiss* with infectious hematopoietic necrosis virus (IHNV), type I IFN transcript levels were significantly correlated with the individual's virus load at 48 and 72 h post infection (hpi) (Purcell et al. 2010). In addition, the mean type I IFN gene expression rate was associated with differences in susceptibility of the rainbow trout families to the infection at 72 hpi (Purcell et al. 2010). Recent studies on rainbow trout infected with viral haemorrhagic septicaemia virus (VHSV) gave very strong evidence that the rapid induction of a type I IFN response is responsible for the resistance to VHSV (Verrier et al. 2012).

Genetic differences in the resistance of different carp breeding lines and crossbreeds to CyHV-3-induced disease have also been reported (Shapira et al. 2005, Dixon et al. 2009, Rakus et al. 2009, Odegard et al. 2010, Piačková et al. 2013); however, very little is known about the genetic factors which could be associated with these differences (Adamek et al. 2014). Recently, we analysed the immune response of 2 genetic carp lines with different susceptibility to KHVD in a transcriptome study based on DNA microarray and real time quantitative PCR (RT-qPCR) analyses, whereby we demonstrated that the differences in the resistance to KHVD between the carp lines can be correlated with differences in expression of immune-related genes (Rakus et al. 2012). However, the analysis brought puzzling results regarding the involvement of IFN type I responses: while the microarray evaluation showed that some ISGs were up-regulated under infection, a subsequent RT-qPCR analysis, designed for measuring the expression of type I IFN encoding mRNA, did not confirm an up-regulation of type I IFN gene transcription (Rakus et al. 2012). Unfortunately, due to a lack of specific tools, we were not able to precisely investigate this discrepancy (Rakus et al. 2012). Recently, several genes from the type I IFN reaction cascade in common carp have been characterised, including genes encoding for pattern recognition receptors, kinases, IFN regulatory factors (IRFs), several IFNs as well as ISGs with antiviral action (Kitao et al. 2009, Feng et al. 2011, Kongchum et al. 2011, Adamek et al. 2012). Using this panel of genes, here we aimed to study the kinetics of type I IFN responses elicited during a CyHV-3 infection in 2 carp organs, viz. skin and head kidney. Skin is considered as one of the portals of entry and primary sites of infection for CyHV-3 (Costes et al. 2009), whereas head kidney is among the major immunological organs of fish (Rombout et al. 2005). We hypothesised that the survival of the fish can be influenced by the speed or magnitude of the type I IFN response in the tissues under study. In order to confirm this, mRNA expression levels of 9 genes including selected type I IFNs, IRFs and ISGs were measured and correlated to the virus load of the particular tissue during a CyHV-3 infection.

MATERIALS AND METHODS

Fish

CyHV-3-free common carp were propagated at the Institute of Ichthyobiology and Aquaculture in Gołysz (Poland) by diallelic crosses of 2 genetically different lines (Polish K and Polish R3). The offspring were kept in a recirculation system with UV-treated

water and bio-filters. For challenge experiments, 2 yr old fish (mean weight ± SD = 120 ± 38 g) were used. Fish were confirmed by PCR to be CyHV-3 free (Rakus et al. 2012, Adamek et al. 2013).

CyHV-3 challenge experiment and sampling protocol

CyHV-3 infection was performed as described by Rakus et al. (2012). In brief, fish were infected with CyHV-3 by bath immersion for 1 h at 22°C in small plastic containers containing tank water with a virus suspension at an infectivity of 3.2×10^2 $TCID_{50}$ ml^{-1}, before being returned to their original tanks. In total, 60 carp were exposed in 15 l of water (see Rakus et al. 2012). The same method was used for the control fish, whereby the virus suspension was replaced by culture medium of uninfected CCB cells. For organ collection, carp were lethally anaesthetised in a 0.5 g l^{-1} tricaine bath (Sigma), and subsequently blood was drawn from the caudal vein. Skin (from a location next to the initial dissection incision on the left side of the body) and head kidney samples were collected in 1 ml of RNAlater (Qiagen) from each carp line (n = 5 fish per sampling point) at 0, 12, 24, 72 and 120 h post infection (hpi). An additional 5 fish from both lines were collected at 72 hpi and 5 fish from line K were collected at 336 hpi. All samples were stored at −80°C until DNA/RNA isolation.

Detection and quantification of CyHV-3

Virus quantification was performed using real-time TaqMan qPCR as described by Gilad et al. (2004) with modifications (Gilad et al. 2004, Rakus et al. 2012). DNA was isolated from 25 mg of head kidney and skin, after mechanical lysis in a QIAgen Tissuelyser II (Qiagen), using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's manual. After isolation, the samples were diluted to 50 ng μl^{-1} and stored at −80°C. The reaction mix contained 1× master mix (Maxima Probe qPCR Mastermix, Fermentas), 0.8 µM of each primer, 0.1 µM of the fluorescent probe (sequences in Table 1) and 250 ng of DNA template. Real-time TaqMan PCR was performed using a Stratagene Mx 3005P thermocycler (Agilent). Plasmid-based virus copy quantification was performed using the product (fragment of CyHV-3 ORF89) amplified with the primers KHV-86F and KHV-163R (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d111p107_supp.pdf) that had been ligated into pGEM–T Easy vectors (Promega) and propagated in JM109 competent *Escherichia coli* bacteria (Promega). The plasmids were isolated with the GeneJET™ Plasmid Miniprep Kit (Fermentas). A standard curve from 10^0 to 10^7 gene copies was prepared and used for quantification of the copy number from each sample. The results are presented as the total number of virus copies per 250 ng of DNA.

RNA isolation and cDNA synthesis

Total RNA was extracted using the TriFast reagent (Peqlab) in accordance with the manufacturer's instructions. Any remaining genomic DNA was digested with 2 U of DNase I (Fermentas) according to the manufacturer's instructions. Synthesis of cDNA was performed from 900 ng of total RNA using the Maxima™ First Strand cDNA Synthesis Kit (Fermentas). A non-reverse transcriptase control was included in the analysis of each sample. cDNA samples were further diluted 1:20 with nuclease-free water prior to RT-qPCR analysis.

RT-qPCR analysis of gene expression

RT-qPCR was used for expression analysis. Reactions were performed in duplicate using the Maxima SYBR Green 2× mastermix (Fermentas) in a Stratagene Mx3005P cycler (Agilent). Briefly, the RT-qPCR mastermix was prepared as follows: 1× Maxima SYBR Green mastermix (with 10 nM of ROX), 0.2 µM of each primer (sequences in Table 1), 5.0 µl of 20× diluted cDNA and nuclease-free water to a final volume of 25 µl. The amplification program included an initial denaturation at 95°C for 10 min, followed by 40 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s. A dissociation curve was performed at the end of each run. The standard curve method for relative quantification of gene mRNA expression rates (Livak 1997, Larionov et al. 2005) was performed using plasmids prepared in the same way as described above. A standard curve from 10^2 to 10^8 gene copies was prepared and used for the quantification of copy numbers of the relevant gene from each sample. For normalisation of expression results, 2 reference genes were tested: 40S ribosomal protein S11 (40S) and β-actin (ACTB). The 40S ribosomal protein S11 was selected as the reference gene, as its mRNA expression was found to be very stable across different tis-

Table 1. Sequences of primers used in this work. Primers marked with 'Q' were used in RT-qPCR expression analyses, primers marked with 'P' were used for the amplification of gene fragments for plasmid based quantification of gene expression

Gene	Primer/probe	Sequence 5′-3′	GenBank ID	Use
Housekeeping genes				
Beta-actin	Cyca_ACTB_qF1	TCACACCACAGCCGAGAG	M24113	P, Q
	Cyca_ACTB_qR1	CAGGGAGGAGGAGGAAGCAG		
40S ribosomal protein S11	q40S.FW1	CCGTGGGTGACATCGTTACA	AB012087	P, Q
	q40S.RV1	TCAGGACATTGAACCTCACTGTCT		
Interferons				
Interferon a1	IFN_I_1_gsp_F1	ACCAAACCCAAATGTGGACGTG	AB376666	P, Q
	IFN_I_1_S_gsp_R1	CCACTTTCTTTAGGTTCCATTTAG		P
	IFN_I_1_S_gsp_qR2	CCACTCATTTCCCGAAGCAGA		Q
Interferon a1S	IFN_S_gsp_F1	AGCGACCTTGAAACACGTTGGAATC	EC393381	P, Q
	IFN_I_1_S_gsp_R1	CCACTTTCTTTAGGTTCCATTTAG		P
	IFN_I_1_S_gsp_qR2	CCACTCATTTCCCGAAGCAGA		Q
Interferon a2	IFN_I_2_gsp_F1	GAAACAAACTCAAATGTGGACATA	AB376667	P
	IFN_I_1_S_gsp_R1	ACTCTTTCCAGGGACTTGTTTGCG		
	IFN_I_2_gsp_qF2	GATGAAGGTGCCATTTCCAAG		Q
	IFN_I_2_gsp_qR3	CACTGTCGTTAGGTTCCATTGCTC		
Interferon regulatory factors				
Interferon regulatory factor 3	CycaIRF3_F1	GGAGACCACTCTGTTTGGAAG	JQ478481	P, Q
	CycaIRF3_R1	CGGCATCGTTCTTGTTGTC		
Interferon regulatory factor 7	CycaIRF7_F2	TCCACTGAGGGTCTGATTGA	JQ698666	P, Q
	CycaIRF7_R2	CGCTGGTGCTGACGAAGA		
Interferon-induced genes				
RNA-specific adenosine deaminase	CycaADAR_F1	GCAGGACGAGGTGTCAGAG	JX481077 and EC392392	P, Q
	CycaADAR_R1	GGCAAAGGGAGCATAACTTC		
Interferon-induced protein 1 like	Cyca927_F1	ATGAAGCAGGACGACAGAG	JQ740891 and EX882412	P, Q
	Cyca927_R1	CCAAGACAGCAAGGGTTACAG		
Protein kinase R	Cyca_PKR_qF1	CCAACATCGTCCGCTACTACTC	JX516101 and EX880666	P, Q
	Cyca_PKR_qR1	GCGTGTCTCCCTCACAAAG		
Viperin	CycaVig1_F2	CGCACCAGAGAGCAGAAAG	EX881775 and EX880905	P Q
	CycaVig1_R2	CTCAATAGGCAGCACGAAC		
Virus genes				
CyHV-3 thymidine kinase	CyHV3_TK_qF1	TGGCTATGCTGGAACTGGTG	DQ177346	P, Q
	CyHV3_TK_R2	GTTACAAGAACGAGGTGGAG		P
	CyHV3_TK_qR3	GCTGGTCTATGGCGTGCTTG		Q
CyHV-3 ORF89	KHV-86F[a]	GACGCCGGAGACCTTGTG	AF411803	P, Q
	KHV-163R[a]	CGGGTTCTTATTTTTGTCCTTGTT		P, Q
	KHV-109P[a]	FAM-CTTCCTCTGCTCGGCGAGCACG-BHQ1		Q

[a]Primers and probe taken from Gilad et al. (2004)

sues and infections in carp (Adamek et al. 2012, 2013, Rakus et al. 2012, van der Marel et al. 2012). The level of gene expression is shown as the copy number of the gene normalised against 1×10^5 copies of the 40S ribosomal protein S11 (normalised copy number) according to the following formula:

Normalised copy number = mRNA copies per PCR for target gene / (mRNA copies per PCR for reference gene / 10^5)

Standard curves for all primer pairs used in RT-qPCR are presented in Fig. S1.

Statistical analysis

SigmaPlot 12 software was used for statistical analysis. The normalised gene expression levels and virus load were transformed to Log10(x). Significant differences ($p \leq 0.05$) in RT-qPCR gene expression and virus load during CyHV-3 infection were assessed using a 3-way ANOVA (at: 0, 12, 24, 72 and 120 hpi) with multiple comparison of means versus a control group using Dunnett's Method (the results from 336 hpi were not considered in this evaluation

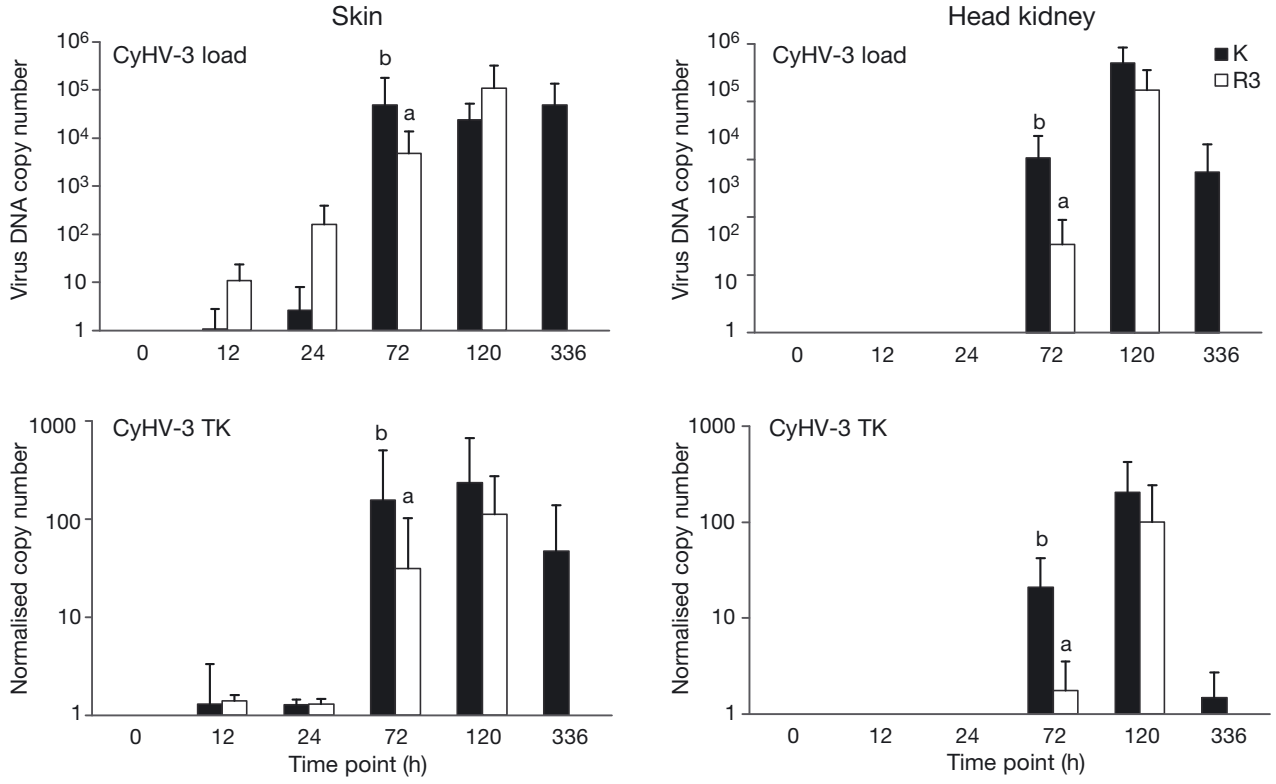

Fig. 1. Cyprinid herpesvirus 3 (CyHV-3) load and replication in skin and head kidney of common carp *Cyprinus carpio* individuals from 2 lines (K and R3). CyHV-3 load was measured by qPCR, and CyHV-3 replication by mRNA expression of the gene encoding the CyHV-3 thymidine kinase (CyHV-3 TK). Virus load data are shown as mean genome copies in 250 ng of isolated DNA (+SD) from 5 fish per line (0, 12, 24, 120 and 336 h post infection, hpi) and 10 fish per line (72 hpi). The mRNA expression levels are shown as mean copy number (+SD) of the gene normalised against 100 000 copies of the carp 40S ribosomal protein S11. Letters (a, b) indicate significant differences at $p \leq 0.05$ between the carp lines at the particular time points. Results from skin of line K are taken from Adamek et al. (2013)

as data were only collected from line K). For the expression levels of carp type I IFN genes and virus load levels, a Pearson correlation was calculated using the simple linear regression model.

RESULTS

CyHV-3 infection and virus replication in skin and head kidney of carp

Two tested carp lines presented a 20 point difference in the cumulative mortality after CyHV-3 infection. The more susceptible line K showed 76% mortality, and the more resistant line R3 showed 56% mortality (Rakus et al. 2012). Viral DNA was detected as early as 12 hpi in skin samples and at 72 hpi in head kidney samples of individuals from both lines. The highest virus loads in skin were observed at 72 hpi in line K (data also presented in Adamek et al. 2013) and at 120 hpi in line R3. The highest virus loads in head kidney were observed at 120 hpi for both lines. At 72 hpi, virus loads in skin and head kidney were significantly higher (10× and 31×, respectively) in fish from line K compared to line R3 (Fig. 1).

Expression of the gene encoding viral thymidine kinase (CyHV-3 TK) was detected in skin at 12 hpi and in head kidney at 72, 120 and 336 hpi. The highest expression level could be detected at 120 hpi in both tissues from the 2 carp lines examined. At 72 hpi, the expression levels of CyHV-3 TK, in both skin and head kidney, were significantly lower in the more resistant line R3 than in the more susceptible line K (Fig. 1).

Interestingly, a significantly higher virus load was found in the skin samples than in the head kidney at 72 hpi (46× higher in line K and 143× higher in line R3). This difference diminished by 120 hpi to 7× for carp from line R3, whereas for carp from line K the levels in head kidney were 2× higher than in skin (Fig. 1, Table S1 in the Supplement at www.int-res.com/articles/suppl/d111p107_supp.pdf).

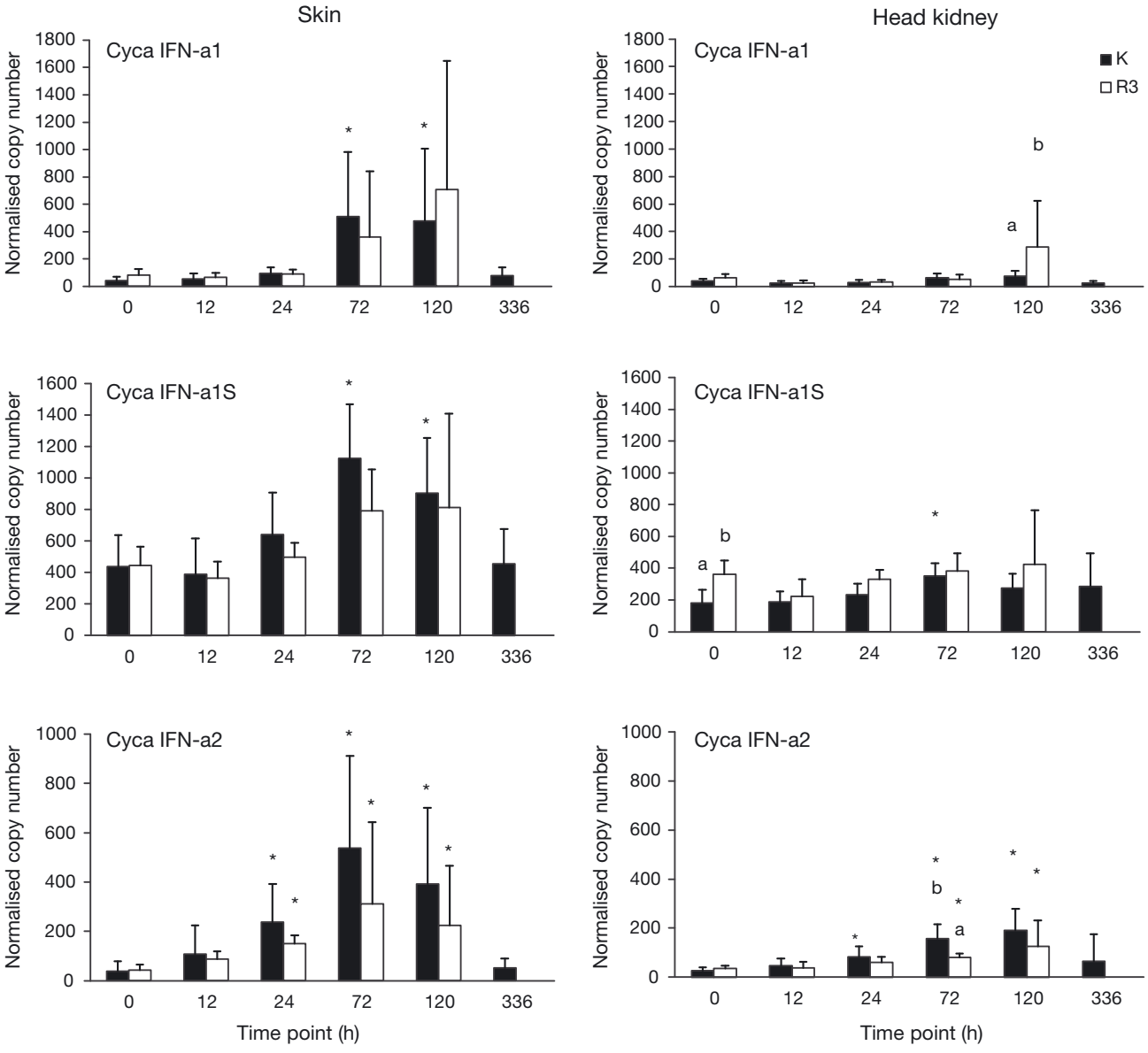

Fig. 2. mRNA expression of genes encoding for common carp *Cyprinus carpio* (Cyca) virus-induced interferons (IFN-a1, IFN-a1S and IFN-a2) in the skin and head kidney during cyprinid herpesvirus 3 infection of individuals from 2 carp lines (K and R3). Expression levels are shown as mean copy number (+SD) of the gene normalised against 100 000 copies of the 40S ribosomal protein S11. Number of individuals per line: n = 5 (0, 12, 24, 120 and 336 h post infection, hpi) and n = 10 (72 hpi). *Significant differences at $p \leq 0.05$ between the control (0 h) and infected individuals. Letters (a, b) indicate significant differences between the carp lines at particular time points. Results from skin of line K are taken from Adamek et al. (2013)

Expression of type I IFN genes in skin and head kidney of carp during CyHV-3 infection

An analysis of the expression in skin revealed that IFN-a1 and IFN-a1S (nomenclature according to Zou & Secombes 2011, Adamek et al. 2012) were significantly up-regulated at 72 hpi (12-fold) and 120 hpi (11-fold) in fish from line K (data from line K were already presented in Adamek et al. 2013), whereas in carp from line R3, only a slight but non-significant up-regulation in the transcription of these genes was observed during the course of the experiment (Fig. 2). The mRNA expression of IFN-a2 was significantly up-regulated in the skin of both carp lines at 24, 72 and 120 hpi (Fig. 2). The highest expression of this gene was measured at 72 hpi, with a 14-fold up-regulation in samples from line K and 7-fold in line R3 relative to the uninfected controls. In the skin of CyHV-3-infected carp, the level of IFN mRNA expression was positively correlated with virus load (with R =

0.65 and 0.76 for IFN-a1 in line K and R3, respectively, R = 0.47 and 0.7 for IFN-a2 in line K and R3, respectively, and R = 0.56 and 0.67 for IFN-a1S line K and R3, respectively; Table S2 in the Supplement). There was no significant difference in the expression level of type I IFN genes in skin between the 2 carp lines.

In head kidney, IFN-a1 transcription was not significantly activated, although some up-regulation was evident in carp from line R3 at 120 hpi. Consequently, at this time point, the expression level of the IFN-a1 gene was significantly (4-fold) higher in samples from line R3 than in carp from line K. In the case of IFN-a1S, a significantly higher expression level in line R3 as compared to line K was observed in non-infected fish on Day 0. After infection with CyHV-3, the transcription of the IFN-a1S gene was only significantly up-regulated in carp from line K at 72 hpi. The transcription of IFN-a2 was significantly up-regulated in carp from line K at 24, 72 and 120 hpi, and in carp from line R3 at 72 and 120 hpi. The expression level of IFN-a2 was significantly higher (2-fold) in line K than in line R3 at 72 hpi (Fig. 2). Similarly to skin, the levels of IFN-a1 and IFN-a2 expression in the head kidney were significantly positively correlated with the virus load (with R = 0.53 and 0.67 for IFN-a1 in line K and R3, respectively, and R = 0.59 and 0.7 for IFN-a2 in line K and R3, respectively; Table S2), whereas expression of IFN-a1S showed a lower correlation with virus load (R = 0.46 and 39 in line K and R3, respectively; Table S2). The expression level of the IFN-a1 and IFN-a2 encoding genes was statistically higher in skin than in head kidney in carp from both lines at 24 and 72 hpi (with a 3-fold difference in carp from line K and a 2-fold difference in carp from line R3 at both time points). Furthermore, the expression of IFN-a1S was higher in skin than in head kidney in carp from line K at 0, 24, 72 and 120 hpi and in carp from line R3 at 72 hpi. At 336 hpi, the transcription of type I IFN genes in skin and head kidney was not different from that of non-infected carp.

Expression of IRFs and ISGs in skin and head kidney of carp during a CyHV-3 infection

In skin and head kidney of both carp lines, the mRNA expression of IRFs 3 and 7 was significantly up-regulated at 72 and 120 hpi, and at 24, 72 and 120 hpi, respectively, for IFR3 and IRF7 (Fig. 3). While in both organs the expression of IRF3 was up-regulated by only 3- to 6-fold, the expression levels of IRF7 in the skin of line K increased 13-fold at 72 hpi and 14-fold at 120 hpi and in the skin of line R3, they increased 21-fold at 72 hpi and 32-fold at 120 hpi. In carp from line K, IRF3 expression was also significantly (3-fold) higher in skin than in head kidney by 72 and 120 hpi with CyHV-3 (Table 2). When the expression level of IRF3 in the skin of carp from the different breeding lines was compared, a significant difference was found after 72 hpi, with a 2-fold higher expression in carp from line K.

The expression of 4 IFN type I-induced genes encoding for antiviral proteins ADAR, IFTM1, PKR and Viperin was studied in skin and head kidney samples of carp infected with CyHV-3 (Fig. 4). The expression of carp adenosine deaminase (ADAR) was significantly up-regulated in the skin of line K carp as early as 24 hpi. The highest expression level of this gene, with an up-regulation of 3- to 5-fold, was measured in skin of both lines at 72 and 120 hpi, respectively (Fig. 4). In head kidney, significant differences in the expression level of ADAR were observed between the 2 carp lines at 0 h, with a higher basal expression level in line R3. For this gene, a significant (3-fold) up-regulation of the expression was detected after CyHV-3 infection only in head kidney of line K, at 72 and 120 hpi.

During the course of a CyHV-3 infection in skin, IFN-induced transmembrane protein 1 (IFITM1) was the most up-regulated gene among all studied genes or all ISGs. In the skin, IFITM1 was up-regulated 40-fold in line K and 65-fold in line R3 at 120 hpi. Surprisingly, the expression of the IFITM1-encoding gene was not significantly altered in head kidney (Fig. 4),

Table 2. Differences in mRNA expression levels of interferon response genes (see Table 1) between skin and head kidney in common carp *Cyprinus carpio* lines K and R3. *Statistically significant differences ($p \leq 0.05$) between skin and head kidney; (–) indicates lack of differences

Time point (h)	IFN-a1		IFN-a2		IFN-a1S		IRF3		IRF7		ADAR		PKR		IFITM1		Viperin	
	K	R3	K	R3	K	R3	K	R3	K	R3	K	R3	K	R3	K	R3	K	R3
0	–	–	–	–	*	–	–	–	–	–	–	*	–	–	*	*	–	–
12	–	–	–	–	–	–	–	–	–	–	*	–	–	–	–	*	–	–
24	*	*	*	*	*	–	–	–	–	–	–	–	–	–	*	*	–	–
72	*	*	*	*	*	*	*	–	–	–	–	*	–	–	*	*	–	–
120	–	–	–	–	*	–	*	–	–	–	–	–	–	–	–	*	–	–

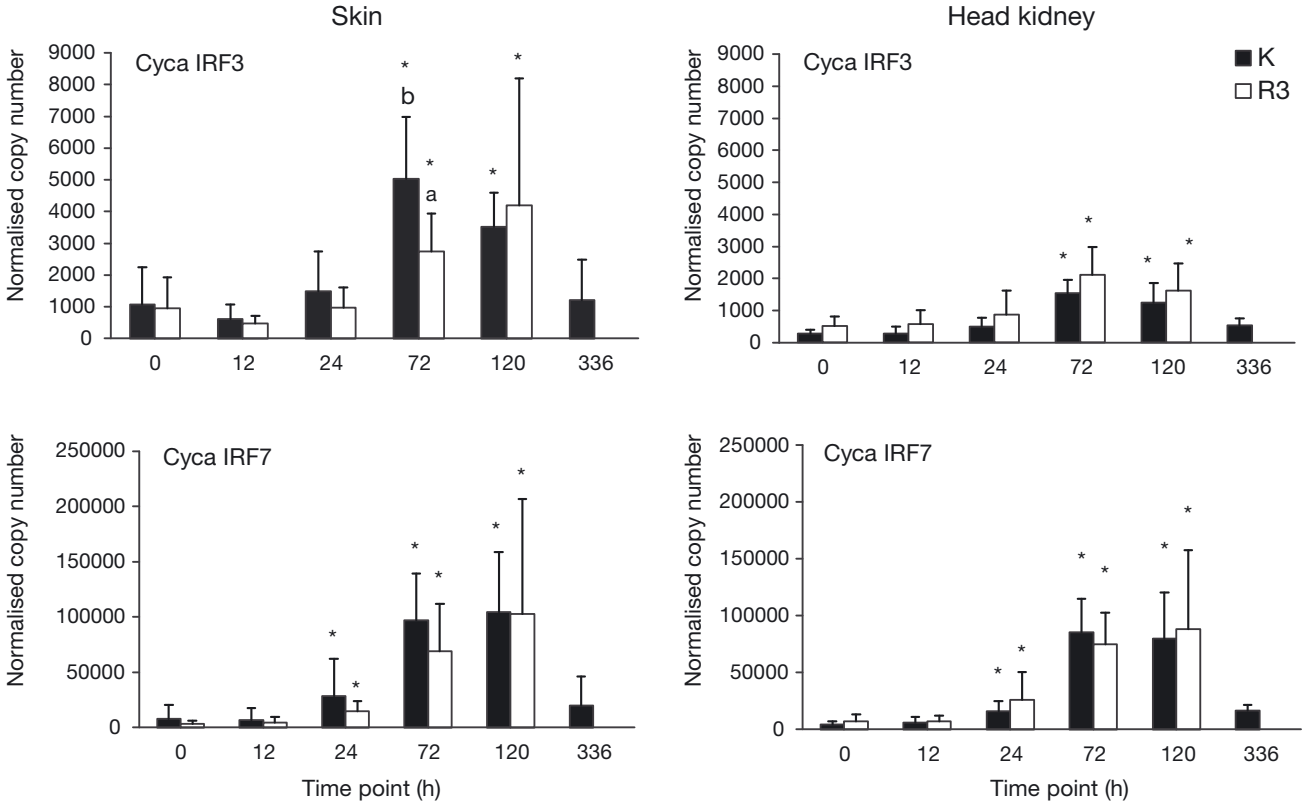

Fig. 3. mRNA expression of genes encoding for common carp *Cyprinus carpio* (Cyca) interferon regulatory factors (IRF3, IRF7) in the skin and head kidney during cyprinid herpesvirus 3 infection of individuals from 2 carp lines (K and R3). Expression levels are shown as mean copy number (+SD) of the gene normalised against 100 000 copies of the 40S ribosomal protein S11. Number of individuals per line: n = 5 (0, 12, 24, 120 and 336 h post infection, hpi) and n = 10 (72 hpi). *Significant differences at $p \leq 0.05$ between the control (0 h) and infected individuals. Letters (a, b) indicate significant differences between the carp lines at certain time points

but the expression level of this gene was significantly higher (from 10- to 1000-fold) in head kidney than in skin at all time points for line R3 and at most time points for line K (Fig. 4, Table 2). When the carp lines were compared, a significantly higher (3-fold) expression of IFITM1 was demonstrated in the skin of line K when compared to line R3 at 72 hpi (Fig. 4).

The expression of protein kinase R (PKR) in skin and head kidney of both lines was significantly up-regulated from 5- to 10-fold at 72 and 120 hpi, respectively (Fig. 4). There was no difference in the expression level between these 2 organs; however, in skin there was a significant difference in PKR expression between the lines, with a nearly 2-fold higher level in line K relative to line R3.

The significant up-regulation of Viperin in skin (12- and 10-fold) and in head kidney (8- and 6-fold) was demonstrated in line K at 72 and 120 hpi, respectively (Fig. 4). In line R3, at the same time points, Viperin was also up-regulated in skin (5- and 7-fold) and in head kidney (4- and 3-fold). There were no significant differences between the lines and the organs (Table 2). In samples of skin and head kidney from infected carp taken at 336 hpi, the expression of IRFs and ISGs was not different from the expression of these genes in samples from non-infected carp.

DISCUSSION

The response of type I IFNs has been shown to be critical for the outcome of many viral diseases (Mossman & Ashkar 2005, Chan et al. 2011). In particular, type I IFNs play an important role in the inhibition of virus replication and in the limitation of virus spreading at an early stage of infection. In the present work, we studied for the first time the systemic type I IFN response of common carp to a CyHV-3 infection, which is now considered as one of the major challenges to carp culture. We used RT-qPCR arrays to monitor changes in the transcription of the genes at 3 levels of the type I IFN pathway, including IRFs, type I IFNs with novel gene-specific primers and several ISGs encoding for the antiviral proteins.

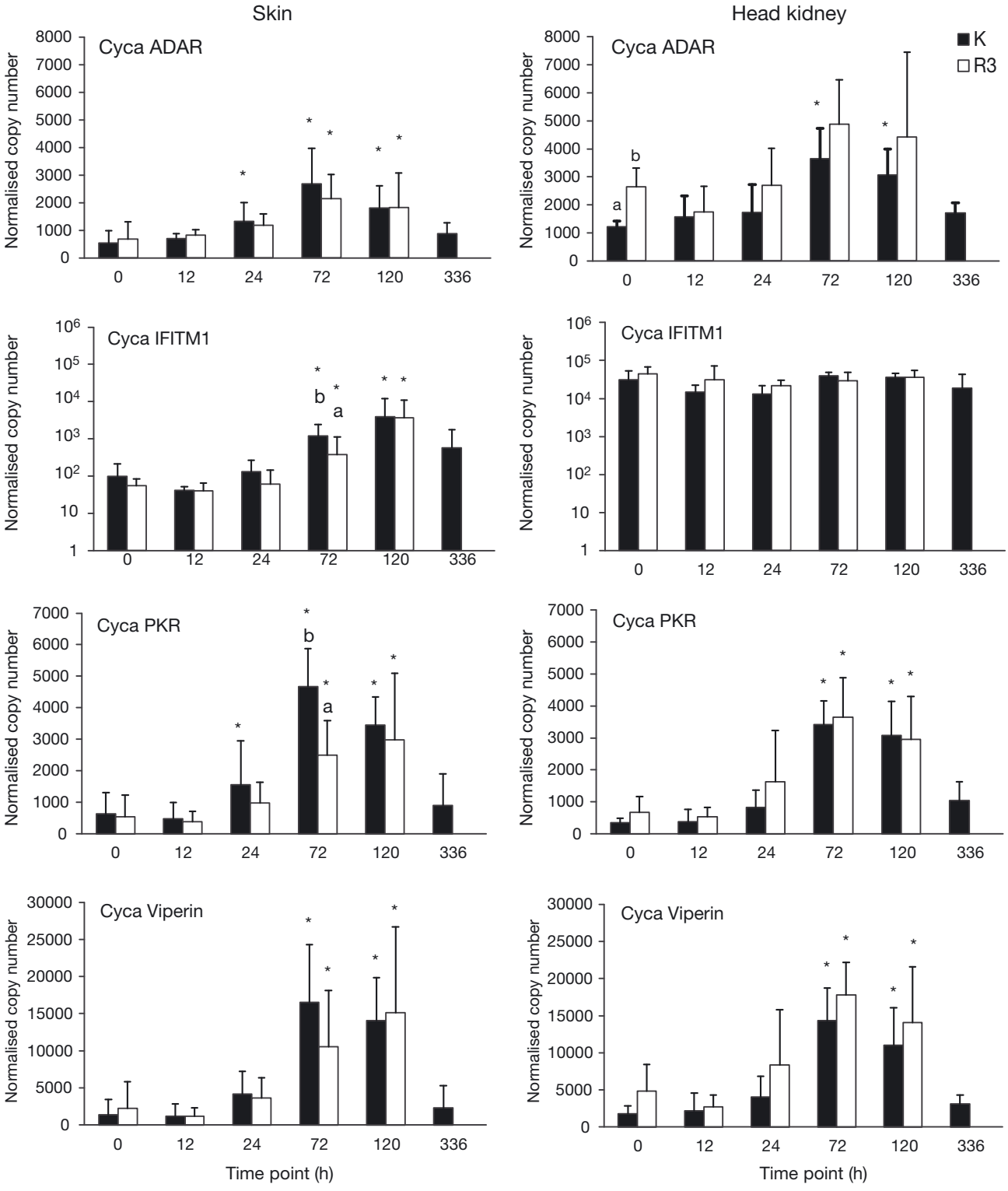

Fig. 4. mRNA expression of genes encoding for common carp *Cyprinus carpio* (Cyca) interferon induced antiviral proteins (ADAR, IFITM1, PKR, and Viperin) in skin and head kidney during cyprinid herpesvirus 3 infection of individuals from 2 carp lines (K and R3). Expression levels are shown as mean copy number (+SD) of the gene normalised against 100 000 copies of the 40S ribosomal protein S11. Number of individuals per line: n = 5 (0, 12, 24, 120 and 336 h post infection, hpi) and n = 10 (72 hpi). *Significant differences at $p \leq 0.05$ between the control (0 h) and infected individuals. Letters (a, b) indicate significant differences between the carp lines at certain time points

CyHV-3 infects carp mainly through the skin, which is not only the major portal of entry for this virus, but also a place of virus replication during the early stages of infection (Costes et al. 2009, Fournier et al. 2012). In both of the studied carp lines, the infection developed first in the skin, but there were differences in the progression of the infection. At 72 hpi, the virus load and the expression levels of the viral gene encoding for CyHV-3 TK were significantly different between the 2 analysed carp lines. Carp from the more resistant line R3 harboured a significantly lower virus load (based on the amount of CyHV-3 specific DNA and thymidine kinase expression) in skin and head kidney than carp from the more susceptible line K. A similar difference was found in the trunk kidney by Rakus et al. (2012). It appears that virus spread from the skin to the internal organs and may suggest a systemic character of the infection in both lines, with a more severe disease progression in line K fish. The higher virus load seen in the internal tissues of carp from this breeding line could be one of the factors responsible for mortality. It has been shown that in the kidney a CyHV-3 infection leads to the degradation of distal tubules (Hedrick et al. 2000, Pikarsky et al. 2004), which causes a massive deterioration of the electrolyte balance (Negenborn 2009).

A study on rainbow trout infected with IHNV showed that the virus load of the trunk kidney during the early stages of the infection (up to 72 hpi) was a good predictor for fish survival or mortality (Purcell et al. 2010). Furthermore, virus load and the speed of replication of the virus in fins was also proposed as a tool for the selection of rainbow trout individuals which were resistant to an infection with VHSV (Quillet et al. 2001, 2007). The differences in virus load could be caused by differences in the magnitude of innate immune responses with type I IFNs as key factors for limiting virus replication. In fact, recent studies showed clearly that a rapid induction of the type I IFN response is one of the mechanisms which provides protection against VHSV infection in resistant fish (Verrier et al. 2012). Also in the case of IHNV, the survival of infected rainbow trout could be related to the magnitude of their type I IFN response to the virus seen at 72 hpi (Purcell et al. 2010). In mice *Mus musculus*, a rapid type I IFN response was correlated with a delayed spread of HSV-1 from the site of inoculation to the peripheral nervous system, which greatly increased the chance of survival (Halford et al. 2004). Therefore, to investigate whether type I IFNs could also be responsible for the observed differences in CyHV-3 load in the carp from our CyHV-3 infection experiment, we analysed the expression of genes involved in type I IFN response in the skin and head kidney. Our results clearly show that CyHV-3 induces an up-regulation of type I IFN, IRF and ISG genes in both tissues, but the differences in virus load which could be detected at 72 hpi between carp from the 2 breeding lines can hardly be explained by differences in the magnitude of the type I IFN response. It appears that in both tissues, an increasing virus load was correlated with a higher level of expression of type I IFNs. It is possible that an effect of the type I IFN expression level on the virus load in the tissue would be more evident when different doses of virus are used for infection. Furthermore, our results indicate that CyHV-3 might be capable of circumventing, or not fully eliciting, a type I IFN response in carp during primary infection (especially in the head kidney), which can explain why Rakus et. al (2012) could not detect type I IFN up-regulation in this tissue, despite the up-regulation of ISGs in the spleen.

Type I IFN responses in the skin were significantly higher than in head kidney, which could also be attributed to the higher virus load in this tissue. Additionally, the significantly higher up-regulation of IRF7 than IRF3 could indicate that similarly to mammals, the TLR9-MyD88-IRF7 pathway is responsible for the induction of type I IFN responses in fish under infection with dsDNA viruses (Kawai et al. 2004, Dai et al. 2011). Interestingly, the more pronounced response of skin cells could be of significant value for future studies using a larger number of carp lines for evaluating the genetic basis of virus resistance, as the skin seems to be the key organ for development of type I IFN responses against this virus. Furthermore, the high up-regulation of IFITM1 expression in skin could indicate cellular immune responses to the infection. In humans, IFITM1 is a marker protein expressed by B cells (Evans et al. 1990, 1993) and T cells (Chen et al. 1984). From our unpublished results, we know that the highest mRNA quantities of IFITM1 are expressed in head kidney, trunk kidney and spleen, and the molecule is not expressed in fibroblastic cell lines. Additionally, under CyHV-3 infection, the expression level in head kidney was not changed significantly and the up-regulation of this gene expression in skin could indicate the influx of leukocytes to the infection site.

Having established that the level of type I IFN, IRF and ISG expression cannot explain the differences in the virus load that were found on Day 3 in individuals from the 2 genetic carp lines, other immune responses must be considered to contribute to the

better survival of one of the lines. As shown by Rakus et al. (2012), increased resistance of R3 carp to CyHV-3 could be associated with increased levels of complement activation, MH class I-restricted antigen presentation and activation of $CD8^+$ T cells in R3 carp compared to carp from line K. Most of the differences in gene expression occurred at 24 and 72 hpi. Therefore, these factors were most likely responsible for delaying the replication of the virus and its spread through the more resistant fish. The importance of a type I IFN induced antiviral state in the cells and its interplay with adaptive immune responses for a control of herpesvirus infections has been demonstrated in mammals (Chan et al. 2011). Currently we are unable to conclude how important the type I IFN response is to support a clearing of the CyHV-3 virus from carp tissues *in vivo*. This will be addressed in our future studies. We showed that the induction of a type I IFN response (by poly I:C) in fibroblasts *in vitro* was able to significantly reduce CyHV-3 infectivity and the speed of spreading of the infection in the tissue culture monolayer, which highlights the importance of this response (Adamek et al. 2012).

Taken together, our results show that CyHV-3 induces a local (mucosal) and systemic type I IFN response in carp. Although observations in mice and rainbow trout indicate that the magnitude of a type I IFN response can influence the outcome of the disease, this could not be documented during our CyHV-3 infection experiment based on the 2 genetically different carp lines. Instead, the magnitude of type I IFN response against CyHV-3 was positively correlated with the virus load of the infected tissue. The significance of the type I IFN response for the control of a CyHV-3 infection in carp still needs to be resolved.

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