

C-reactive protein and complement as acute phase reactants in common carp *Cyprinus carpio* during CyHV-3 infection

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ABSTRACT: Cyprinid herpesvirus 3 (CyHV-3) is the aetiological agent of a highly virulent and lethal disease of common carp *Cyprinus carpio* and its ornamental koi varieties. However, specific knowledge about immune mechanisms behind the infection process is very limited. We aimed to evaluate the effect of the CyHV-3 infection on the profile of 2 major components of the common carp immune acute phase response: the C-reactive protein (CRP) and the complement system. Common carp were infected with CyHV-3 by bath immersion. Fish were sampled before the infection and at 6, 12, 24, 72, 120 and 336 h post-infection for serum and head kidney, liver, gill and spleen tissues. CRP levels and complement activity were determined from the serum, whereas CRP- and complement-related genes (*crp1*, *crp2*, *c1rs*, *bf/c2*, *c3*, *masp2*) expression profiles were analysed in the tissues by quantitative PCR. Both CRP levels and complement activity increased significantly up to 10- and 3-fold, respectively, in the serum of infected fish during the challenge. Analysis revealed distinct organ- and time-dependent expression profile patterns for all selected genes. These results suggest that CRP and complement behave as acute phase reactants to CyHV-3 infection in common carp with an organ- and time-dependent response.

KEY WORDS: CRP · Complement · Cyprinid herpesvirus 3 · KHV · Koi herpesvirus · Carp · Biomarker

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INTRODUCTION

Although viral pathogens and associated diseases are less common in aquaculture than bacterial, parasitic and fungal diseases, some viruses cause major economic losses resulting in high mortality and/or morbidity rates in a very short period of time post-infection (Crane & Hyatt 2011). In the late 1990s, a highly contagious and virulent disease began to

cause significant mortality of common carp *Cyprinus carpio* and its ornamental koi varieties *C. carpio koi* worldwide (Bretzinger et al. 1999, Hedrick et al. 2000, Haenen et al. 2004, Pokorova et al. 2005). The causative agent of the disease was cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV) or carp interstitial nephritis and gill necrosis virus (CNGV). CyHV-3, a member of the genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpes-*

virales (Davison et al. 2009), has a genome comprising 295 kb, linear, double-stranded DNA predicted to contain 155 potential protein-coding open reading frames (ORFs) (Davison et al. 2013). The genome is enclosed within an icosahedral capsid surrounded by a tegument and a host-derived lipid envelope, which contains virus glycoproteins (Michel et al. 2010). The mode of entrance of the virion into a fish has been demonstrated to occur primarily via the skin (Costes et al. 2009, Raj et al. 2011) and the pharyngeal periodontal mucosa (Fournier et al. 2012), although the gills and the gut may also serve as other possible infection routes (Dishon et al. 2005). The mortality rate can be as high as 80 to 100% in susceptible populations (Hedrick et al. 2000), with death occurring 6 to 7 d post exposure (Dixon et al. 2009, Rakus et al. 2009). External and internal disease symptoms have been well characterised and include severe gill lesions with the appearance of necrosis, bleeding gills, sunken eyes, and pale skin patches (Pokorova et al. 2005), as well as abnormal adhesions in the body cavity and a mottled appearance of internal organs (Hedrick et al. 2000, Goodwin 2003, Perelberg et al. 2003). Infection with CyHV-3 leads to severe leucocytosis shortly after infection (Negenborn 2009), and recent microarray and quantitative (qPCR) analyses have shown up-regulation of several immune-related genes indicating increased inflammation, type I IFN and T cell responses (Rakus et al. 2012, Adamek et al. 2013). Furthermore, fish were shown to develop protective antibodies to CyHV-3 after 40 d of exposure (Adkison et al. 2005). However, some critical aspects of the carp innate immune response against CyHV-3 such as the acute phase proteins have not been extensively analysed and yet may provide protection during the very first days of infection when an adaptive immune response against CyHV-3 may not be functional. For example, Rakus et al. (2012) noted a potential role of classical complement activation in the resistance of carp against this virus.

The acute phase response (APR) is an important constituent of the host's innate immune defence in response to injury, tissue trauma and pathogen invasion. This systemic response consists of a series of specific physiological reactions that involve metabolic changes in the host (Bayne & Gerwick 2001). Among the different plasma proteins mobilised during an APR, the complement system and C-reactive protein (CRP) play a significant role during bacterial infections (MacCarthy et al. 2008, Dumrongphol et al. 2009, Mohanty & Sahoo 2010, Pionnier et al. 2013). However, during viral infections, APRs are

usually lower (Gruys et al. 2005), and CRP serum levels have often been exploited to discriminate between viral and bacterial infections (Costalonga et al. 2009). Nevertheless, when severe cellular destruction occurs as a result of virus spread through the organism, a full APR can be observed (Gruys et al. 2005). CRP, an evolutionary conserved protein found in the body fluids of both invertebrates and vertebrates, is commonly associated with the APR (Pepys et al. 1978) and has been proposed as a biomarker for infection and/or health status in cultured carp (Ramos & Smith 1978, MacCarthy et al. 2008). Interestingly, recent studies performed in healthy carp or fish infected with *Aeromonas salmonicida* also revealed a widespread constitutive expression of CRP-related genes with differential expression in the gut, liver and head kidney, suggesting different biological activities of the related isoforms (Falco et al. 2012, Pionnier et al. 2013). Serum CRP levels increase by up to 6-fold in common carp within 40 h of infection with *A. hydrophila* (MacCarthy et al. 2008) and up to 18-fold within 2 to 4 d in channel catfish *Ictalurus punctatus* exposed to turpentine (Szalai et al. 1994). Complement, another essential component of the fish APR, is a cascade comprised of numerous membrane-bound and soluble proteins leading to the formation of a membrane attack complex which destroys the pathogen (Nakao et al. 2011). Complement activation through the classical and/or alternative pathways is commonly evaluated in the serum of infected fish. Recent studies have concentrated on complement-related genes in fish during bacterial (Dumrongphol et al. 2009, Mohanty & Sahoo 2010), viral (Yasuike et al. 2007, Encinas et al. 2010, Rakus et al. 2012) or parasitic (Gonzalez et al. 2007, Forlenza et al. 2009) infections. These investigations have revealed extra-hepatic expression of complement components and suggest an important role of complement in the regulation of local immune responses (Forlenza et al. 2009, Nakao et al. 2011).

The aim of the present study was to determine the association between the APR proteins of common carp and viral infection. Building upon the preliminary investigations of Rakus et al. (2012) on carp complement activation, we report the role of the APR in common carp during a CyHV-3 infection. The expression profile of 2 carp CRP-like genes (*crp1* and *crp2*), recently described by Falco et al. (2012), and several carp complement component genes (*c1rs*, *bf/c2*, *c3* and *masp2*), described by Forlenza et al. (2009), were analysed in the liver, head kidney, spleen and gill of CyHV-3- or sham-infected carp as markers of the APR.

MATERIALS AND METHODS

Experimental animals

Common carp *Cyprinus carpio* from the Polish K line (Białowas et al. 2008) were kept at $21 \pm 1^\circ\text{C}$ in UV treated re-circulating systems under a 12:12 h light:dark cycle at the Institute of Ichthyobiology and Aquaculture in Golysz, Poland. Fish were fed daily with commercial carp pellets (Aller Aqua) at 3 to 5% body weight. Carp ($n = 70$) with an average weight of 120 g were transported to the Laboratory of Fish Disease, National Veterinary Research Institute, Pulawy, Poland, and allowed to acclimatise in 800 l tanks at $21 \pm 1^\circ\text{C}$ for 4 wk. Feeding was carried out with commercial carp pellets at 3% body weight per day.

Virus preparation

CyHV-3 was prepared as described by Rakus et al. (2012). Briefly, *Cyprinus carpio* brain (CCB) cells (Neukirch et al. 1999), cultured in minimum essential medium, were inoculated with CyHV-3 virus isolated in the Laboratory of Fish Disease, National Veterinary Research Institute in Pulawy, Poland, from infected common carp in 2005 (passage no. 4). The medium from infected cells, which included the virus, was harvested and used for fish infection. The virus suspension titre, given as tissue culture infective dose ($\text{TCID}_{50} \text{ ml}^{-1}$), was determined by the method of Reed & Muench (1938) at $8 \times 10^4 \text{ TCID}_{50} \text{ ml}^{-1}$.

CyHV-3 challenge and fish sampling

Carp used within this trial were from the same cohort as described by Rakus et al. (2012). Carp ($n = 35$) were infected with CyHV-3 by bath immersion challenge. Fish were bathed for 1 h at 22°C in small plastic containers containing 15 l of aquarium water and the virus suspension ($320 \text{ TCID}_{50} \text{ ml}^{-1}$ in the final volume of water). In addition, 35 carp were sham-infected following the same protocol with the difference that medium was harvested from uninfected CCB cultures. Control and infected fish were returned to their respective tanks, and 5 carp from both groups were sampled at 6, 12, 24, 72, 120 and 336 h post-infection (hpi). Five additional fish from the control group were sampled for time point 0, i.e. just before the exposure to the virus. Carp were lethally anaesthetized by submersion in 0.5 g l^{-1} tri-

caine (Sigma); blood was obtained from the caudal vein, allowed to clot overnight at 4°C , centrifuged ($2500 \times g$, 10 min at 4°C) and the serum stored at -80°C . Liver, head kidney, spleen and gills were removed at 24, 72, 120 and 336 hpi and stored in RNAlater (Qiagen) at -80°C . Tissues were selected for the following reasons: the liver as the main production site for CRP, the head kidney and the spleen as primary immune organs, and the gills as one of the organs prone to infection. Samples (organs and serum) were sent on ice to Keele University (UK) for further analysis.

Quantification of free-phase carp CRP (cCRP) in serum

Free-phase CRP from carp serum (cCRP) was quantified (MacCarthy et al. 2008, Pionnier et al. 2013) with a competitive enzyme-linked immunosorbent assay (ELISA) using CRP extracted and purified from healthy carp serum with a 2-step affinity chromatography procedure (Cartwright et al. 2004, MacCarthy et al. 2008). Briefly, high-density lipoprotein complexes containing apolipoprotein A-I, a major contaminant in the affinity isolation of carp CRP (Cartwright et al. 2004) were removed from the serum before the collection of purified free-phase cCRP which was stored at 4°C in a calcium buffer (10 mM CaCl_2 , 50 mM Tris base, 15 mM NaCl_2 , pH 8) until required.

Determination of CRP levels in the serum of experimental and control fish was carried out using a competitive ELISA. Briefly, individual wells of a high-binding capacity 96-well plate (Corning) were coated with $0.2 \mu\text{g}$ of cCRP diluted in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) and incubated for 20 h at 4°C . Unbound cCRP was removed by washing 3 times with a PBS-T buffer (PBS with 0.2% Tween-20), and any remaining binding sites were blocked by incubating at 37°C for 30 min in PBS containing 5% of dried milk powder (Marvel). Any excess of blocking agent was removed by washing 3 times with PBS-T. Carp serum (diluted 1:10 in PBS) was incubated with $160 \mu\text{g} \mu\text{l}^{-1}$ of rabbit anti-native cCRP IgG (produced by Cartwright et al. 2004) for 30 min at 20°C . A standard curve was produced by replacing carp serum with purified free phase cCRP serially diluted in PBS (0 to $0.5 \mu\text{g} \text{ ml}^{-1}$). After incubation with either carp serum or standards, $100 \mu\text{l}$ of the treated rabbit anti-cCRP IgG solution was applied to the cCRP-coated wells and incubated for 1 h at 37°C . Excess antibody was

removed by washing 3 times with PBS-T, and 100 μ l of the secondary antibody, a goat anti-rabbit IgG peroxidase conjugate (Sigma, dilution 1:8000 in PBS), was applied to the wells and incubated for 1 h at 37°C. After washing 5 more times with PBS-T, 100 μ l of *o*-phenylenediamine dihydrochloride (Sigma) were added to each well. The plate was incubated for 1 h at 37°C in the dark before adding 25 μ l of 2.5 M HCl to stop the enzymatic reaction. The optical density (OD) of each sample was read at 492 nm (Labsystems Multiskan MS plate reader). The cCRP concentration of the individual serum samples was determined with the GraphPad Prism v5 software by comparison between the sample absorbance value and those from the standard curve for which the free phase cCRP concentration is known.

Alternative complement pathway activity assay in carp serum

Serum alternative complement pathway (ACP) activity was analysed according to previously described methods (Matsuyama et al. 1988, Yano et al. 1988, Yano 1996, Selvaraj et al. 2005, 2006) with slight modifications as reported by Pionnier et al. (2013). Briefly, sheep red blood cells (SRBC; TCS Biosciences) were washed with 0.85% saline solution and resuspended in gelatin veronal buffer (GVB; 0.1% gelatin, 250 mM NaCl, 50 mM barbitone, 40 mM sodium barbitone, 10 mM MgCl₂, 2 mM CaCl₂; all from Sigma), and a standard working concentration of 8×10^6 SRBC ml⁻¹ was prepared. Carp serum was subjected to serial dilution (from 7% to 0.5%) and incubated for 1 h at 20°C (0.1 to 15 μ l of serum, 100 μ l 10 mM EGTA-GVB, 100 μ l SRBC). For each dilution, a positive control reflecting 100% haemolysis was also analysed (0.1 to 15 μ l of serum, 100 μ l distilled water, 100 μ l SRBC), and a negative control in which sera were subjected to heat inactivation (56°C for 30 min) was also included.

Samples were then subjected to centrifugation at $400 \times g$ (5 min at 4°C) and kept on ice at 4°C in order to stop the lytic reaction. Supernatant (200 μ l) was transferred to a 96-well plate, and the haemoglobin release was measured by OD reading at 410 nm (Labsystems Multiskan MS plate reader). The rate of haemolysis was calculated for each serum dilution as the OD value for the sample test divided by the OD value for the positive control. Graphs were built with GraphPad Prism v5 software by plotting the log(dilution) against the log(rate of haemolysis); the ACH₅₀ (in ACH₅₀ units ml⁻¹), corresponding to the serum

dilution giving 50% SRBC haemolysis, was determined from the graph.

Quantification of CyHV-3 DNA

Virus DNA quantification was performed using real-time TaqMan qPCR (Gilad et al. 2004). DNA was isolated from 25 mg of liver, spleen and gill tissues after mechanical lysis by means of a QIAgen Tissuelyser II (Qiagen) using the QIAamp DNA Mini Kit (Qiagen) following the manufacturer's instructions. After isolation, the quantity of DNA obtained was evaluated with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Real-time TaqMan qPCR was performed to amplify a fragment of the CyHV-3 ORF 89 and ORF 90 genes (Table 1) by the use of the KHV-86F and KHV-163R primers and the KHV-109P probe (Table 1). The PCR reaction mix contained 1 \times master mix (Maxima Probe qPCR Kit, Fermentas), 800 nM of each primer and 100 nM of fluorescent probe. The reaction was performed using a Stratagene Mx 3005P thermocycler (Agilent). In order to quantify virus genome copies, a recombinant plasmid to be used as standard was obtained by ligating a PCR product amplified with the primers KHV-86F and KHV-163R into the pGEM-T Easy vector (Promega). The PCR was performed using high fidelity polymerase (Advantage 2 PCR kit, Clontech), and the plasmids were propagated in JM109 competent *Escherichia coli* (Promega). Plasmids were isolated with the GeneJET™ Plasmid Miniprep Kit (Fermentas), and a standard curve from 10⁰ to 10⁸ gene copies was prepared and used for quantification of the copy numbers from each sample. The results are presented as the total number of virus copies per 250 ng of DNA.

Gene expression by real-time PCR

RNA isolation and cDNA synthesis

RNA was extracted and purified from head kidney, liver, spleen and gill tissues using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions, and concentrations were determined by Nanodrop 1000 (Thermo Scientific). RNA was diluted to a standard concentration with diethyl pyrocarbonate (DEPC)-treated water (Invitrogen) and DNase treated (Promega) to remove any trace of remaining genomic DNA. RNA was then transcribed to cDNA using Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase according to the manufacturer's in-

Table 1. Oligonucleotides used in this study. CyHV-3: cyprinid herpesvirus 3; CRP: C-reactive protein

Gene	Gene function	Oligo type	Sequence (5'–3')	GenBank acc. no.	Source
KHV-86F	Quantification of CyHV-3 DNA	Forward	GAC GCC GGA GAC CTT GTG	AF411803	Gilad et al. (2004)
KHV-163R		Reverse	CGG GTT CTT ATT TTT GTC CTT GTT		
KHV-109P		Probe	FAM-CTT CCT CTG CTC GGC GAG CAC G-BHQ1		
ORF72	CyHV-3 capsid triplex protein	Forward	GCT TTC TCG ACA CGG AAT GG	DQ177346	Adamek et al. (2012)
		Reverse	GGT TGG CCT CTG TGG ACT TG		
40S	Housekeeping gene	Forward	CCG TGG GTG ACA TCG TTA CA	AB012087	Huttenhuis et al. (2006)
		Reverse	TCA GGA CAT TGA ACC TCA CTG TCT		
<i>crp1</i>	CRP response	Forward	AGC AAT GCA ACA TTT TTC CGT C	JQ010977	Falco et al. (2012)
		Reverse	ACT TGC GTC AAA GCC ACC CAC		
<i>crp2</i>	CRP response	Forward	GAT GCT GCA GCA TTT TTC AGT C	JQ010978	Falco et al. (2012)
		Reverse	CTC CGC ATC AAA GTT GCT CAA AT		
<i>c1rs</i>	Classical complement pathway	Forward	CAA GCC CAT CTT GGC TCC TGG	AB042609	Forlenza et al. (2009)
		Reverse	GTC CAG ATC AAG CGG GGA CGT		
<i>bf/c2</i>	Classical and alternative complement pathways	Forward	CGG TCA TGG GAA AAA GCA	AB047361	Forlenza et al. (2009)
			Reverse		
<i>c3</i>	Central component of the complement system; marker of the 3 pathways	Forward	GGT TAT CAA GGG GAG TTG AGC TAT	AB016215	Forlenza et al. (2009)
			Reverse		
<i>masp2</i>	MB-lectine complement pathway	Forward	CAA GCT GTC CAA GGT GAT TG	AB234294	Forlenza et al. (2009)
			Reverse		

structions (Invitrogen). Briefly, 0.5 µg RNA was added to 0.5 mM dNTPs, 5 mM MgCl₂, 5× PCR Buffer II, 1.25 µM random hexamers, 20 units of RNase inhibitor, DEPC-treated water and 25 units of M-MuLV and incubated in a GeneAmp[®] PCR System 9700 thermal cycler (Applied Biosystems) using the following program: 25°C for 10 min, 42°C for 30 min and 95°C for 5 min. cDNA samples were diluted (1:20) in DEPC-treated water before being stored at –20°C.

Real-time RT-PCR of APR-related genes

Real time reverse transcription (RT)-PCR assays were performed using the ABI PRISM[®] 7000 Sequence Detector System (Applied Biosystems). A volume of cDNA corresponding to 10 ng of RNA was added to a final concentration of 900 nM of each primer (Table 1) and 1× Power SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 20 µl. Complement component genes were selected based upon on their functional role and their position in the complement pathways, i.e. *c1rs* for the classical, *bf/c2* for the classical and alternative, *masp2* for the lectin and *c3* for its central role in the complement cascade (Gonzalez et al. 2007). Thermal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s

at 95°C and 1 min at 60°C. A melting curve was created for each run and checked to ensure the amplification of a single product. Results were analysed according to the 2^{-ΔΔCt} method (Livak & Schmittgen 2001) and normalised against the expression of the 40S ribosomal protein S11 reference gene (Huttenhuis et al. 2006). The results are presented as x-fold change in relation to the control treatment.

Real-time RT-qPCR for CyHV-3 capsid triplex protein

Virus replication was detected based upon mRNA expression of the CyHV-3 capsid triplex protein (CTP) using a plasmid-based quantification RT-qPCR assay as described by Adamek et al. (2012, 2013). A standard plasmid containing the CTP gene was constructed using the method described above for the virus genome plasmid and the primers listed in Table 1. A standard curve ranging from 10² to 10⁷ gene copies was prepared and used to quantify the copy number of the gene transcripts in each sample. qPCR assays for each sample were performed using a StepOnePlus thermal cycler (Applied Biosystems). Each reaction contained cDNA corresponding to a volume of 10 ng of RNA, 200 nM of each primer

(Table 1), 1× Power SYBR Green PCR Master Mix in a total volume of 20 µl. Samples were analysed using the following thermal profile: polymerase activation at 50°C for 2 min, initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s (Adamek et al. 2013). The expression levels of the CyHV-3 CTP gene are presented as the gene copy number normalised for 1×10^5 copies of the gene encoding for the 40S ribosomal protein S11 (normalised copy number) using the following equation: Normalised copy number = mRNA copy number of the CyHV-3 CTP gene / (mRNA copy number of 40S ribosomal protein S11 / 1×10^5).

Confirmation of positive results was performed by comparison against a melting curve generated during each PCR run.

Statistical analysis

All data are given as means \pm SEM of 4 or 5 independent individuals. Statistical analysis was carried out using GraphPad Prism v5 and PASW Statistics 18 software. Data were tested for normality and homoscedasticity prior to further analysis. A 2-way analysis of variance (ANOVA) and post hoc Bonferroni multiple comparisons test were performed on serum cCRP levels and complement activity to determine significant differences between the infected and the control groups. Gene expression data were normalised using a log-transformation prior to a 2-way ANOVA and subsequent Bonferroni post hoc test analysis. In order to investigate whether serum cCRP levels and serum complement ACP activity were correlated, Pearson correlation analyses were performed. Additional post hoc Pearson correlation analyses were performed to investigate whether variations observed in serum levels or activities were correlated to specific related gene expression. Significance was defined as $p \leq 0.05$.

RESULTS

Course of infection

CyHV-3 DNA was detected as early as 24 hpi in the spleen (Fig. 1) and then in all analysed tissues from 72 hpi. The highest virus loads for each organ were recorded at 120 hpi with 9187, 61 766, and 435 252 copies per 250 ng of DNA isolated from liver, spleen and gill tissues, respectively. Although significant

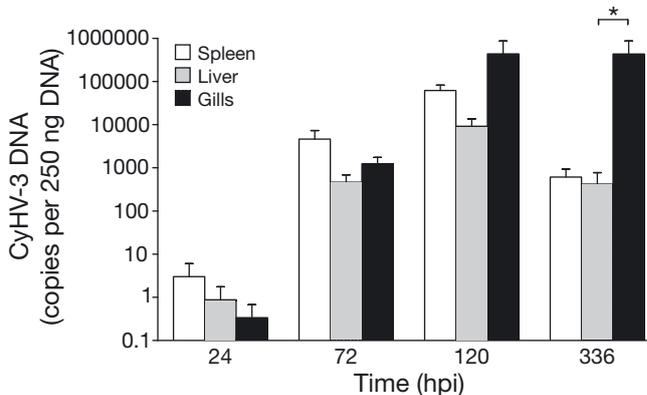


Fig. 1. *Cyprinus carpio*. Cyprinid herpesvirus 3 (CyHV-3) load in the spleen (white bars), liver (gray bars) and gill (black bars) tissues of carp measured by TaqMan qPCR. Data are means \pm SEM genome copies for 250 ng of isolated DNA for $n = 5$ fish per time point. *Significant difference ($p \leq 0.05$)

differences were not noted between organs at 24, 72 and 120 hpi, the viral loads were significantly higher in the gills compared to the liver ($p < 0.05$) at 336 hpi (432 789 and 418 copies, respectively). Viral DNA was not detected in control fish.

The presence of CyHV-3 CTP mRNA expression was first seen at 72 hpi in the spleen in infected fish (Table 2). The highest expression levels were recorded in all tissues at 120 hpi (150 normalised copies in the gills, 210 normalised copies in the head kidney, 260 normalised copies the liver and 1270 normalised copies in the spleen). Virus replication also occurred at 336 hpi in the head kidney. Viral mRNA was not found to be expressed in control fish.

Serum acute phase proteins profiles

Serum cCRP levels (Fig. 2A) were significantly different between control and infected groups ($F =$

Table 2. *Cyprinus carpio*. Cyprinid herpesvirus 3 (CyHV-3) capsid triplex protein (CTP) mRNA expression in the liver, head kidney, spleen and gills measured at 24, 72, 120 and 336 h post infection (hpi). Data are mean copy numbers (\pm SEM, $n = 5$) of the CyHV-3 CTP gene normalised against 100 000 copies of 40S ribosomal protein S11. No Ct: CyHV-3 CTP mRNA was not detected

Time (hpi)	Normalised copy number in tissue			
	Liver	Head kidney	Spleen	Gill
24	No Ct	No Ct	No Ct	No Ct
72	No Ct	No Ct	3.3 ± 2	No Ct
120	147.2 ± 83	210.6 ± 95	1267 ± 370	147.2 ± 83
336	No Ct	29.6 ± 26	No Ct	No Ct

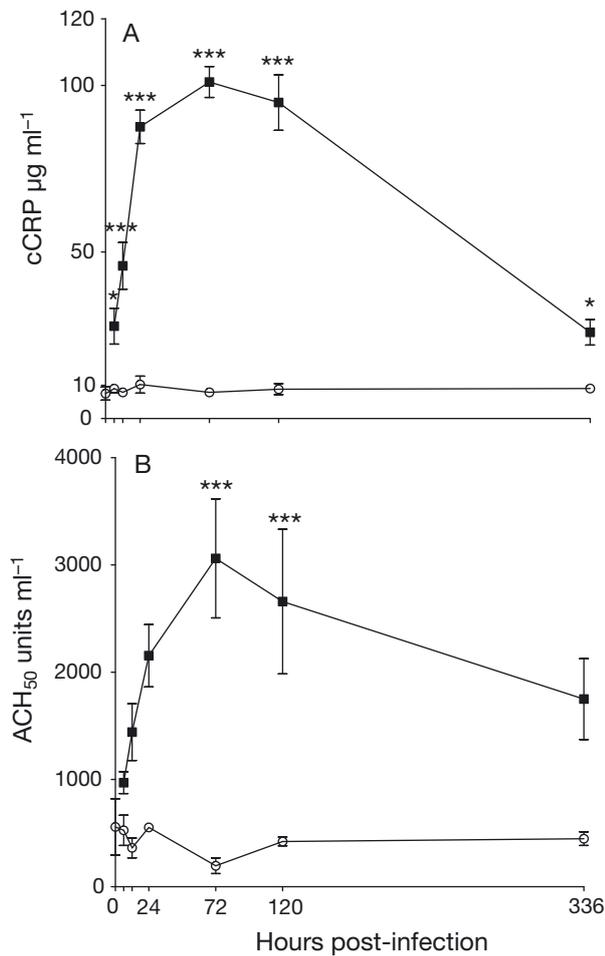


Fig. 2. *Cyprinus carpio*. (A) C-reactive protein (CRP) levels and (B) alternative complement pathway (ACP) activity in the serum of carp infected with cyprinid herpesvirus 3 (CyHV-3) by bath immersion. Data are means \pm SEM ($n = 4$ or 5). Statistical significance as determined by Bonferroni post hoc 2-way ANOVA when comparing data from infected fish (■) to those obtained from the control fish (○) at their respective sampling points indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

629.6, $df = 1$, $p < 0.0001$), over time since treatment ($F = 30.7$, $df = 6$, $p < 0.0001$), and also when time/treatment interactions were considered ($F = 31.0$, $df = 6$, $p < 0.0001$). A Bonferroni post hoc analysis revealed that serum cCRP levels were significantly higher in the infected fish compared to control fish during the whole duration of the challenge. Indeed, whilst cCRP levels in control fish were constantly below $10 \mu\text{g ml}^{-1}$, cCRP levels in infected carp were already significantly increased by 6 hpi ($27.8 \mu\text{g ml}^{-1}$, $p \leq 0.05$) and reached a plateau from 24 hpi ($87.6 \mu\text{g ml}^{-1}$, $p \leq 0.001$) until 120 hpi ($95 \mu\text{g ml}^{-1}$, $p \leq 0.001$) after which a decrease occurred at 336 hpi ($25.9 \mu\text{g ml}^{-1}$, $p \leq 0.05$).

Serum ACP activity (Fig. 2B) was abolished by heat inactivation and was also significantly different between control and infected groups ($F = 59.9$, $df = 1$, $p < 0.0001$). Although the duration of infection did not appear to have an effect ($F = 2.0$, $df = 6$, $p = 0.0864$), the time/treatment interaction was significantly affected ($F = 2.7$, $df = 6$, $p = 0.0316$). A Bonferroni post hoc analysis revealed that the serum ACP activity was significantly higher in infected fish at 72 hpi ($3060 \text{ ACH}_{50} \text{ units ml}^{-1}$, $p \leq 0.001$) and 120 hpi ($2658 \text{ ACH}_{50} \text{ units ml}^{-1}$, $p \leq 0.001$) whilst the ACP activity in control fish remained stable at these sampling points (approximately $500 \text{ ACH}_{50} \text{ units ml}^{-1}$). It is interesting to note that, as with serum cCRP levels, serum ACP activity increased in infected fish from 6 hpi onwards and reached a plateau between 72 and 120 hpi before it finally decreased at the end of the challenge (336 hpi).

Pearson correlation statistical analyses revealed that CRP levels and complement activity were not correlated in control fish but were significantly positively correlated in infected fish ($r = 0.6996$, $p < 0.0001$), indicating complement ACP activity and CRP levels both increasing at the same time in those carp.

CRP- and complement-related gene expression profiles

The constitutive expression levels of CRP (*crp1* and *crp2*) and complement (*c1rs*, *bf/c2*, *c3* and *masp2*) genes in the control group (Table 3) clearly indicated that all of the analysed genes were synthesised in the liver. Except for *crp2* and *bf/c2*, the threshold cycle (Ct) values were lower in the liver compared to the other tissues. It is interesting to note, however, that extra-hepatic expression was also clearly noticeable for *bf/c2* in the head kidney, spleen and gill tissues, suggesting a wide expression of this *bf/c2* gene.

In the liver (Fig. 3A), all genes except *crp1* were up-regulated in response to CyHV-3 infection ($F = 7.2$ – 30 , $df = 1$, $p \leq 0.0117$). Time also significantly affected the expression of all genes analysed ($F = 4.6$ – 11.7 , $df = 3$, $p \leq 0.0086$), except for *masp2* ($F = 2.7$, $df = 3$, $p = 0.0647$).

Interestingly, post hoc analysis revealed that the CyHV-3 infection caused several significant down-regulations for the expression of *crp1* at 24 hpi (0.1-fold, $p \leq 0.01$), 72 hpi (0.26-fold, $p \leq 0.01$) and 120 hpi (0.12-fold, $p \leq 0.01$) except for a significant up-regulation at 336 hpi (2.3-fold, $p \leq 0.05$). Although

Table 3. *Cyprinus carpio*. Ct values of C-reactive protein (CRP; *crp1* and *crp2*) and complement (*c1rs*, *bf/c2*, *c3* and *masp2*) gene expression levels from the control group in the different tissues. Significant differences when comparing the Ct values from the liver to those from the other organs are indicated as follows: ** $p \leq 0.01$, *** $p \leq 0.001$. Data are means \pm SEM (n = 5)

Gene	Ct value in tissue			
	Liver	Head kidney	Spleen	Gill
<i>crp1</i>	24.7 \pm 0.3	34.2 \pm 0.2***	33.3 \pm 0.1***	35.4 \pm 0.6***
<i>crp2</i>	35.6 \pm 0.3	31.7 \pm 0.4***	34.5 \pm 0.4	31 \pm 0.4***
<i>c1rs</i>	23.5 \pm 0.6	31.4 \pm 0.3***	30.8 \pm 0.2***	31.9 \pm 0.2***
<i>bf/c2</i>	27.9 \pm 0.5	24.5 \pm 0.4***	26.6 \pm 0.3	30 \pm 0.3**
<i>c3</i>	19.2 \pm 0.3	35.6 \pm 0.8***	30.5 \pm 0.2***	32.3 \pm 0.5***
<i>masp2</i>	24.4 \pm 0.4	36.7 \pm 0.3***	37.3 \pm 0.2***	37.6 \pm 0.4***

crp2 expression was also up-regulated (6.4-fold, $p \leq 0.01$) at 336 hpi, in contrast to *crp1*, there was also an increase at 72 hpi (3.6-fold, $p \leq 0.05$). In addition, a pattern of significant up-regulations was also shown for complement-related gene expression at 72 hpi, i.e. 4.9-fold for *c1rs* ($p \leq 0.01$), 8.2-fold for *bf/c2* ($p \leq 0.001$), 5.8-fold for *c3* ($p \leq 0.001$) and 2.8-fold for *masp2* ($p \leq 0.05$). Significant up-regulations were also detected at 120 hpi for *bf/c2* (8.1-fold, $p \leq 0.001$) and *c3* (3.4-fold, $p \leq 0.01$), and at 336 hpi for *c1rs* (4.1-fold, $p \leq 0.05$), and a significant down-regulation was observed at 24 hpi for *c1rs* expression (0.12-fold, $p \leq 0.05$).

In contrast to the liver, viral infection significantly affected the gene expression of *c3*, *masp2* and *crp1* in the head kidney (Fig. 3B) ($F = 5.8$ – 8.7 , $df = 1$, $p \leq 0.022$) but not the expression of *c1rs*, *bf/c2* and *crp2* ($F = 1.3$ – 3.37 , $df = 1$, $p \geq 0.0756$). With the exception of *c1rs* and *masp2* ($F = 1.6$, $df = 3$, $p = 0.2047$ and $F = 2.3$, $df = 3$, $p = 0.0957$, respectively), duration of infection significantly affected CRP- and complement-related gene expression ($F = 3.262$ – 8.303 , $df = 3$, $p \leq 0.0341$).

Post hoc analysis revealed a significant up-regulation of *crp1* at 336 hpi in the head kidney (6.3-fold, $p \leq 0.01$) similar to the one observed in the liver, although down-regulations were not detected at the early sampling points. Interestingly, *crp2* expression was significantly affected by the duration of the infection with up-regulation detected at 24 hpi (1.8-fold, $p \leq 0.05$) followed by a subsequent down-regulation at 72 and 336 hpi (0.33- and 0.25-fold, respectively, $p \leq 0.05$). As with the liver, a pattern of significant up-regulation of complement-related gene expression was detected in the head kidney at 72 hpi for *bf/c2* (2.35-fold, $p \leq 0.001$), *c3* (25.74-fold, $p \leq 0.001$) and *masp2* (5-fold, $p \leq 0.05$). Finally, the fourth com-

plement-related gene analysed, *c1rs*, was significantly up-regulated at 336 hpi (2.9-fold, $p \leq 0.05$).

In the spleen (Fig. 3C), expression of *bf/c2*, *c3*, *crp1* and *crp2* was significantly affected by CyHV-3 infection ($F = 6.5$ – 29.6 , $df = 1$, $p \leq 0.016$) but not the expression of *c1rs* and *masp2* ($F = 1.6$, $df = 1$, $p = 0.2183$ and $F = 0.01$, $df = 1$, $p = 0.9201$, respectively). As in the liver and head kidney, expression of the 2 CRP-related genes was significantly affected by time ($F = 3.5$, $df = 3$, $p = 0.0275$ for *crp1* and $F = 3.9$, $df = 3$, $p = 0.0169$ for *crp2*). However,

for the complement-related genes, duration of infection only affected the expression of *bf/c2* ($F = 7.9$, $df = 3$, $p = 0.0004$).

Post hoc analysis revealed significant up-regulations in CRP-related gene expression, i.e. up-regulation of *crp1* at 120 hpi (3.2-fold, $p \leq 0.01$) and up-regulations of *crp2* at 24 hpi (3.4-fold, $p \leq 0.01$) and 336 hpi (3.3-fold, $p \leq 0.01$). Regarding complement-related genes, the expression profiles of *c1rs* and *masp2* were not significantly affected during the duration of the challenge whilst the expression of *bf/c2* was substantially up-regulated at 72 hpi (12.4-fold, $p \leq 0.001$) and 120 hpi (8.5-fold, $p \leq 0.01$) and up-regulation was detected in *c3* expression at 336 hpi (8.2-fold, $p \leq 0.01$).

Finally, in the gills (Fig. 3D), the expression of all genes analysed was significantly affected by the viral infection ($F = 4.8$ – 25 , $df = 1$, $p \leq 0.0364$), with the exception of *c3* ($F = 1.6$, $df = 1$, $p = 0.2111$). In contrast to the head kidney and liver, the effect of duration of infection was found to be significant only for *bf/c2* ($F = 4.6$, $df = 3$, $p = 0.0087$) and *crp2* ($F = 9.3$, $df = 3$, $p = 0.0001$) expression.

Interestingly, post hoc analysis revealed that *crp1* expression was not affected by the infection in the gills, but a pattern of up- and down-regulation of *crp2* expression was detected, i.e. up-regulation at 24 hpi (3.5-fold, $p \leq 0.001$), then down-regulation at 72 and 120 hpi (0.3-fold, $p \leq 0.01$ and 0.36-fold, $p \leq 0.05$ respectively), and finally up-regulation at 336 hpi (2.7-fold, $p \leq 0.05$). Regarding complement-related genes, whilst the expression of *c3* and *masp2* were not significantly affected during the challenge period, *c1rs* was significantly up-regulated at 120 hpi (2.2-fold, $p \leq 0.05$) and *bf/c2* was significantly up-regulated at 24 hpi (3.1-fold, $p \leq 0.05$), 72 hpi (7.8-fold, $p \leq 0.05$), 120 hpi (7.6-fold, $p \leq 0.01$) and 336 hpi (20.7-fold up-regulation, $p \leq 0.001$).

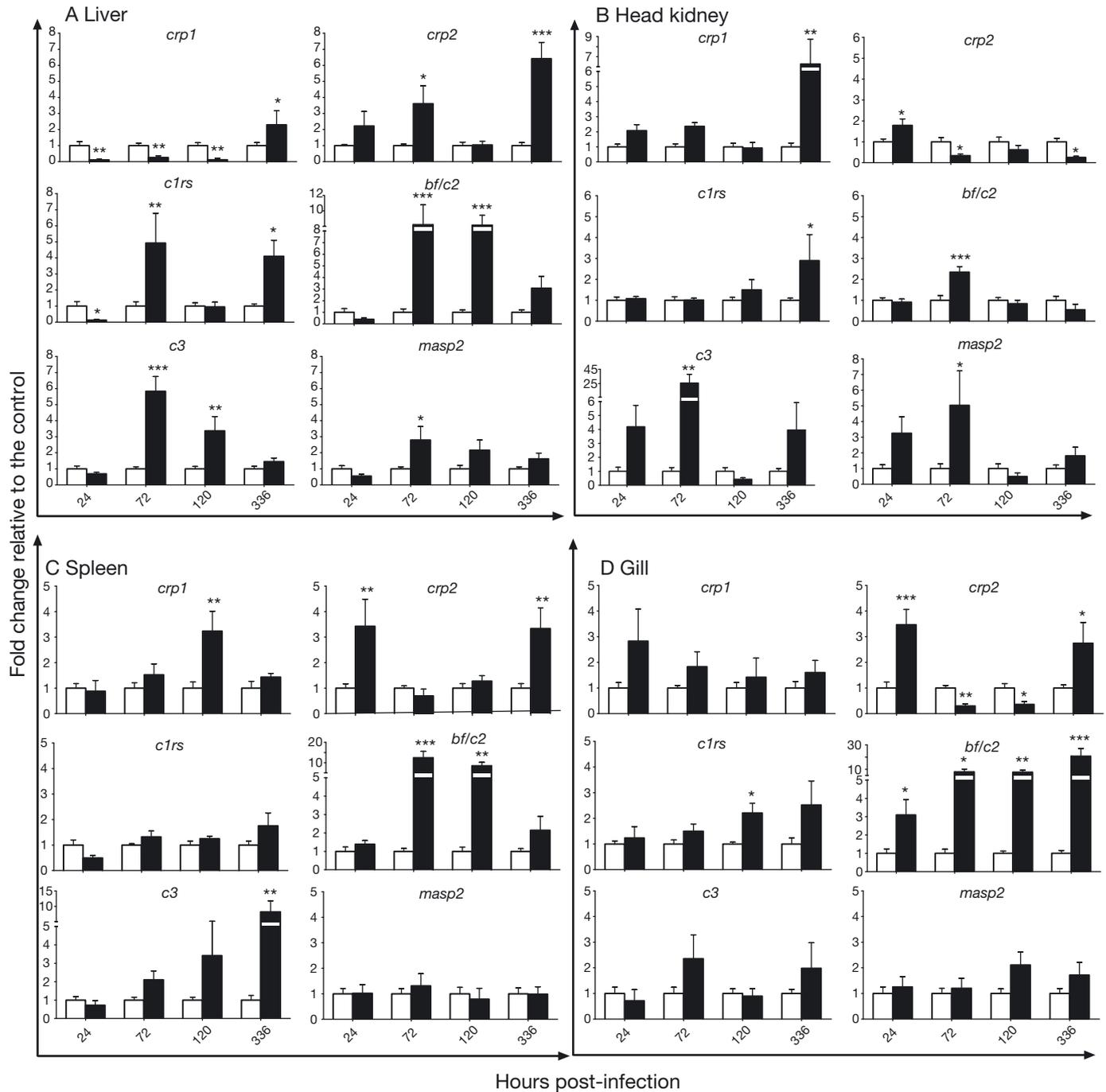


Fig. 3. *Cyprinus carpio*. C-reactive protein (CRP; *crp1* and *crp2*) and complement (*c1rs*, *bf/c2*, *c3* and *masp2*) related gene transcript profiles in (A) liver, (B) head kidney, (C) spleen and (D) gills of carp infected with cyprinid herpesvirus 3 (CyHV-3) by bath immersion. Expression levels of the infected groups (black bars) are shown as x-fold change compared to the control group (white bars) at each time point. Significant differences when comparing the infected groups to their respective time point control group are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001. All data are means \pm SEM (n = 5)

DISCUSSION

The present study reveals that both CRP and complement responses, monitored by either serum activity/levels or gene expression in tissues, significantly changed in common carp challenged with CyHV-3

by bath infection. Although a viral load was noted at 24 hpi, a significant increase in viral DNA occurred after 72 hpi in all the tissues analysed (spleen, liver and gills). In addition, significant increases in serum CRP levels and complement activity were recorded during the first 72 hpi in the infected carp, indicating

a strong and rapid immune acute phase response in these fish. Interestingly, although we noted the absence of viral gene expression at 336 hpi, Adamek et al. (2013) suggested that this may occur after this period of infection.

The rapid and significant increase in serum cCRP levels (from 6 hpi to a 10-fold increase in 72 h) has revealed for the first time that CRP behaves as a positive acute phase reactant in common carp challenged with CyHV-3. This immediate response is remarkable, since at this point, no pathological changes were recorded. The promptly elevated levels of serum cCRP that reach a maximum between 72 and 120 hpi may be related to the systemic character of the infection and therefore could support the putative role of cCRP in the induction of the leukocytosis as observed by Negenborn (2009). In addition, the viral load and replication as recorded in all tissues analysed at 72 and 120 hpi combined with previous results from Rakus et al. (2012) and Adamek et al. (2013) indicate that the virus spreads throughout the whole body, reaching multiple organs within 72 h after pathogen exposure. This leads to significant up-regulations in the synthesis of several immune-related genes at 72 hpi, including genes involved in the regulation of CRP expression (Szalai et al. 2000) such as IL-6, IL-1 α , IL-1 β , TNF- α and C3 and C5a complement components (Rakus et al. 2012). Furthermore, a significant increase in the classical pathway complement activity and in the expression of its related genes (*c1rs* and *bf/c2*) at 120 hpi may contribute to cCRP activation.

Interestingly, these elevated levels of serum cCRP do not appear to be correlated with CRP-related gene expression profiles when considering all tissues analysed, in particular the liver. Results revealed different patterns in all tissues examined; for example, levels of *crp1* were decreased in liver, but were enhanced in spleen and kidney. In contrast, *crp2* gene expression was reduced in head kidney but elevated in liver and spleen. However, due to the substantial difference in *crp1* and *crp2* Ct values in the liver of control fish (i.e. a 1024-fold difference in expression), the amount of CRP1 mRNA produced in infected fish was still significantly higher than the amount of CRP2 mRNA even if there was a 0.12-fold reduction in *crp1* and a 6-fold up-regulation of *crp2*. Previous studies also suggested that additional CRP-related genes might code for different common carp CRP isoforms (Falco et al. 2012, Pionnier et al. 2013). As the rabbit anti-cCRP IgG used to detect CRP levels in the carp serum reacts with native carp CRP, this could explain the differences observed between cCRP serum levels and tissue-related

gene expression. In addition, the antibody used in this study reacts with native carp CRP, but we do not know yet if CRP is encoded predominantly by *crp1* or *crp2*. These results suggest an organ- and time-dependent response which could be due to different biological and functional properties of the synthesised proteins in the different tissues as hypothesised by Falco et al. (2012). However, it is interesting to note that *crp1* and *crp2* have been found to be expressed constitutively in a mucosal site (gills) as well as in systemic sites (spleen, head kidney and liver), thus confirming the widespread CRP expression in a range of tissues in carp reported by Falco et al. (2012). The observation that CyHV-3 infection had a significant effect on cCRP-related gene expression in the gills may be particularly noteworthy, as gills are severely affected by the virus (Perelberg et al. 2003), and the highest viral loads were detected in the gills. Despite the fact that, in mammals, increasing serum CRP levels are more characteristic of a bacterial infection rather than a viral infection (Cray et al. 2009), primarily mediated through the direct binding of CRP to phosphocholine residues of bacterial C-polysaccharides (Volanakis & Kaplan 1971), our results suggest that the CRP response may also be of great importance during viral infections in carp. In that case, CRP, as an acute phase reactant, could play a role in the general activation of other acute phase immune components such as the complement system as observed, for example, in mammals (Agrawal et al. 2001) and in the late recognition and clearance of apoptotic, damaged or necrotic cells (Hack et al. 1997). The former is in accordance with simultaneously enhanced mRNA levels of apoptosis-inducing genes during the CyHV-3 infection (J. J. Miest et al. unpubl.), which indicate induced apoptosis at 120 hpi.

CyHV-3 infection also induced a complement response in carp, which was inactivated by heat treatment, and resulted in a 6-fold increase in serum ACP activity beginning at 6 hpi and reaching a maximum of approximately 3000 ACH₅₀ units ml⁻¹ between 72 and 120 hpi. This is in accordance with the results obtained for the ACP-related gene expression profiles (i.e. *bf/c2* and *c3*). Indeed, significant up-regulations of *bf/c2* were detected throughout the challenge in the 4 tissues examined, with substantial up-regulations occurring at 72 and 120 hpi, e.g. around 8-fold in the liver and in the gills, and up to 12.4-fold in the spleen. Such significant up-regulations of *bf/c2* were also found by Forlenza et al. (2009) in the hepatopancreas of common carp challenged with the parasite *Trypanoplasma borreli*. The results of our study indicate that CyHV-3 infection significantly induced the

production of Bf/C2 in a variety of systemic sites, i.e. spleen, liver and head kidney, but also at a possible site of infection, i.e. gills, suggesting that the complement alternative pathway was stimulated in response to the virus. However, the literature on the relationship between complement-related gene expression and virus infection in fish is inconsistent. Whilst some studies have shown that neutralisation of some fish viruses such as the infectious haematopoietic necrosis virus (IHNV) and the viral haemorrhagic septicaemia virus (VHSV) are complement-independent in several fish species (LaPatra 1996, Boshra et al. 2006), other investigations have revealed that the hosts' immune responses could be complement-dependent (LaPatra 1996, Lorenzen & LaPatra 1999). According to Ellis (2001), this could depend on the virus type, i.e. if the virus possesses an envelope, the complement system will be activated and if not, no changes would be detected in complement response. As CyHV-3 possesses an envelope, our results would support this theory. The expression profile of the other complement-related gene analysed, viz. *c3*, which is also involved in the ACP, revealed a significant up-regulation in the head kidney, liver and spleen. The up-regulation of the *c3* gene detected in the head kidney (25-fold) and in the liver (6-fold) at 72 hpi could be related to a need for C3 production in order to maintain the complement activity at a high level. Such *c3* up-regulations in response to virus infection in fish were also observed in the Japanese flounder *Paralichthys olivaceus* challenged with VHSV (Byon et al. 2006) and in rainbow trout *Oncorhynchus mykiss* challenged with IHNV (Overturf & LaPatra 2006). Finally, up-regulation (from 2- to 5-fold) of the genes encoding *c1rs* and *masp2* occurred in the head kidney, liver and gills but not in the spleen. This suggests that the classical and lectin complement pathways could have been stimulated, potentially through CRP, although no significant increase was detected in the serum complement classical pathway activity of those carp (Rakus et al. 2012). The fact that the expression of these classical and lectin pathway-related genes (*c1rs* and *masp2*, respectively) were less affected than the expression of the alternative pathway-related genes (*bf/c2* and *c3*) is in agreement with previous studies suggesting that the alternative pathway has a greater role than the classical or the lectin pathways in fish immune response to viral and bacterial infections (Yano 1996, Ellis 2001, Boshra et al. 2006).

In carp, the acute phase response to CyHV-3 infection has also been found to be organ dependent. The increase in CRP and complement responses could be related to different tissue damages observed, for in-

stance, in the gills, kidney, spleen and liver of carp infected by CyHV-3 (Perelberg et al. 2003). Our results are in accordance with other studies performed on the same individual fish, revealing that the expression of several immune-related genes was affected in the head kidney and spleen (Rakus et al. 2012), and that the regulation of apoptosis-related genes was also organ- and time-dependent (J. J. Miest et al. unpubl.). In our study, gene expression of CRP- and complement-related genes was especially affected at 72 and 120 hpi in the head kidney and liver. However, these effects were less prominent in the gills and spleen. These observations are supported by Pearson correlation analyses, which revealed significant correlations between serum cCRP levels and *crp1* and *crp2* gene expression but only in the liver of infected fish ($r = 0.28$, $p = 0.0007$ and $r = 0.48$, $p = 0.047$, respectively). Despite the fact that teleost complement components and CRP have been found to be primarily expressed in the liver (Boshra et al. 2006, Nakao et al. 2011), our results are in accordance with those obtained in previous studies performed on common carp (Falco et al. 2012, Pionnier et al. 2013) and also in cod *Gadus morhua* (Lange et al. 2004a) and halibut *Hippoglossus hippoglossus* (Lange et al. 2004b), suggesting a widespread tissue distribution of complement and CRP in fish as observed in mammals (Sahu et al. 1998). Nevertheless, statistical analyses revealed the greatest infection and time effects in the liver, where the expression of 5 of the 6 genes analysed was significantly affected by either the infection or the duration of the infection, highlighting the importance of the liver in acute phase reactant production in carp. Interestingly, data analysis also revealed that the expression of some CRP- and complement-related genes in the gill tissue was significantly affected by the infection with, for example, up to 20-fold up-regulation of *bf/c2* and both up- and down-regulation in *crp2*. These results indicate that the gills may be an important mucosal site involved in the carp immune APR as suggested in previous studies (Perelberg et al. 2003, Dishon et al. 2005).

In summary, an acute phase immune response has been identified in common carp infected by CyHV-3 via a bath immersion route. This stimulation, i.e. increased serum cCRP levels and ACP activity and up-regulation of complement- and CRP-related genes in analysed immune organs, has shown that these 2 innate protein systems, i.e. CRP and complement, behave as typical acute phase reactants to a viral infection in carp. Hence it could be possible to use CRP and complement as immediate biomarkers to detect infections in fish as it is used in humans.

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