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Title: Differential effects of Alloherpesvirus CyHV-3 and Rhabdovirus SVCV on apoptosis in fish cells

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Abstract

Whilst *Herpesviridae*, which infect higher vertebrates, actively influence host immune responses to ensure viral replication, it is mostly unknown if *Alloherpesviridae*, which infect lower vertebrates, possess similar abilities. An important antiviral response is clearance of infected cells via apoptosis, which in mammals influences the outcome of infection. Here, we utilise common carp infected with CyHV-3 to determine the effect on the expression of genes encoding apoptosis-related proteins (p53, Caspase 9, Apaf-1, IAP, iNOS) in the pronephros, spleen and gills. The influence of CyHV-3 on CCB cells was also studied and compared to SVCV (a rhabdovirus) which induces apoptosis in carp cell lines. Although CyHV-3 induced iNOS expression *in vivo*, significant induction of the genetic apoptosis pathway was only seen in the pronephros. *In vitro* CyHV-3 did not induce apoptosis or apoptosis-related expression whilst SVCV did stimulate apoptosis. This suggests that CyHV-3 possesses mechanisms similar to herpesviruses of higher vertebrates to inhibit the antiviral apoptotic process.

Keywords

CyHV-3, KHV, SVCV, carp, CCB, apoptosis, *Alloherpesviridae*

Abbreviations

CyHV-3: *Cyprinid Herpesvirus 3*

KHV: Koi herpesvirus

SVCV: *Spring viremia of carp virus*

MCP: major capsid protein

GP: glycoprotein

CCB: common carp brain cell line

1. Introduction

Throughout evolution viruses have developed various strategies to evade the immune system of the host and thus ensure their replication. One of these strategies targets the induction of apoptosis in infected cells which, when it occurs early in the infection, prevents viral replication and distribution (Hay and Kannourakis, 2002). The viral strategy can, for example, result in the inhibition of cellular apoptosis to ensure viral replication in the host cells, or the active induction of apoptosis in order to impair the immune response or to release progeny at the later stage of viral replication (Best and Bloom, 2004; Leu et al., 2013; Tschopp et al., 1998). The association between apoptosis and viral infection is therefore complex, either increasing or reducing host cell death. Which of these outcomes predominates during an infection seems to, at least partially, depend on the virus type, i.e. viruses with large genomes appear to have a higher capacity to actively influence the apoptotic process compared with viruses with small genomes (e.g. Roulston et al., 1999). In general RNA viruses, such as the rhabdovirus *Spring viremia of carp virus* (SVCV), have a small genome which does not appear to encode genes with the ability to influence the apoptotic process of the host. In contrast, DNA viruses, such as the herpesviruses, have large genomes, and are known to interfere with the host's immune response and apoptosis pathway by expressing homologue genes to their host (Ahne et al., 2002; van Beurden et al., 2011). This phenomenon has been intensively studied in mammalian herpesviruses (i.e. *Herpesviridae*). As reviewed by Lagunoff and Carroll (2003) it has been shown that many sequenced *γ-Herpesviridae*, such as *Epstein-Barr virus* (EBV) and *Human herpesvirus 8* (HHV-8) express a homologue of the anti-apoptotic protein Bcl-2. In addition, HHV-8 has been shown to express proteins such as LANA and vIL6 that prevent p53 and IFN-α induced

apoptosis. In contrast, Herpes simplex viruses, which belong to the *α-Herpesviridae*, trigger apoptosis earlier in the infection but inhibit this process later in the infection process by expressing a variety of anti-apoptotic genes. This ability to actively influence host apoptosis has been suggested by Leu et al. (2013) to be directly correlated to the virulence of the virus since it facilitates viral replication and virus survival.

Although there have been many studies on the association between viral infections and apoptosis in mammalian systems very little is known about these mechanisms in lower vertebrates, particularly fish. Common carp (*Cyprinus carpio* L.) is a host for two highly contagious viruses: *Cyprinid herpesvirus 3* (CyHV-3) and *Spring viraemia of carp virus*. CyHV-3, commonly called Koi herpesvirus (KHV) (Hedrick et al., 2000), is a member of the *Alloherpesviridae* family of herpesviruses (Waltzek et al., 2005), it is a double stranded DNA virus, with a genome size of 295 kb, encoding 155 predicted open reading frames (Davison et al., 2013). CyHV-3 genome encodes proteins potentially involved in immune evasion mechanisms such as tumour necrosis factor receptor homologues (encoded by ORF4 and ORF12) and an interleukine-10 (IL-10) homologue (encoded by ORF134) (Aoki et al., 2007; Ouyang et al., 2013). In recent publications the effect of this virus on the innate immune response of its primary host *C. carpio* were highlighted (Adamek et al., 2012; 2014a; 2014b; 2013; Pionnier et al., 2014; Rakus et al., 2012; Syakuri et al., 2013). As part of these studies it was shown that CyHV-3 inhibits *in vitro* up-regulation of type I interferons (Adamek et al., 2012), the cytokines, which have been closely associated with the induction of apoptosis in mammals (Tanaka et al., 1998). We therefore hypothesised that CyHV-3 could influence host apoptosis and thus facilitate its replication.

SVCV causes mortality in farmed and wild carp in Europe and North America, and also affects other cyprinids in which it tends to be less virulent (Garver et al., 2007). This

109 virus has been identified as a member of the *Rhabdoviridae* family in the order of the
110 *Mononegavirales* and the genus *Spirivivirus* (ICTV 2013). SVCV, in accordance with most
111 members of the *Rhabdoviridae* family, has a genome that is composed of one molecule of
112 non-segmented, linear, single stranded negative-sense RNA encoding 5 genes (Ahne et al.,
113 2002). Although two independent studies have shown that SVCV infection of the EPC cell
114 line *in vitro* induces apoptosis at the morphological level (Björklund et al., 1997; Kazachka et
115 al., 2007), the mechanism by which SVCV induces apoptosis at the molecular level still
116 requires elucidation.

117

118 This manuscript aimed to study for the first time the influence CyHV-3 on the apoptotic
119 process both *in vivo* and *in vitro*. It was shown that unlike SVCV, CyHV-3 did not induce
120 apoptosis in CCB cells. Moreover, *in vitro* CyHV-3 infection did not induce genes encoding
121 for classical apoptosis-related proteins (i.e. p53, Caspase 9, Apaf-1, IAP) as well as iNOS and
122 type I IFN, whilst *in vivo* the genetic apoptosis pathway was only induced 14 days post
123 infection. Possible factors influencing the differential apoptosis response during CyHV-3 and
124 SVCV infections are discussed.

125

2. Material & Methods

2.1 Fish

Common carp (*Cyprinus carpio* L.) of the Polish line K (Irnazarow, 1995) were reared in the facilities at the Laboratory of Fish Disease at the National Veterinary Research Institute in Pulawy, Poland. Carp were kept in two 800 L tanks at 21 ± 1 °C under a 12/12 h light/dark cycle and were allowed to acclimate for 4 weeks prior to the infection. Feeding occurred daily with commercial carp pellets (Aller Aqua, Poland) at 3 % body weight/day. No mortality occurred during this acclimatisation period.

2.2 *In vivo* CyHV-3 challenge

CyHV-3 (local Polish isolate) was isolated at the Laboratory of Fish Disease, National Veterinary Research Institute in Pulawy, Poland from infected common carp in 2005 (passage No. 4) as described by (Rakus et al., 2012). The virus was propagated in cells of the *C. carpio* brain (CCB) cell line (Neukirch et al., 1999; Neukirch and Kunz, 2001), which were cultured in minimum essential medium (MEM) (Gibco, Germany) enriched with 4.5 g/L glucose (D-glucose monohydrate), 10 % fetal calf serum, penicillin (200 i.u./ml), streptomycin (0.2 mg/ml), and 1 % non-essential amino acid solution (all Sigma Aldrich, Germany). Culturing was carried out at 22 °C with 5 % CO_2 in a humid atmosphere (Thermo Scientific Heraeus CO_2 Incubator).

Fish (mean weight ± SD = 120 ± 38 g) were divided into a control and an infection group. The latter group was infected with the CyHV-3 virus by immersion (Rakus et al., 2012). Briefly, fish were exposed to the CyHV-3 suspension in aquarium water containing 3.2 x 10^2 $TCID_{50}$/ml in small plastic containers for 1 h at 22 °C and then returned to their respective tank. Control fish were treated in the same way but medium from uninfected CCB cultures

149 was used instead of the virus suspension. For gene expression analysis five control fish and
150 five infected fish were sampled at of 1, 3, 5, and 14 days post infection (p.i.). Fish were killed
151 with a lethal dose of 0.5 g/L tricaine (Sigma Aldrich, Germany) and organ samples
152 (pronephros, gills, spleen) were removed, placed into sterile tubes containing RNA later
153 (Qiagen, Germany) and stored at -80 °C until RNA extraction.
154

155 **2.3 *In vitro* infection with CyHV-3 and SVCV.**

156 **2.3.1. Cells and viruses**

157 CCB cells were cultured in minimum essential medium (MEM) with Earle's salts
158 supplemented with Non-Essential Amino Acids (NEAA), 10 % foetal bovine serum, 0.35 %
159 glucose, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cultures were incubated at 25 °C
160 in a humidified atmosphere containing 2 % CO_2. All culture ingredients were supplied by
161 Sigma Aldrich, Germany.
162 CyHV-3 (Israel isolate KHV I, FLI Germany) (Hedrick et al., 2000) was re-isolated from the
163 skin of carp, which had been infected by intraperitoneal injection with the virus, according to
164 a standard protocol (Thoesen, 1994). SVCV (isolate 56 – 70) was kindly donated by Prof. N.
165 Fijan in 1979 to the Veterinary University Hanover, Germany. Both viruses were taken from
166 the stock prepared for earlier studies published by Adamek et al., 2012.
167

168 **2.3.2. *In vitro* infection protocols**

169 The *in vitro* infections were performed as described earlier (Adamek et al., 2012). Briefly:
170 CCB cells were cultured in 24 well plates (Nunc, Germany) and grown to a monolayer. For
171 the CyHV-3 infection cells were incubated at 25°C, while cells for the SVCV infection were
172 kept at 20°C. CCB cells (6 replicates) were exposed to culture medium as a control or to

173 medium containing either 1 x 10^3 $TCID_{50}$ SVCV or 1.5 x 10^2 $TCID_{50}$ CyHV-3 for 1 hour or 2
174 hours respectively. These incubation temperatures, doses and infection times were chosen in
175 order to induce a lytic infection in the monolayer. The medium with the virus was then
176 removed and cells were cultured in fresh medium for 4 (SVCV) or 9 (CyHV-3) days.
177 Monolayers were observed daily for CPE, cells were removed by trypsinisation (0.1x
178 Trypsin-EDTA; Sigma Aldrich Germany) and concentrated by centrifugation (1000 x *g*, 5
179 min). Sampling took place at 1, 2, 3, 4, 5, 6 and 9 days post infection (p.i.). Three of the 6
180 replicates were then used for the analysis of apoptosis levels by acridine orange staining,
181 while the other 3 replicates were used for gene expression analysis. For the latter analysis the
182 pellet was reconstituted in lysis buffer (RNeasy Mini kit, Qiagen UK) and samples stored at -
183 80 °C prior to RNA extraction. For the visual determination of apoptosis levels CCB
184 suspensions were mixed 1:1 with a 10 µg/ml acridine orange solution (Sigma Aldrich, UK)
185 and analysed using a UV microscope (Zeiss Axiophot) with FITC filter. The proportion of
186 apoptosis was determined by noting the number of cells with nuclear fragmentation in a
187 population of 200 cells as described by Miest and coworkers (Miest, 2013; Miest et al., 2013;
188 Miest et al., 2012). Analysis of apoptosis-related gene expression supplemented this
189 morphological analysis.
190

191 **2.4 RNA extraction and cDNA synthesis**

192 RNA was extracted from CCB cells, the pronephros, spleen and gills using the RNeasy Mini
193 Kit (Qiagen, UK) following the manufacturer's instructions. RNA concentration was
194 determined by Nanodrop 1000 (Thermo Scientific, UK) and normalized to a common
195 concentration with DEPC treated water (Invitrogen, UK) before subjecting the samples to
196 DNase 1 treatment. 500 ng RNA were transcribed to cDNA using the M-MuLV reverse

transcriptase system with random hexamers according to the manufacturer's protocol (Invitrogen). All reactions were carried out in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems). cDNA samples were diluted 1:10 (*in vitro*) or 1:5 (*in vivo*) with DEPC treated water and stored at -20 ºC.

2.5 Analysis of gene expression with real time PCR (qPCR)

Primers (Table 1) specific for the apoptotic process in carp were used. These genes mainly target the intrinsic apoptosis pathway due to a lack of sequences available for genes involved in the extrinsic pathway. Ribosomal 40S protein was utilised as the reference gene (Huttenhuis et al., 2006), and was selected based on the highest stability among a variety of host-genes tested (40S, 18S, β-Actin; results not shown) according to the BestKeeper software (Pfaffl et al., 2004). This is in accordance with earlier results showing the highest stability of 40S protein expression (Adamek et al., 2012; Adamek et al., 2013; Rakus et al., 2012). For the real-time PCR 2 µl of cDNA, corresponding to 5 ng of RNA in *in vitro* samples or 10 ng in *in vivo* samples, were added to 1x Power SYBR® Green Master Mix (Applied Biosystems) and 900 nM forward and reverse primer. The volume was adjusted to 20 µl with DEPC treated water. qPCR was carried out in an ABI 7000 real-time cycler (Applied Biosystems) with 2 min at 50 ºC, 10 min at 95 ºC and 40 cycles of 15 sec at 95 ºC and 1 min at 60 ºC. After each run, dissociation curves of PCR products were obtained.

Analysis of gene expression was carried out according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Target genes were normalized against the reference gene 40S, and x-fold change calculated in relation to the control group of each time point.

220 **2.6 Confirmation and quantification of viral replication**

221 Viral replication of CyHV-3 was confirmed by analysis of viral innate major capsid protein
222 (MCP) gene expression (CyHV-3 ORF 92). For replication of SVCV the gene expression of
223 glycoprotein (GP) was targeted. The MCP and GP detection (for primers see table 1) was
224 performed using a recombinant plasmid based virus genome copy quantification assay,
225 established by Adamek et al. (2012), with slight modifications. Briefly, cDNA was
226 synthesized, the PCR reactions set up as described above, and the qPCR assays were
227 performed using a StepOnePlus thermal cycler (Applied Biosystems). A similar thermal
228 profile was used as that described for the apoptosis-related gene expression with cycles
229 consisting of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. In order to obtain copy numbers
230 of MCP and GP, a standard curve with known plasmid copy numbers of MCP or GP was run
231 in parallel. In brief, amplicons of 40S and MCP/GP were amplified using an Advantage 2
232 PCR kit (Clontech, USA) and ligated into p-GEM-T Easy vectors (Promega, USA).
233 Subsequently plasmids were inserted into JM109 competent *Escherichia coli* bacteria
234 (Promega, USA). After overnight incubation plasmids were isolated using the GeneJet^{TM}
235 Miniprep Kit (Fermentas, Germany). A standard curve ranging from 10^7 to 10^2 copy numbers
236 was prepared and used for quantification of MCP and GP. For this purpose MCP and GP
237 values were normalized against copy numbers of 40S and are presented as the gene copy
238 number normalised for 1 x 10^5 copies of the gene encoding for the 40S ribosomal protein S11
239 (normalised copy number) using the following equation:
240 Normalised copy number = mRNA copy number of the CyHV-3 CTP gene / (mRNA copy
241 number of 40S ribosomal protein S11 /1x10^5).
242
243 **2.7 Statistical analysis**

All data are presented as mean ± SEM. Statistical data analysis was carried out using GraphPad Prism 5 and SPSS 19 (IBM). Data were tested for normality and equal distribution of variances. When necessary gene expression data were normalized using log_{10}-transformation while percentage data (apoptosis level) were arc-sin transformed prior to analysis. A two-way ANOVA was performed to test for significant differences between time points and treatments with subsequent Bonferroni post-hoc analysis. Significance was defined as $p \leq 0.05$.

3. Results

3.1 Gene expression study during CyHV-3 infection of C. carpio

The expression of viral major capsid protein in the gills (Fig. 1), measured as normalised copy number of MCP was significantly affected by the infection ($F = 11.23$, $df = 1$, $p \leq 0.05$) and was significantly heightened on day 5 p.i. (86 ± 68 copies, $p \leq 0.001$). In comparison to other organs studied, apoptosis-related mRNA levels in the gills were least affected by infection with CyHV-3. In this organ a significant increase, in comparison to control fish, occurred in the expression of the genes encoding the pro-apoptotic protein Apaf-1 and iNOS only at 14 days p.i. (Apaf-1: 2.7 ± 0.7-fold expression, $p \leq 0.05$; iNOS: 10.7 ± 7.1-fold expression, $p \leq 0.01$).

In contrast, in the spleen (Fig. 2), where virus replication was also detected on day 5 p.i. (349 ± 123 copies, $p \leq 0.001$ with $F = 41.25$, $df = 1$, $p \leq 0.0001$), expression of studied genes was strongly influenced by the presence of CyHV-3. iNOS mRNA levels were enhanced by approximately 10-fold at day 3 and day 5 p.i. ($p \leq 0.01$ and 0.001 respectively) as compared to the non-infected control. At day 3 p.i. the expression of the gene encoding the anti-

apoptotic protein IAP decreased by approximately 55 % ($p \leq 0.001$) and expression of the gene encoding the pro-apoptotic protein Apaf-1 was enhanced on day 14 p.i. (1.7 ± 0.2 –fold, $p \leq 0.05$).

mRNA levels of major capsid protein of CyHV-3 were also significantly increased in the pronephros (Fig. 3) due to the CyHV-3 infection ($F = 10.48$, $df = 1$, $p \leq 0.05$) on day 5 p.i. (68 ± 32 copies, $p \leq 0.001$). In comparison to the gills and spleen, the greatest effect of infection on the expression of those genes studied was recorded in pronephros. The genes encoding the three pro-apoptotic proteins p53, Caspase 9 and Apaf-1 were up-regulated, primarily at 14 days p.i., approximately 1.6-fold (p53 = 1.6 ± 0.2, $p \leq 0.05$; Caspase 9 = 2.2 ± 0.2-fold, $p \leq 0.001$; Apaf-1 = 7.4 ± 1.0-fold, $p \leq 0.05$) compared to the non-infected control fish. The progress of the infection also affected the gene encoding the anti-apoptotic protein IAP. During the early stage of infection, i.e. 3 d p.i., the expression of this gene was approximately half of the expression in the control samples (0.5 ± 0.1 –fold, $p \leq 0.05$), whilst it was enhanced 1.9 ± 0.2-fold ($p \leq 0.001$) during the late stage, i.e. 14 d p.i., of the infection. When compared to the control group iNOS expression in pronephros was enhanced on day 5 p.i. (11.2 ± 4.1, $p \leq 0.01$) and 14 p.i. (10.5 ± 2.1, $p \leq 0.05$).

3.2 Gene expression study during in vitro CyHV-3 infection of CCB cells

Expression analysis of the viral major capsid protein (MCP) gene in CCB cells (Fig. 4) revealed differences in virus replication between the treatments ($F = 3856$, $df = 1$, $p < 0.0001$). The non-infected samples were negative for the presence of the virus, whilst the amount of MCP copy numbers increased steadily over the time-course of the infection and reached $9 \times 10^7 \pm 2 \times 10^7$ copies on day 9 p.i.. CCB cells that were infected with CyHV-3

290 also showed cytopathic effects (CPE) from 5 d p.i. onwards and on day 9 p.i. the monolayer
291 was completely destroyed. Interestingly, this destruction of the monolayer was not associated
292 with an increase of cells with apoptosis related morphology, i.e. fragmented nuclei, as
293 detected with acridine orange. Instead fluorescence microscopic analysis revealed the
294 presence of giant cells (GC) and multinucleated (syncytial) giant cells (MGC) (Fig. 4) which
295 most likely occurred when infected cells fused forming syncytia.

296 The analysis of apoptosis-related gene expression (Fig. 5) corroborates the above
297 observations that apoptosis is not induced since only one of the pro-apoptotic genes analysed,
298 *p53*, was significantly up-regulated ($p \leq 0.05$), and then only at 6 days p.i. (5.2 ± 1.2 –fold
299 expression compared to the control). Interestingly, the gene of the anti-apoptotic protein IAP
300 and the gene encoding the antiviral protein type I IFN were also not affected by the infection
301 and iNOS was significantly down-regulated on day 5 and 6 p.i., when the gene expression
302 was only 10 % of the control non-infected cells ($p \leq 0.05$) (Fig.5).

303

304 ***3.3. Gene expression study during in vitro SVCV infection of CCB cells***

305 Infection of cells was confirmed by immunocytochemistry and by significantly increased
306 copy numbers of the glycoprotein ($6.4 \times 10^6 – 2.9 \times 10^7$ normalised copies, $p \leq 0.001$)
307 encoding gene throughout the duration of the experiment ($F = 5288$, $df = 1$, $p \leq 0.0001$) (Fig.
308 4). This viral infection induced heightened mRNA levels of antiviral IFN Type I on all four
309 days of the infection peaking on 4 d p.i. (254 ± 75 –fold, $p \leq 0.001$) (Fig. 6) and significantly
310 elevated apoptosis levels on day 3 (8.7 ± 2.5 %) and 4 p.i. (13.2 ± 1.7 %) as detected with
311 acridine orange (Fig. 4).

312 The infection with SVCV (Fig. 6) also increased levels of iNOS gene expression, which
313 peaked on day 1 p.i. (7.3 ± 1.2 -fold) and declined on subsequent days reaching a minimum
314 on day 4 (2.2 ± 0.6 -fold). Gene expression levels of the pro-apoptotic Caspase 9 was
315 enhanced on day 2 p.i. (5.2 ± 2.4-fold, $p \leq 0.05$) and p53 were increased on day 3 (3.3 ± 0.1-
316 fold, $p \leq 0.001$) and day 4 p.i.(2.4 ± 0.6-fold, $p \leq 0.05$). The other pro-apoptotic gene
317 analysed, i.e. *apaf-1*, was not significantly affected by the infection. In addition, the
318 expression profile of the gene encoding the anti-apoptotic protein, IAP, was significantly
319 increased by the infection, i.e. day 3 and 4 post-SVCV infection, a 2.5 – 3-fold increase in
320 IAP mRNA levels ($p \leq 0.001$ and 0.01 respectively) was observed.

321

322 **4. Discussion**

323 The present study is part of a series of publications exploring various aspects of innate
324 immune responses during a CyHV-3 infection in common carp (Adamek et al., 2013;
325 Adamek et al., 2014a; Pionnier et al., 2014; Rakus et al., 2012; Syakuri et al., 2013) which
326 allows an unique, holoistic analysis of the systemic immune response against this virus.

327 The CyHV-3 infection was confirmed by monitoring viral replication using thymidine kinase
328 and MCP expression in skin and pronephros (Adamek et al., 2013; Adamek et al. 2014a;
329 Rakus et al., 2012) and MCP expression in various organs used in this study. It was noted that
330 the MCP levels differed between the two studies, which probably reflects the differential
331 influence of the infection on the organs studied.

332 The CyHV-3 infection triggered an immune response in *C. carpio*, which resulted in up-
333 regulation of expression of a wide array of immune-related genes including those encoding
334 for cytokines and T-cell markers (Rakus et al., 2012), as well as CRP and complement-

335 related genes (Pionnier et al., 2014) in pronephros and spleen. In addition, up-regulation of
336 iNOS expression levels was observed in the skin (Adamek et al., 2013), intestine (Syakuri et
337 al., 2013) as well as in the pronephros, spleen and gills noted in our study. Based on this
338 holistic immune response, it can be concluded that the CyHV-3 infection induced the
339 activation of a systemic innate immune response in a similar manner as the response induced
340 by other herpesviruses in mammals (Brockman and Knipe, 2008; Da Costa et al., 1999).

341 Besides the humoral arm of the innate immune response apoptosis is also an important factor
342 during the immune response and in the pathogenicity of viruses (Leu et al., 2013), including
343 herpesviruses (e.g. Aubert and Blaho, 2001; Henderson et al., 1991). Our analysis of the
344 expression of genes encoding pro-apoptotic proteins of the intrinsic pathway (Apaf-1, p53,
345 and Caspase 9) during *in vivo* CyHV-3 infection showed that apoptosis may have occurred,
346 predominantly in the pronephros during the later stages of infection, i.e. day 14 p.i.. In
347 contrast, in the other organs examined only expression of Apaf-1 was slightly increased,
348 which may indicate that apoptosis did not occur extensively in the gills and spleen of infected
349 fish. The up-regulation of apoptosis-related genes at 14 d p.i. in the pronephros, and possibly
350 apoptosis itself, could be caused by various factors. A study by (Perelberg et al., 2008)
351 demonstrated that specific CyHV-3 antibodies are produced between day 7 and 14 of the
352 infection. This antibody production indicates the induction of the specific immune response,
353 which could lead to apoptosis in viral infected cells mediated by cytotoxic T-cells (Murphy et
354 al., 2008). This process of T-cell induced apoptosis has been extensively documented in
355 mammals, but also appears to occur in fish (Uribe et al., 2011). The finding by Rakus et al.
356 (2012) that the gene expression of various T-cell markers in the spleen of the same fish
357 utilised in our investigation is only up-regulated during the latter stages of the infection (i.e.
358 14 d p.i.) corroborates the assumption that the observed apoptosis-related gene expression

might be connected to the specific immune response. Lack of pro-apoptotic gene expression in gills, spleen and pronephros during the first five days post infection may suggest that replication of CyHV-3 suppresses the anti-viral apoptotic response in carp in the first stages of the infection. However without knowledge of apoptosis levels in the host cells it is not possible to exclude the possibility that apoptosis occurred via the extrinsic pathway.

In order to investigate the impact of CyHV-3 infection on apoptosis in more detail we used the *in vitro* system in which we compared CCB cell responses to CyHV-3 and SVCV infections. Morphological analysis of CCB cells infected with CyHV-3 confirmed that apoptosis did not occur during the time-course of the viral infection, which is in accordance with the observed absence of up-regulation of apoptosis-related gene expression. The lack of apoptosis during CyHV-3 infection is in stark contrast to the pro-apoptotic antiviral default program of the cell as seen in the pro-apoptotic effects observed during infections with SVCV. The latter is also in line with previous reports which indicate that members of the *Rhabdoviridae* family (e.g. SVCV, *Viral haemorrhagic septicaemia virus* (VHSV) and *Infectious pancreatic necrosis virus* (IPNV)) induce apoptosis *in vivo* and *in vitro* (e.g. Björklund et al., 1997; Eléouët et al., 2001; Kazachka et al., 2007).

Nitric oxide is an anti-viral agent (e.g. Saura et al., 1999) and has been linked to host apoptosis and limitation of RNA-virus replication (Lin et al., 1997; Ubol et al., 2001). SVCV infection led to increased levels of the inducible NO synthase (iNOS), whilst during CyHV-3 infection iNOS gene expression was down-regulated. The lack of iNOS gene expression during CyHV-3 infection could be an indicator of immune evasion mechanisms by the virus, and that these probably influence the antiviral host response on various levels.

The increased levels of apoptosis during the SVCV infection were also reflected in elevated mRNA levels of p53 and a trend to elevated levels of Caspase 9. It cannot be excluded that the extrinsic apoptosis pathway was also involved in the induction of apoptosis during SVCV

384 infection. Nevertheless, in order to establish the detailed mechanisms of SVCV induced
385 apoptosis further knowledge on the signaling pathways of apoptosis in carp and its related
386 gene sequences is needed. It is apparent however that the pro-apoptotic effects seem to
387 compensate the inhibitory effects of anti-apoptotic IAP as apoptosis levels increased
388 throughout the experiment. The execution of apoptosis is also aided by the down-regulation
389 of cytoprotective heme oxygenase-1, which was observed in EPC cell cultures and *in vivo* in
390 carp (Yuan et al., 2012). This gene is involved in the protection of cells against oxidative
391 damage, and thus its down-regulation leaves the cell more vulnerable to damage caused by
392 nitric oxide (Yuan et al., 2012).

393 Viruses can adopt a range of strategies to escape host apoptosis, either by inhibiting the cell
394 death pathway or by avoidance through completing replication before the onset of apoptosis
395 (Koyama et al., 2000). Whilst SVCV seems to pursue the latter strategy since high virus
396 replication was detected 24 h p.i., CyHV-3 seems to inhibit apoptosis. These differential
397 effects of CyHV-3 and SVCV on the fish cell line used may be due to the properties of the
398 individual virus. RNA viruses such as SVCV have a small genome with less complexity as
399 the much larger DNA viruses such as CyHV-3. The SVCV genome consists of only five
400 genes and thus may lack genes that can actively interfere with the host response (Ahne et al.,
401 2002). Reports on apoptosis induced by *Vesicular stomatitis virus* (VSV), another
402 rhabdovirus, suggest that apoptosis is induced via two independent pathways. One pathway is
403 via host-induced apoptosis during the immune response, whilst the second pathway is
404 associated with the expression of viral M-protein (e.g. Gaddy and Lyles, 2005). The more
405 complex CyHV-3 genome (295 kbp) encodes a much larger number of proteins (155
406 predicted ORFs), and throughout its evolutionary association with its hosts has probably
407 acquired a number of host genes which it uses to manipulate the immune response.

Additionally CyHV-3 also appeared to induce the formation of giant cells, some of which were multinucleated after fusion of few infected cells into syncytia. This observation not only confirms the findings of Neukirch et al. (1999), but supports the general observations that giant cells or syncytia are a common phenomenon during infections with members of the *Herpesviridae* and *Alloherpesviridae* (e.g. Guo et al., 2009; Hanson et al., 2011; Secchiero et al., 1998). Although it is not known what causes these cellular changes, anti-apoptotic effects induced by a herpesvirus could affect the host cell cycle and hence, induce unregulated cell growth as demonstrated by (Secchiero et al., 1998) for *Human herpesvirus 7*. The formation of giant cells in a viral infection may provide the virion with a haven to replicate unrecognized by the immune system. This hypothesis is supported by the finding that many herpesviruses, such as the *Epstein-Barr virus* and *Human herpesvirus 8*, are associated with papilloma and tumour formation (e.g. Carrillo-Infante et al., 2007). The tumour suppressor protein p53 functions as a cell cycle regulator that can induce apoptosis upon DNA damage or unscheduled DNA synthesis as occurs during viral replication. In our studies however, even though giant cells were observed and viral replication occurred, only slight up-regulation of p53 gene expression was noted in infected cell cultures. It is possible that p53 activation is blocked by anti-apoptotic mechanisms induced by the virus, which might be mediated by a range of factors (Roulston et al., 1999). For example, herpesviruses with anti-apoptotic features often express host-derived genes which inhibit apoptosis (Hardwick, 1998). Although a preliminary screen of the CyHV-3 genome did not detect any viral anti-apoptotic genes such as Bcl-2 and IAP (Miest, 2013), the presence of Bcl-2 has been noted in the genome of AngHV-1 (van Beurden et al., 2010). In addition, the common clinical signs during *Alloherpesviridae* infections related to dysfunctional cell division (syncytia, papilloma, carcinoma) (Hanson et al., 2011), hints at common anti-apoptotic characteristics among *Alloherpesviridae*, which might have been acquired individually throughout viral

433 evolution. Even though a classical member of the apoptotic pathway was not identified within
434 the CyHV-3 genome, it is known that this virus expresses vIL-10 (Ouyang et al., 2013;
435 Sunarto et al., 2012; van Beurden et al., 2011). IL-10 has immunosuppressive functions,
436 including the inhibition of IFN-γ, TNFα and, by impairing the activation of macrophages,
437 also inhibits the production of ROS and NO (Redpath et al., 2001). IL-10 could therefore
438 limit the induction of apoptosis via the extrinsic TNFα-dependent pathway additionally to the
439 intrinsic pathway due to oxidative stress.

440 It is however interesting to note that various other pathways of apoptosis-inhibition have been
441 suggested for the *Herpesviridae* family, including inhibition of interferon (IFN) response as
442 well as inhibition of the activation of TNFα and Fas induced pathways (Lagunoff and Carroll,
443 2003; Roulston et al., 1999). The protective anti-viral mechanisms of IFN include the
444 initiation of apoptosis in infected cells (Tanaka et al., 1998). Interestingly, in our *in vitro*
445 studies CyHV-3 infection did not induce up-regulation of type I IFN, which corroborates the
446 recent findings by Adamek et al. (2012). In contrast, SVCV induced a type I IFN response
447 during *in vitro* infection of CCB cells. Hence, it is possible that the lack of type I IFN
448 induction was involved in the absence of apoptosis observed during the CyHV-3 infection.

449 In conclusion, CyHV-3 possesses mechanisms to counteract the antiviral pro-apoptotic
450 response in fish cells, which may explain some of the pathology associated with the disease.
451 Additionally, this is the first time that a member of the *Alloherpesviridae* family has been
452 shown to possess anti-apoptotic properties similar to the *Herpesviridae* indicating that viral-
453 host apoptotic interactions may have been evolutionary conserved.

454

455

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639

640 **Figure & table captations**

641 **Table 1:** Primers utilized for gene expression analysis by real-time PCR

Gene name and Genbank ID/Reference		Primer type	Sequence (5' → 3')	Gene function
40S	AB012087/ (Huttenhuis et al., 2006)	Forward	CCGTGGGTGACATCGTTACA	Housekeeping gene
		Reverse	TCAGGACATTGAACCTCACTGTCT	
CyHV-3 Major capsid protein	DQ177346/ (Adamek et al., 2012)	Forward	AGCCACCTCTTGGTCGTG	Viral replication
		Reverse	ACTCCCTGTCCCAGCACTC	
SVCV Glyco-protein G	Z37505.1 (García-Valtanen et al., 2014)	Forward	GCTACATCGCATTCCTTTTGC	Viral replication
		Reverse	GCTGAATTACAGGTTGCCATGAT	
p53	(Cols Vidal, 2006)	Forward	CCAAACGCAGCATGACTAAAGA	Pro-apoptotic Intrinsic pathway
		Reverse	CGTGCTCAGTTTGGCCTTCT	
Caspase 9	EC394517.1	Forward	CGAGAGGGAGTCAGGCTTTC	Pro-apoptotic Intrinsic pathway
		Reverse	TCAGAAGGGATTGGCAGAGG	
Apaf-1	EU490407	Forward	CGCTCACAGGTCACACTAGAACTG	Pro-apoptotic Intrinsic pathway
		Reverse	AGATACTCACCGGTCCTCCACTT	
IAP	(Cols Vidal, 2006)	Forward	CGTGGAGTGGAGGATATGTCTCA	Anti-apoptotic Intrinsic pathway
		Reverse	TCCTGTTCCCGACGCATACT	
iNOS	AJ242906	Forward	TGGTCTCGGGTCTCGAATGT	NO production Intrinsic pathway
		Reverse	CAGCGCTGCAAACCTATCATC	
IFN Type I	AB376667/ (Adamek et al., 2012)	Forward	GATGAAGGTGCCATTTCCAAG	Anti-viral response
		Reverse	CACTGTCGTTAGGTTCCATTGCTC	

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647 **Table 1:** Primers utilized for gene expression analysis by real-time PCR

648 **Figure 1**: Gene expression in the gills of *C. carpio* after infection with CyHV-3

649 Carp were infected with 3.2 x 10^2 $TCID_{50}$/ml CyHV-3 by bathing and samples were taken 1,
650 3, 5, and 14 d p.i.. MCP (major capsid protein of CyHV-3) is represented as normalised copy
651 number and only the infected group is shown (grey box). Controls were all negative for viral
652 replication. Genes encoding carp proteins (iNOS, Caspase 9, Apaf-1, p53, IAP) are depicted
653 as x-fold gene expression relative to the control. White bars represent the non-infected
654 control and black bars the viral infected fish. The graphs present Mean ± SEM, n = 5.
655 Asterisks depict significant differences between infection treatment and the associated control
656 with *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

657

658 **Figure 2:** Gene expression in the spleen of *C. carpio* after infection with CyHV-3

659 Carp were infected with 3.2 x 10^2 $TCID_{50}$/ml CyHV-3 by bathing and samples were taken 1,
660 3, 5, and 14 d p.i.. MCP (major capsid protein of CyHV-3) is represented as normalised copy
661 number and only the infected group is shown (grey box). Controls were all negative for viral
662 replication. Genes encoding carp proteins (iNOS, Caspase 9, Apaf-1, p53, IAP) are depicted
663 as x-fold gene expression relative to the control. White bars represent the non-infected
664 control and black bars the viral infected fish. The graphs present Mean ± SEM, n = 5.
665 Asterisks depict significant differences between infection treatment and the associated control
666 with *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

667

668 **Figure 3:** Gene expression in the pronephros in *C. carpio* after infection with CyHV-3

669 Carp were infected with 3.2 x 10^2 $TCID_{50}$/ml CyHV-3 by bathing and samples were taken 1,
670 3, 5, and 14 d p.i.. MCP (major capsid protein of CyHV-3) is represented as normalised copy
671 number and only the infected group is shown (grey box). Controls were all negative for viral
672 replication. Genes encoding carp proteins (iNOS, Caspase 9, Apaf-1, p53, IAP) are depicted
673 as x-fold gene expression relative to the control. White bars represent the non-infected
674 control and black bars the viral infected fish. The graphs present Mean ± SEM, n = 5.
675 Asterisks depict significant differences between infection treatment and the associated control
676 with *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

677

678 **Figure 4:** Apoptosis and viral replication in CyHV-3 and SVCV infected CCB cells

679 Left: Virus replication in relation to apoptosis (A). Levels of apoptosis were measured by
680 observing acridine orange stained cells and depicted as bars. Viral replication was analysed as
681 normalised copy numbers of MCP (CyHV-3) and glycoprotein G (SVCV) and is displayed as
682 ●. The graph displays Mean ± SEM of n = 3, control groups are not shown in graph.
683 Asterisks depict significant differences of apoptosis levels and + indicates significant
684 differences of viral copy numbers between infection treatment and the associated control

between with */+: $p \leq 0.05$, **/++: $p \leq 0.01$, ***/+++: $p \leq 0.001$. Right: Morphological analysis of cells infected with CyHV-3. CCB cells were infected with 1.5 x 10^2 $TCID_{50}$/ml CyHV-3 and stained with acridine orange. Giant cells (B) and giant multinucleated cells (C) were observed and set in relation to normal sized cells (white arrows).

Figure 5: Gene expression in CCB cells after infection with CyHV-3

CCB cells were infected with a dose of 1.5 x 10^2 $TCID_{50}$/ml CyHV-3 and samples were taken at 1, 2, 3, 4, 5, 6 and 9 d p.i.. Data are depicted as x-fold gene expression relative to the control. White bars represent the non-infected control and black bars the viral infected cell cultures. The graphs present Mean ± SEM, n = 3. Asterisks depict significant differences between infection treatment and the associated control with *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

Figure 6: Gene expression in CCB cells after infection with SVCV

CCB cells were infected with a dose of 1 x 10^3 $TCID_{50}$/ml SVCV and samples were taken at 1, 2, 3, and 4 d p.i.. Carp genes are depicted as x-fold gene expression relative to the control. White bars represent the non-infected control and striped bars the viral infected cell cultures. The graphs present Mean ± SEM, n = 3. Asterisks depict significant differences between infection treatment and the associated control with *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

704 **Highlights**

705 - CyHV-3 inhibits the apoptotic process *in vivo* and *in vitro*

706 - SVCV induces apoptosis-related gene expression *in vitro*

707 - Similar cellular apoptosis-related anti-host strategies exist among *Herpesviridae* and

708 *Alloherpesviridae*

709

710

Figure 1

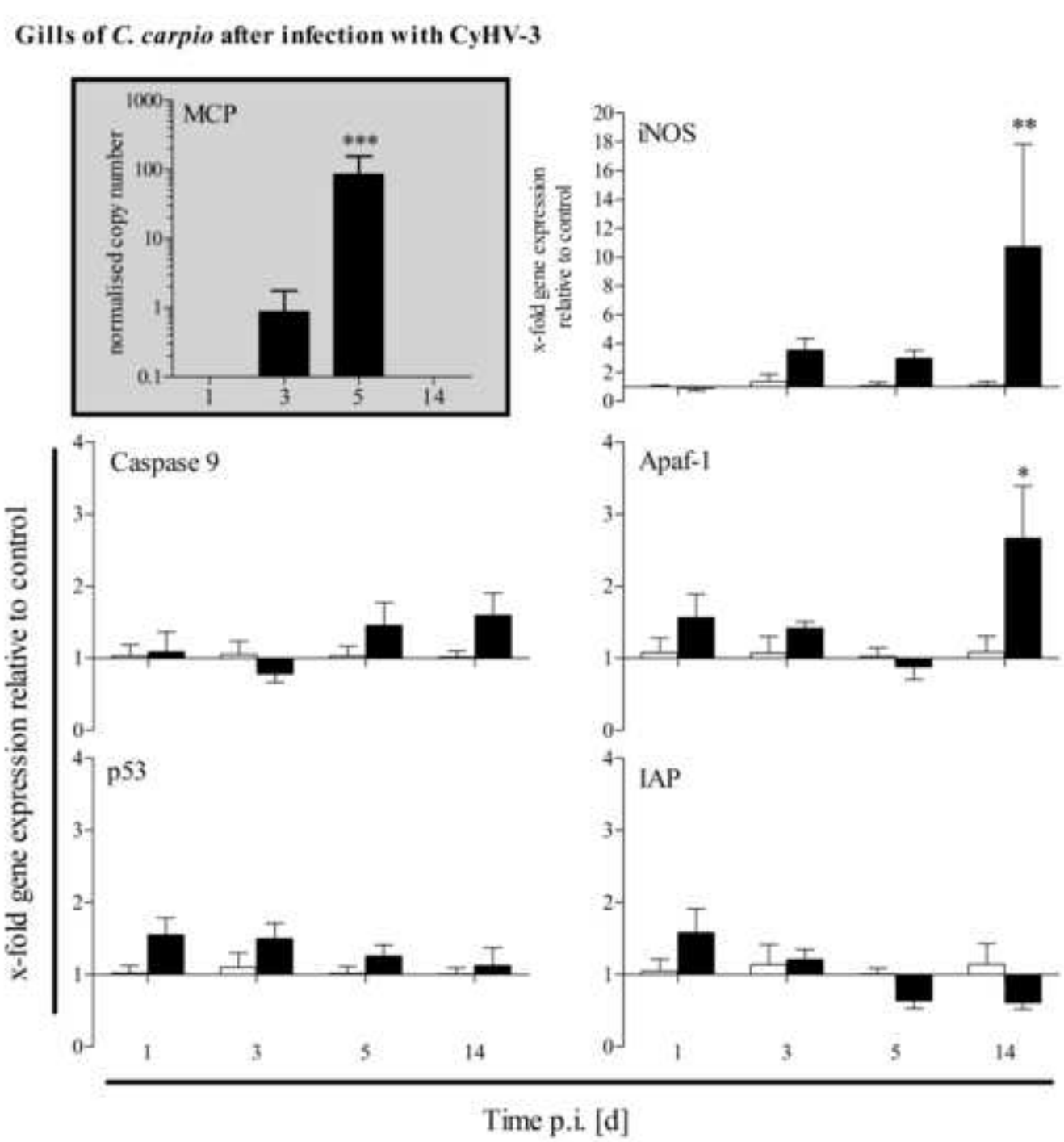
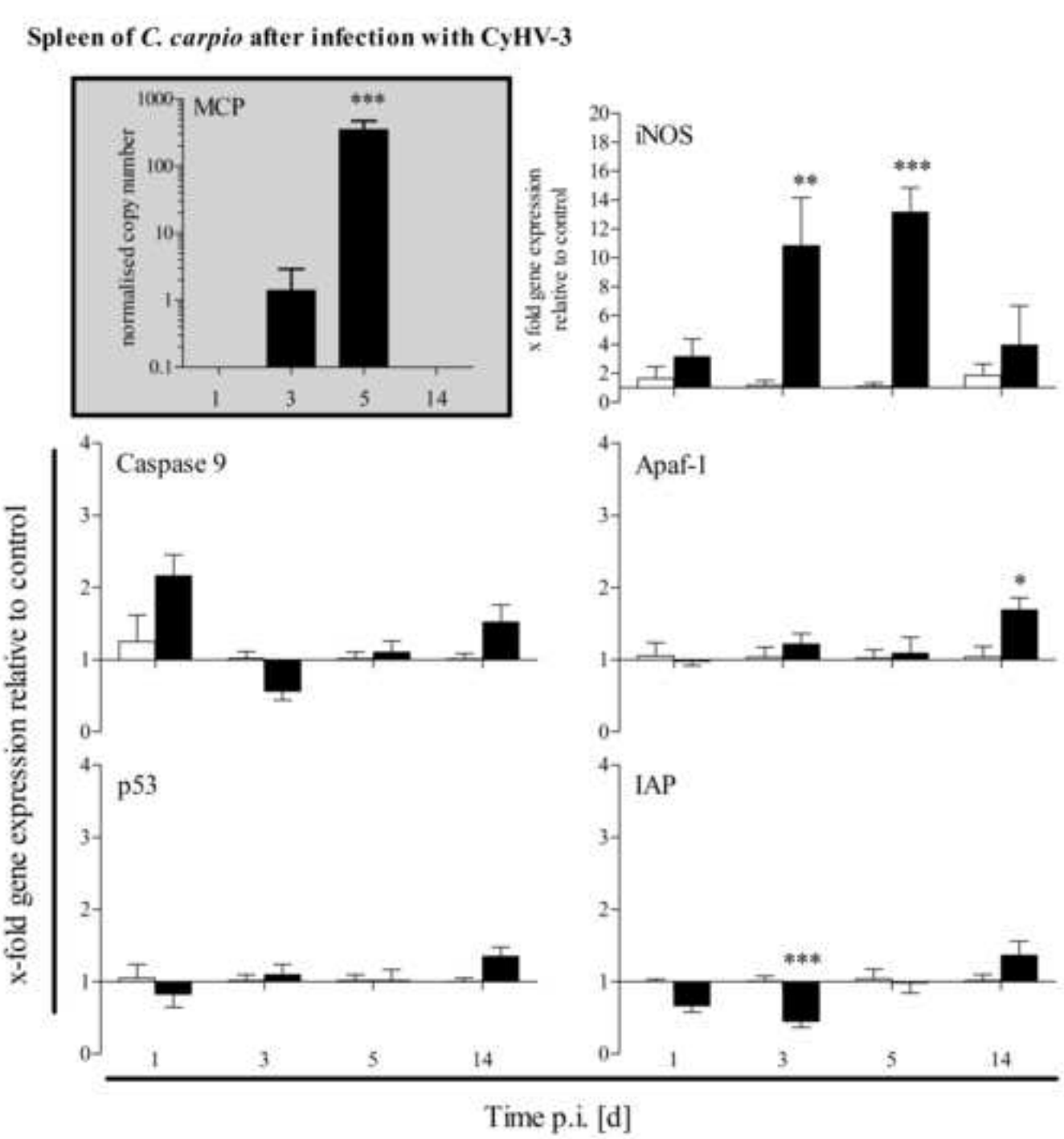

Gills of C. carpio after infection with CyHV-3
MCP
normalised copy number
1000
100
10
1
0.1
***
1
3
5
14
iNOS
x-fold gene expression relative to control
**
Caspase 9
Apaf-1
*
p53
IAP
x-fold gene expression relative to control
Time p.i. [d]

Figure 2


Spleen of C. carpio after infection with CyHV-3
MCP
normalised copy number
1000
100
10
1
0.1
***
1
3
5
14
iNOS
x fold gene expression relative to control
20
18
16
14
12
10
8
6
4
2
0
**
***
Caspase 9
Apaf-1
p53
IAP
*
***
x-fold gene expression relative to control
Time p.i. [d]