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Cholesterol-rich lipid rafts play an important role in the *Cyprinid herpesvirus 3* replication cycle[☆]



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ABSTRACT

The *Cyprinus herpesvirus 3* (CyHV-3) is a member of the new *Alloherpesviridae* virus family in the *Herpesvirales* order. CyHV-3 has been implicated in a large number of disease outbreaks in carp populations causing up to 100% mortality. The aim of this study was to investigate the requirement of cholesterol-rich lipid rafts in CyHV-3 entry and replication in carp cells. Plasma membrane cholesterol was depleted from common carp brain (CCB) cells with methyl- β -cyclodextrin (M β CD). Treated and non-treated cells were infected with CyHV-3 and virus binding and infection parameters were assessed using RT-qPCR, immunocytochemistry and virus titration. The effect of cholesterol reduction severely stunted virus entry *in vitro*, however after cholesterol replenishment virus entry and subsequent replication rates were similar to the control infection. Furthermore, cholesterol depletion did not significantly influence virus binding and the subsequent post-entry replication stage, however had an impact on virus egress. Comparative analysis of the lipid compositions of CyHV-3 and CCB membrane fractions revealed strong similarities between the lipid composition of the CyHV-3 and CCB lipid rafts. The results presented here show that cholesterol-rich lipid rafts are important for the CyHV-3 replication cycle especially during entry and egress.

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1. Introduction

The *Alloherpesviridae* is a new virus family in the *Herpesvirales* order. It is comprised of both piscine and amphibian herpesviruses (McGeoch et al., 2006) and is evolutionary distinct from the other families of the order *Herpesvirales* (Davison et al., 2009; Hanson et al., 2011; Waltzek et al., 2009). Members of the *Alloherpesviridae* family are increasingly recognised as pathogens in aquaculture. One important pathogen is the *Cyprinid herpesvirus 3* (CyHV-3), a herpesvirus from the *Cyprinivirus* genus which infects common carp, *Cyprinus carpio* and its coloured variety, the koi (Waltzek et al., 2009). CyHV-3 infections may cause severe outbreaks of the so called koi herpesvirus disease (KHVD) leading to up to 100% mortalities in infected populations, consequently causing a severe negative impact on carp aquaculture and the ornamental koi trade.

Characteristic features of the virus include a large 295 kbp long linear genome with 156 potential ORFs (Aoki et al., 2007) encoding for at least 40 proteins building the mature viron (Michel et al., 2010). The CyHV-3 virion has an icosahedral capsid, an amorphic protein tegument and a lipid envelope containing virus glycoproteins (Dishon et al., 2005; Hutoran et al., 2005) which it acquires during the budding step from infected cells. This process is similar to the mechanism observed for mammalian herpesviruses (Mettenleiter, 2002). In electron microscopical studies on infected cells, CyHV-3 nucleocapsids appear to bud from the inner nuclear membrane into the perinuclear space. When the nucleocapsids cross the outer nuclear membrane into the cytoplasm, its primary envelope is lost and a second envelope is acquired through budding into cytoplasmic vesicles (Hanson et al., 2011; Miwa et al., 2007).

In enveloped viruses, the viral membrane is required for the critical steps of entry into the target cell and fusion with the host's cellular membrane in order to deliver the viral capsid into the cell cytosol. The classical endocytotic pathway, which many viruses use as the primary step of internalisation, is clathrin-dependent endocytosis (Marsh and Helenius, 2006). Instead, many viruses use the lipid raft/caveola-dependent entry route, which is

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characterised by the importance of high levels of cholesterol and sphingolipids ((Doherty and McMahon, 2009) and references therein). In the plasma membrane of cells (chole) sterol-sphingolipids are particularly enriched in dynamic nanoscale liquid ordered microdomains, ordered assemblies of proteins and lipids, which are also termed as lipid rafts (Simons and Toomre, 2000). These microdomains are associated with a range of important cellular and physiological functions including cell signalling, membrane and protein trafficking and sorting and nutrient transport. Lipid rafts have been extensively studied in mammals. In fish, lipid rafts have been isolated from rainbow trout (Zehmer and Hazel, 2003), Atlantic cod (Gylfason and Asgeirsson, 2008), and common carp (Brogden et al., 2014), furthermore the presence of functional lipid rafts in goldfish macrophages was shown (Garcia-Garcia et al., 2012). The lipid raft model is used to investigate a large range of processes including, protein trafficking (Ikonen, 2001), metabolic diseases (Maalouf et al., 2010) and cell signalling (Magee et al., 2002; Varma and Mayor, 1998). In addition, lipid rafts and cell membrane cholesterol were found to be involved in various stages of the viral life cycle, such as entry (Nguyen and Hildreth, 2000; Ono and Freed, 2001), assembly and budding (Chazal and Gerlier, 2003). Many studies demonstrated that several enveloped viruses enter host cells in a cholesterol-dependent manner, including coronavirus (Choi et al., 2005), poxvirus (Chung et al., 2005), paramyxovirus (Martin et al., 2012) and herpesvirus (Bender et al., 2003).

At the time of writing, no knowledge is available regarding the host cell entry and egress of fish specific herpesviruses from the *Alloherpesviridae* family, which are evolutionary distinct from mammalian herpesviruses (Davison et al., 2009) and can cause great economic losses in the aquaculture industry and ornamental fish trade. Currently, CyHV-3 infection is considered the most serious disease affecting carp aquaculture worldwide (Rakus et al., 2013). Knowledge of how this virus penetrates the host's cell membrane helps understanding the infection process and allows us to improve antiviral strategies against this serious pathogen of common carp.

In the present communication we therefore examine the role of cholesterol in CyHV-3 entry and egress based on the common carp derived fibroblastic CCB cell line. We demonstrate that depletion of cellular cholesterol by methyl- β -cyclodextrin (M β CD) reduces CyHV-3 cell entry and budding. Furthermore, the CyHV-3 lipid profile showed a greater similarity to the cholesterol rich, lipid ordered membrane microdomains suggesting a role for lipid rafts in virus trafficking in the cell. To our knowledge, this is the first communication which addresses the role of lipid membrane components during the infection of fish cells with an aquatic herpesvirus.

2. Materials and methods

2.1. Cell and virus cultivation

Common carp brain (CCB) cells (Neukirch et al., 1999) were cultured in minimum essential medium (MEM) with Earle's salts as described earlier by Adamek et al., (2012).

CyHV-3 strain KHV I (Hedrick et al., 2000) was re-isolated from the skin of intraperitoneally infected carp using CCB cells according to a standard protocol (see Adamek et al., (2012)), propagated in CCB cells for 2 passages and stored at -80°C until further use.

2.2. Establishment of a cholesterol reduction assay using methyl-beta-cyclodextrin

In order to determine the effects of M β CD (Sigma, Germany) CCB cells were plated in 24-well plates, treated with M β CD

concentrations at 5 mM, 7.5 mM, 10 mM and 15 mM for 1–6 h. Cell morphology was assessed by microscopic observation and cell viability by using crystal violet. A significant number of cells died in wells treated with 15 mM M β CD, however the cell morphology remained unchanged and the cell monolayer remained intact for 10 mM and lower (data not included), therefore 10 mM M β CD with a 1 h incubation time was used for future studies. In subsequent studies CCB cells were seeded in 24 well plates and cultured until confluent. The cells were then treated in triplicates for 1 h with 10 mM M β CD (dissolved in RPMI 1640 medium, Sigma), 2.5 mM M β CD plus cholesterol (cholesterol dissolved in ethanol at 80 mM) or RPMI culture medium. The cells were then washed twice with PBS to remove all traces of M β CD and excess cholesterol and RPMI medium was added. After the cells had been incubated for 1, 2 or 48 h in RPMI, the cells were lysed with 1% Triton X-100 and stored at -20°C for future processing.

2.3. Influence of cholesterol reduction on virus entry, replication and egress

To assess the influence of cholesterol reduction on CyHV-3 infection, CCB cells were treated with M β CD as described above. After removal of the medium containing M β CD and washing with PBS, the cells were infected with CyHV-3 and incubated for a further 1 h. Then the virus containing medium was removed and the cells were washed twice before fresh untreated medium was added.

For an analysis of the effect of cholesterol depletion on virus replication and virus protein expression, M β CD treated cells were infected with CyHV-3 at a multiplicity of infection (MOI) of 0.002, which gave optimal results for investigations into viral spreading in CCB cells (Adamek et al., 2012). Infected cells were incubated at 25°C and at 48 h and 120 h post infection cells were harvested from 6 wells of each treatment type by replacing the medium with TriFast reagent (PiqLab) and frozen at -80°C for later analysis of viral gene expression. As controls, cell cultures not treated with M β CD and cultures treated with 10 mM M β CD plus 2.5 mM cholesterol were infected and harvested as described above. For an analysis of virus protein expression, M β CD treated cells, M β CD plus cholesterol treated cells as well as untreated cells were infected with CyHV-3 as described above, incubated at 25°C for 4 and 7 days, removed from the tissue culture vessels, placed on glass slides, air dried and then fixed with ice-cold acetone for 10 min. In addition, cell cultures were treated with M β CD or M β CD plus cholesterol, incubated for 3 days at 25°C , then infected with CyHV-3 and cultivated for a further 7 days. The cells were transferred to glass slides and then fixed as described above.

To assess the influence of cholesterol on virus entry and post entry replication, CCB cells pre-treated with M β CD and untreated cells (as infection control and post infection treatment with M β CD) were incubated with CyHV-3 at an MOI of 1 at 25°C for 1 h and then washed with PBS. Cells treated with M β CD prior to infection and cells for the infection control were then incubated with medium at 25°C for 1 h. Additional cell cultures were incubated with 10 mM M β CD post infection with CyHV-3 for 1 h at 25°C . Cells were incubated for 48 h and frozen at -20°C . Thereafter, the number of infective virus particles from both experiments was quantified in terms of a 50% tissue culture infective dose (TCID₅₀) assay according to Reed and Muench, (1938). For an analysis of the effect of cholesterol reduction on virus binding, CCB cells were incubated with CyHV-3 at an MOI of 10 for 1 h on ice, washed twice and then frozen at -20°C in 150 μl PBS until quantification of infective virus particles bound to the cells by means of a TCID₅₀ assay.

To investigate the effect of cholesterol depletion on virus egress, CCB cells were infected with CyHV-3 at an MOI of 1 and incubated

for 24 h, then treated with M β CD for 1 h, washed with PBS and subsequently the medium was replenished. Thereafter, 100 μ l of medium was collected at 1 h, 2 h and 3 h post medium replenishment and the virus genome copy number was quantified with PCR.

2.4. Lipid isolation and analysis

2.4.1. Virus purification

Production and purification of CyHV-3 for the determination of virion lipid composition was done according to the method described by Gilad et al. (2002).

Isolated virus was shown to be pure from cell debris by negative staining with 2% phosphotungstic acid, based on a method described by Hayat, 1989, and subsequent visualisation using a Zeiss EM 10C electron microscope (Zeiss, Oberkochen, Germany).

2.4.2. Isolation and extraction of detergent resistant and detergent-soluble membrane fractions

Detergent resistant and soluble membrane fractions were isolated based on the method previously described by Brogden et al. (2014). The two fractions were stored at -20°C for later analysis.

2.4.3. Lipid extraction and preparation

Lipids were extracted from cell membrane and from virus samples as described previously with minor modifications (Brogden et al., 2014). Obtained lipid samples were vacuum dried and stored at -20°C for future analysis.

2.4.4. Lipid analysis and quantification

Lipid samples were re-suspended in chloroform/methanol and analysed by high performance thin layer chromatography (HPTLC) on silica gel 60 plates (Merck, Germany) as described earlier (Brogden et al., 2014; Neumann et al., 2014). Bands found in the samples were compared to a lipid standard solution and analysed using the CP ATLAS software (Lazarsoftware) (Brogden et al., 2014; Neumann et al., 2014). The band intensity was determined by measuring the area under the curve and subtracting the background.

2.5. Virus quantification

2.5.1. Expression of viral genes

For checking the expression dynamics of CyHV-3 in CCB cells, genes encoding for thymidine kinase (TK), major capsid protein (MCP) and DNA polymerase (DP) were analysed.

For this, total RNA was extracted from collected samples using TriFast reagent (PeqLab, Germany) and cDNA was synthesised from 300 ng of total RNA after DNase digestion using Maxima RT

chemistry (Fermentas, Germany) as described previously (Adamek et al., 2012). A non-reverse transcriptase control was included for each sample. cDNA samples were diluted 20 \times prior to real time quantitative PCR analysis. Primer descriptions are shown in Table 1.

Real time quantitative PCR (qPCR) was used for expression analysis. Reactions were performed using the Maxima SYBR Green 2x mastermix (Fermentas, Germany) in a Stratagene Mx3005P cyclor (Agilent, USA) as described previously by Adamek et al., (2012). The expression of genes was quantified as described previously (Adamek et al., 2012). Briefly, a standard curve based quantification was performed using serially diluted recombinant plasmids (10^1 to 10^7 copies; standard curves are presented in Supplementary file 1). For normalisation of the results, the expression of carp 40S ribosomal protein S11 served as a reference gene. The level of gene expression was shown as copy number of the gene normalised for 1×10^4 copies of the reference gene.

2.5.2. Quantification of virus genome copies

Number of CyHV-3 copies was measured with qPCR as described by Gilad et al., (2004). Briefly: virus DNA was isolated from 100 μ l of medium using QIAamp DNA Mini Kit and protocol for DNA purification from body fluids (Qiagen, Germany).

For quantification of viral DNA qPCR was used. Reactions were performed using the Maxima Probe 2x mastermix (Fermentas, Germany) in a Stratagene Mx3005P cyclor (Agilent, USA) as described previously by Adamek et al., (2013).

2.5.3. Fluorescent immunocytochemistry (FICC)

Cells were collected at 4 and 7 days post infection (dpi) from $n = 3$ cell cultures per time point per treatment. Furthermore cells which were infected 3 days post M β CD treatment and cultivated for 7 dpi were also collected.

For FICC analysis of virus protein expression, infected CCB cells were labelled with the mouse monoclonal antibody (MAB) P14 KHV (Aquatic Diagnostics, UK), subsequently with fluorescence isothiocyanate conjugated rabbit anti mouse antiserum (Dako Cytomation, Denmark) and assessed by UV microscopy (Zeiss, Germany) as described earlier (Adamek et al., 2012). For controls (i) non infected cells were stained in the same way as samples from the infection experiment, (ii) the primary antibody was replaced by blocking solution, (iii) the primary antibody was replaced by control mouse IgG.

2.6. Statistical analysis

SigmaPlot 12 software was used for statistical analysis. Differences between the treatments were considered as statistically significant at $P \leq 0.05$. The percentages of virus positive cells

Table 1
Primers used in this work.

Gene	Primer	Sequence 5'–3'	GenBank ID	Use	Ref.
40S ribosomal protein S11	q40S.FW1	CCGTGGGTGACATCGTTACA	AB012087	P, Q	(Gonzalez et al., 2007)
	q40S.RV1	TCAGGACATTGAACCTCACTGTCT			
CyHV-3 DNA polymerase	CyHV3_OFR79_F1	CCGTGAGGTTGATCGAGGAG	DQ177346	P	(Adamek et al., 2012)
	CyHV3_OFR79_R1	GTCTATCGCGACCTTGCTG			
	CyHV3_O79_qF1	CGCCGACTTTACCTTTCTCG		Q	
	CyHV3_O79_qR1	AACCCCTCTTGCTTGTC			
CyHV-3 major capsid protein	CyHV3_OFR92_F1	ACGAGTTCTCCCTCGCTTC	DQ177346	P	(Adamek et al., 2012)
	CyHV3_OFR92_R1	GACCCAGTCCCTTGACGTTG			
	CyHV3_O92_qF1	AGCCACCTCTTGGTCGTG		Q	
	CyHV3_O92_qR1	ACTCCCTGTCCAGCACTC			
CyHV-3 thymidine kinase	CyHV3_TK_qF1	TGGCTATGCTGAACTGGTG	DQ177346	P, Q	(Adamek et al., 2012)
	CyHV3_TK_R2	GTTACAAGAACGAGGTGGAG			
	CyHV3_TK_qR3	GCTGGTCTATGGCGTGCTTG		Q	

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.

(assessed with FICC) were subjected to arcsin transformation before statistical analyses. The normalised expression levels of viral genes expression and virus genome copies were transformed with $\log(10)$. The differences in between the treatments in the percentages of FICC virus positive cells and viral gene expression were assessed using two-way ANOVA with a pairwise multiple comparison using Holm–Sidak's procedure.

3. Results

3.1. Treatment of CCB cells with M β CD induces a concentration dependent depletion of cholesterol

To investigate the importance of cholesterol and lipid rafts during virus entry, a cholesterol depletion and replenishment assay was established *in vitro* using the common carp derived CCB cell line. The aim of the cholesterol depletion assay was to remove a significantly high concentration of cholesterol whilst keeping a viable cell monolayer. For this, CCB cells were incubated with 0, 5, 7.5, 10 and 15 mM of M β CD. The results in Fig. 1a show that there was an M β CD concentration dependent reduction in cellular levels of cholesterol after 1 h of incubation. Interestingly,

free fatty acids were also depleted by similar levels; however the levels of phosphatidylcholine remained relatively stable, excluding the cells incubated with 15 mM M β CD (Fig. 1a). At this concentration a higher level of cell death was observed as described in Section 2.2 (data not shown). Incubation with 7.5 and 10 mM of M β CD led to a significant reduction in cholesterol levels whilst not disrupting the cell monolayer, furthermore no changes in cell morphology could be observed. Subsequently, the kinetics of M β CD induced cholesterol depletion was examined including the rate of cholesterol replenishment. Incubation of CCB cells with 10 mM M β CD for 1 h led to an almost 70% reduction in the cholesterol content of the cells at 1 and 2 h post incubation (Fig. 1b). At 48 h post incubation, the cellular cholesterol was replenished to the pre-treatment level. In untreated cells and the cells incubated with both 10 mM M β CD and 2.5 mM cholesterol, the cholesterol levels were maintained at the pre-treatment level (Fig. 1b). In all treatments, the cell monolayers remained intact and the cells remained viable. Therefore for further experimentation, 10 mM M β CD and an incubation period of 1 h were selected.

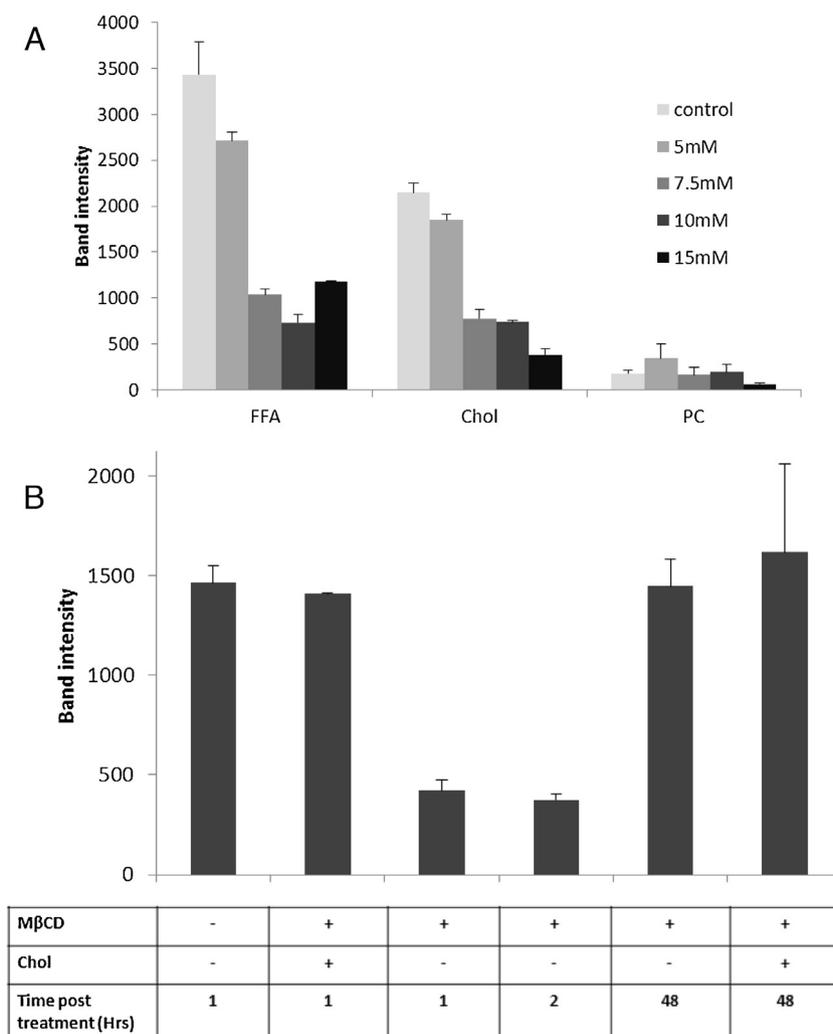


Fig. 1. Effect of Methyl-beta-cyclodextrin treatment on cellular lipid levels of CCB cells. (A) Concentration of cholesterol (Chol), free fatty acids (FFA) and phosphatidylcholine (PC) of cells incubated for 1 h with M β CD. (B) The levels of cholesterol in cells treated with 10 mM of M β CD for 1 h and analysed 1 h after incubation. Both diagrams show mean values (\pm SD) of $n = 3$ separate experiments.

3.2. Effects of M β CD on virus entry, replication and egress

In order to address the effect of M β CD on virus entry and replication cycle, first the progression of virus infection in M β CD

treated cells was monitored. The mRNA expression of CyHV-3 genes which were used as a marker for CyHV-3 replication, TK, MCP and DP all showed significantly lower expression levels in the M β CD treated cells at 2 and 5 days post infection. In infected cell

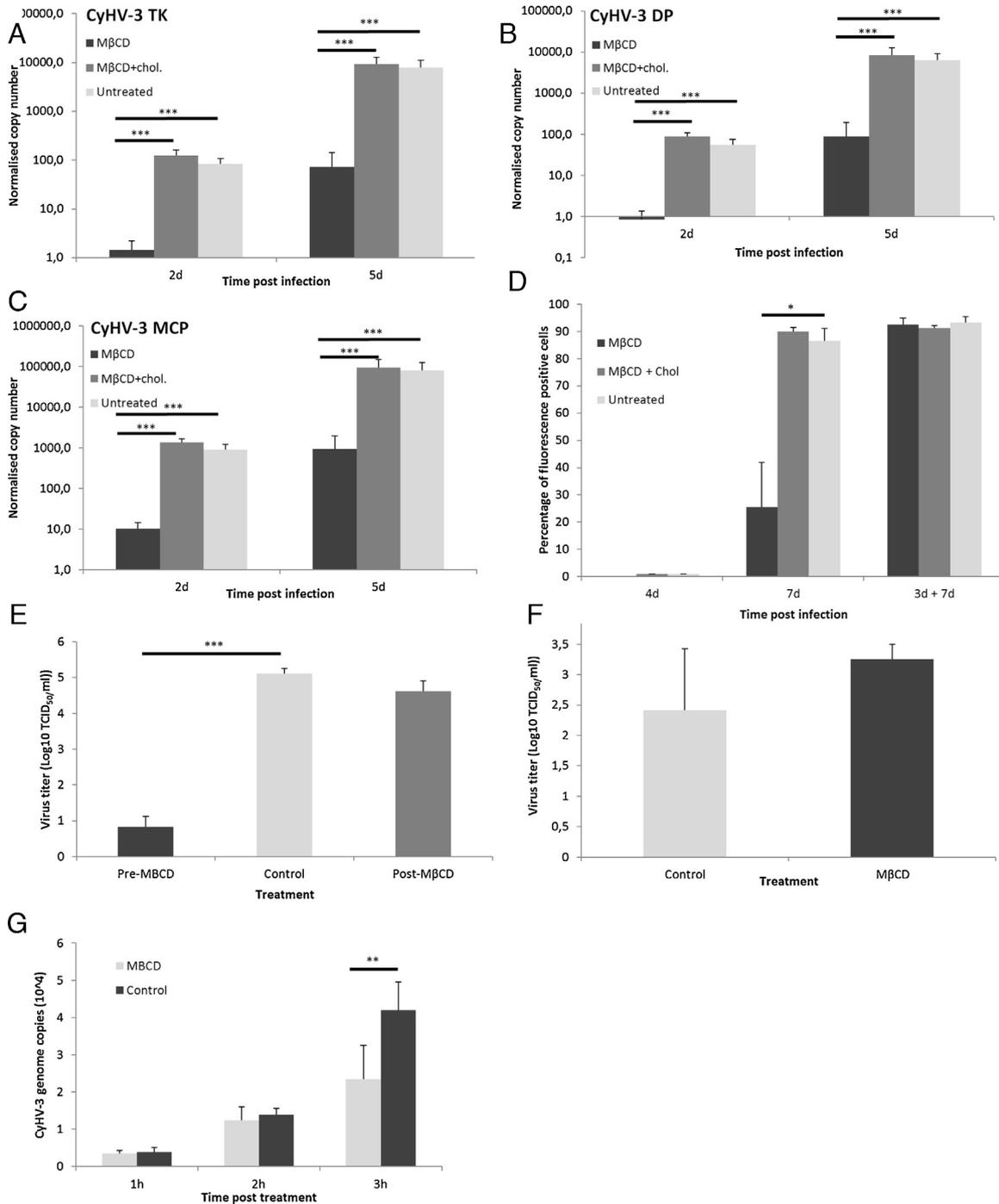


Fig. 2. Effect of methyl-beta-cyclodextrin treatment on virus binding, entry, spread and egress. (A–C) Expression profiles of *Cyprinid herpesvirus 3* genes in CCB cell cultures during CyHV-3 infection. CCB monolayers were treated with M β CD, M β CD and cholesterol or left untreated (CyHV-3) prior to infection with the virus. The mRNA expression of thymidine kinase TK (A), DNA polymerase DP (B), and major capsid protein MCP (C), were used as marker for CyHV-3 replication. Expression levels are shown as mean copy number (\pm SD) of the gene normalised for 100 000 copies of 40S ribosomal protein S11 ($n=6$). (D) Immunocytochemical detection of CyHV-3 in infected CCB cells. Cells were treated with or without M β CD, M β CD and cholesterol co-treatment and after cholesterol reduction and replenishment. Depicted is the percentage of CCB cells positive for CyHV-3 glycoprotein on 4 and 7 days post infection (dpi). No virus associated protein was detected in M β CD treated cells at 4 dpi. Results are displayed as percentage (\pm SD) from $n=3$ separate cell cultures. (E and F) Role of cholesterol in CyHV-3 binding, entry and post entry. Number of infective CyHV-3 particles in CCB cells, which were treated with M β CD prior (Pre- M β CD, non-infected) or post (post- M β CD) CyHV-3 infection (E). Number of CyHV-3 particles binding to CCBs depleted with M β CD followed by CyHV-3 infection on ice (F). In both experiments, the number of infective virus particles was quantified in terms of a TCID₅₀ assay. (G) Virus egress determined by measuring CyHV-3 genome copies post infection (24 h) and subsequent M β CD (1 h) treatment, at 1, 2 and 3 h post cholesterol reduction. CyHV-3 genome copy was quantified by qPCR. Data are presented as mean \pm SD from 4 separate cell culture. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

cultures, the expression levels of these genes increased during the time of infection, and therefore the expression levels of these genes

corresponds to the progress of infection in an *in vitro* setting (Adamek et al., 2012).

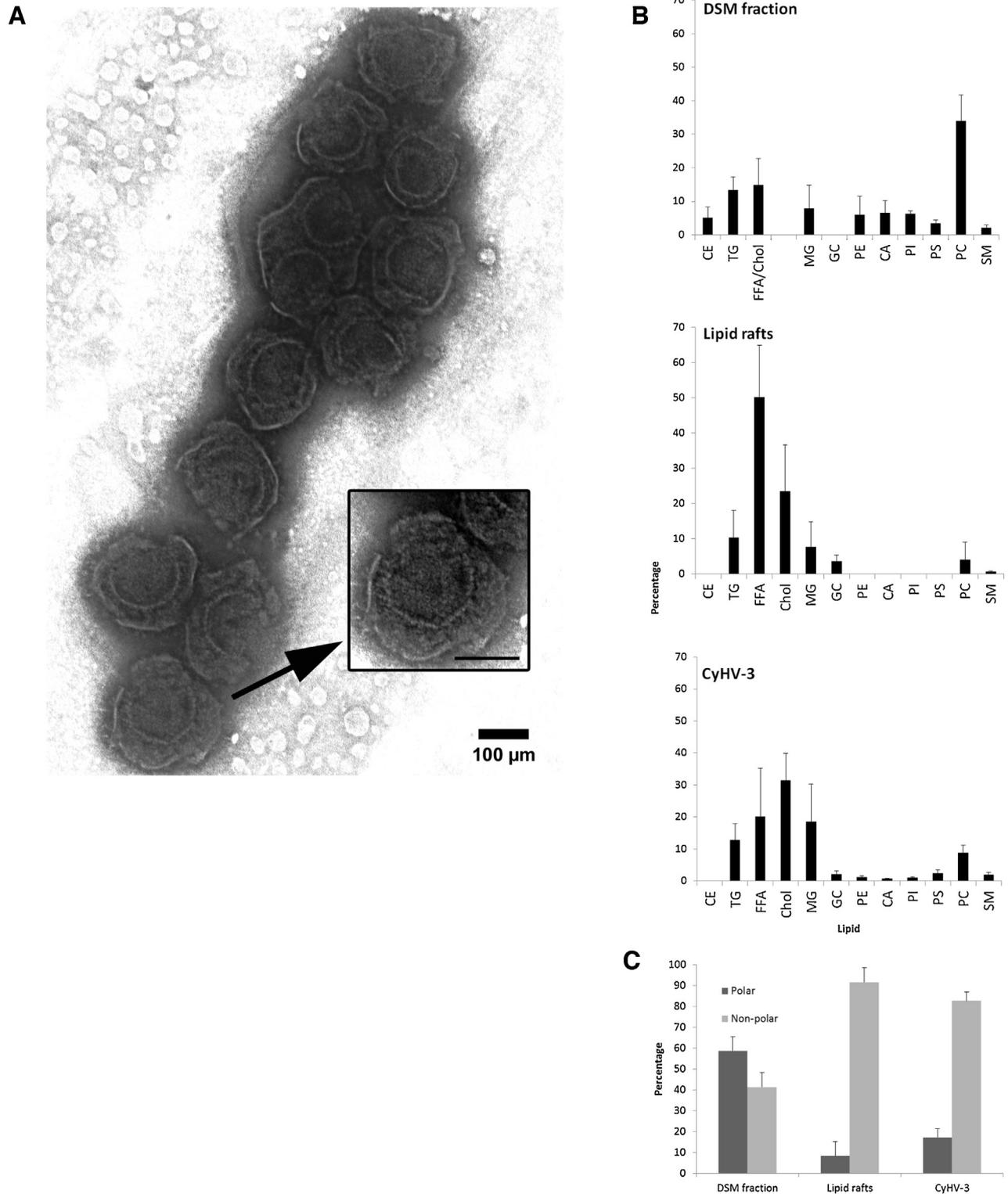


Fig. 3. Purification and analysis of CyHV-3 lipid composition. (A) Negative staining of purified CyHV-3 visualised by transmission electron microscopy. Images show a virus isolate free from cell debris contamination. The magnified image shows a single virion comprising of a lipid envelope and capsid proteins. (B) Lipid composition of CyHV-3 virions and CCB membrane fractions. Concentration of the lipids CA: cardiolipin, CE: cholesterol-ester, Chol: cholesterol, FFA: free fatty acids, GC: galactoceramide, MG: monoacylglycerol, PC: phosphatidylcholine, PE: phosphoethanolamine, PI: phosphatidylinositol, PS: phosphatidylserine, SM: sphingomyelin, and TG: triglycerides displayed as a total percentage of all lipids isolated from CyHV-3 virions, detergent soluble membrane (DSM) and lipid rafts (LR) membrane fractions of CCB cells analysed using HPTLC. In the DSM fraction, cholesterol and FFA were not separated and accounted for a combined total of 14.9%. $n = 3$. (C) Percentage of polar and non-polar lipids in CCB cell DSM and lipid raft membrane fractions and CyHV-3 virions. $n = 3$ independent preparations.

The viral gene expression levels were significantly lower in M β CD treated cells when compared to cells co-treated with M β CD and cholesterol as well as in non-treated cells in both time points ($P \leq 0.001$). At 2 days p.i., the mRNA expression in M β CD treated cells was 56 \times lower for CyHV-3 TK, 65 \times for CyHV-3 DP and 85 \times lower for CyHV-3 MCP compared to non-treated cells (Fig. 2a). The differences were even higher at 5 days p.i. (from 73 \times for CyHV-3 DP to 110 \times for CyHV-3 MCP) (Fig. 2a–c). Interestingly, this assay also showed (similarly to FICC) that the M β CD and cholesterol co-treated cells displayed slightly higher expression of viral genes (from 1.2 \times to 1.6 \times) than the non-treated cells (Fig. 2a–c).

In support of the gene expression data, FICC results presented a similar trait to the viral gene expression analysis. Cell monolayers infected after treatment with M β CD also contained a significantly lower percentage of cells expressing virus proteins (infected cells) at both 4 and 7 days p.i., when compared to M β CD plus cholesterol and untreated medium. The cells incubated with a combination of M β CD and cholesterol, or left untreated had similar levels of infected cells of 85.5% and 90% respectively, whereas the cells treated with M β CD showed a significantly lower ($P \leq 0.001$) average of 25.5% of cells presenting virus proteins (Fig. 2d). Critically, cells depleted of cholesterol and then naturally replenished over a 3 day period, showed comparable levels of infection to the untreated samples. These data show that both replication rate and spread of the virus in the culture was lower in M β CD treated cells when compared to the two control groups.

In order to establish if cholesterol is required for virus binding, entry or subsequent post entry stages of replication, two assays were performed. In a virus entry assay, cells treated with 10 mM M β CD prior to CyHV-3 infection had a by 4 orders of magnitude, significantly lower ($P \leq 0.001$) virus titre compared to mock treated controls (Fig. 2e). Cells infected with CyHV-3 and subsequently treated with 10 mM M β CD gave the same virus titre as the control (Fig. 2e). This result indicates that CyHV-3 requires cholesterol to enter the cell, but not the following post entry stages of replication. In a further experiment, the importance of cholesterol on virus binding was investigated, where the removal of cholesterol via M β CD treatment prior to infection led to no significant difference ($P \leq 0.05$) in the virus titre when compared to the untreated control (Fig. 2f).

Lastly, to investigate the importance of cholesterol in virus egress, CCB cells were first infected with CyHV-3, then cultivated for 24 h, washed and subsequently treated with M β CD to deplete cholesterol. Finally the virus genome copy number was quantified after 1, 2 and 3 h post cholesterol depletion (Fig. 2g). Virus egress was shown to be lower at 1 and 2 h post M β CD treatment. Interestingly, egress was significantly ($P \leq 0.01$) hindered at 3 h post M β CD treatment. This result indicates that cholesterol plays an important role during CyHV-3 egress from CCB cells.

3.3. CyHV-3 virions have a high concentration of cholesterol and a similar lipid composition to lipid rafts from host cells

In order to examine a possible association of the virus with the cell membrane and in particular with specific plasma membrane microdomains, the lipid profiles of detergent soluble membrane fractions (DSM), lipid raft or detergent resistant membrane fractions (DRM) and CyHV-3 were determined.

Firstly, the virus was purified on a sucrose density gradient and to ensure the preparation was free-from membrane contaminants, the virus was visualised by negative staining and analysed by electron microscopy (Fig. 3a). The image shows that the isolation was successful and that the virus was isolated without cell debris remnants.

HPTLC analysis revealed that the CyHV-3 contained a wide variety of lipids. Eleven individual lipid species were identified,

with high concentrations of triglycerides, free fatty acids, cholesterol, monoacylglycerol and phosphatidylcholine, with trace amounts of galactocerebroside, phosphatidylethanolamine, cardiolipin, phosphatidylinositol and sphingomyelin detected (Fig. 3b). In CyHV-3 the ratio of polar/non-polar lipids was 17:83 (Fig. 3c).

In cell membrane fractions separated by Triton X-100 treatment into DRM or lipid raft fraction and DSM fractions, two distinctly different lipid profiles were observed. The DSM fraction contained a broader range of 10 lipids present at measurable concentrations. This fraction was particularly rich in phospholipids with a very high concentration of phosphatidylcholine (Fig. 3b). In the lipid raft fraction, seven lipids were detected at measurable concentrations, with high levels of free fatty acids and cholesterol present, however many phospholipids were below the minimum detection level (Fig. 3b). In the DSM fraction, a ratio of polar/non-polar lipids of 59:41 was found while in the lipid raft fraction the ratio of polar/non-polar lipids was 8:92, which was similar to the 17:83 ratio found in CyHV-3 (Fig. 3c).

4. Discussion

Herpesviruses are increasingly recognised as pathogens in cyprinid fishes. The development of effective treatment strategies for these virus infections requires knowledge of their basic replication traits. Entry into a host cell for a virus is the critical step for initiating infections. Knowledge of virus entry pathways is therefore important for understanding viral pathogenesis, target cell population and exploring effective treatment strategies which block virus infection before entering the cell. Cyprinid herpesviruses, together with other herpesviruses from fish and amphibians differ in gene contents from mammalian herpesviruses (McGeoch et al., 2006) and therefore were assigned to the family of *Alloherpesviridae* within the order *Herpesvirales* (www.ictvonline.org; (Davison et al., 2009; Waltzek et al., 2009)). Mammalian herpesviruses have been shown to use different pathways for cell entry. In addition to endocytosis, mammalian herpesviruses can enter cells via cholesterol dependent fusion at the plasma membrane (Rahn et al., 2011). Cholesterol, a major component of lipid raft fractions in the cell membrane, has been shown to be critically important in the replication cycle of herpesviruses (Bender et al., 2003; Ren et al., 2011) and several enveloped viruses from different families (Bavari et al., 2002; Huang et al., 2011; Ren et al., 2008). The present study provides evidence that cholesterol is also essential in the replication cycle of CyHV-3, a herpesvirus from a fish host.

The importance of cholesterol in the cellular membrane in establishing an infection with CyHV-3 in carp cells was shown in the present work by several lines of evidence. Firstly, M β CD-treatment of cells reduced the virus multiplication and synthesis of virus associated proteins in infected cells. Secondly, the effect of cholesterol removal was reversible: when cholesterol was replenished in the cell or when additional cholesterol was added to M β CD-treated cells, the virus multiplication was restored. Thirdly, M β CD treatment of cells prior to virus infection resulted in a lower virus load relative to mock-treated cells or cells treated post virus infection. Fourthly, M β CD-treatment decreased the number of virus particles released from the cells. Finally, lipids isolated from the virus envelope are similar in composition to that of the lipid raft fraction of CCB cells and dissimilar to the detergent soluble non-raft fraction.

Cholesterol is thought to keep the raft assembly in the cell membrane together (Simons and Toomre, 2000) and a sequestration of cholesterol by pore forming agents such as saponin or by M β CD allows for the manipulation of the constituents of lipid rafts (Simons and Toomre, 2000). In particular, M β CD is widely used to

study the importance of cholesterol in the infection process of several viruses (Bender et al., 2003; Chung et al., 2005; Martin et al., 2012; Ren et al., 2011), since it captures cholesterol and removes it from the cell membrane. As a result, lipid microdomains are disrupted with concomitant blocking of biological processes that depend on them (Zidovetzki and Levitan, 2007). Here we could show that incubation with increasing amounts of M β CD resulted in a substantial decrease of cholesterol levels in carp derived CCB cells whilst still retaining an intact monolayer of viable cells. The incubation of goldfish kidney derived macrophages with M β CD caused a shifting of flotillin-1, a protein commonly associated with lipid rafts in mammalian cells, from the lipid raft fraction to the non-lipid raft associated fraction of the membrane (Garcia-Garcia et al., 2012). This indicates that like in mammalian cells, the incubation of fish derived cells with M β CD leads to a disruption of lipid rafts in a fashion similar to mammalian cells. At the same time, unlike other cholesterol binding agents that become incorporated into membranes, M β CD is strictly surface-acting and can be rapidly removed when cells are incubated with fresh medium. In CCB cells, the cholesterol level remained low until at least 2 h post M β CD treatment. During this period of time, a reduced entry of CyHV-3 into CCB cells and subsequent multiplication and virus protein expression was observed. The virus binding/virus entry assays indicated that cholesterol was critical for virus entry but not for binding and for the post entry stages of the replication cycle. A similar effect of cholesterol depletion was also observed in bovine herpesvirus type 1 infection of MDBK cells (Zhu et al., 2010). The data reported here also shows that the lower CyHV-3 replication rate seen in M β CD-treated CCB cells was not an indirect result of M β CD treatment on host cell gene regulation or lipid trafficking. Interestingly, cholesterol was not shown to be required during the binding process to the plasma membrane. However, by 48 h post M β CD incubation, the cholesterol levels of CCB cells had been completely replenished and then the virus replication and spread followed a similar trend to that of the untreated infected cells.

During morphogenesis, CyHV-3 acquires its envelope through budding into cytoplasmic vesicles (Hanson et al., 2011; Miwa et al., 2007). Due to the morphogenesis process, the lipid composition of the virus is therefore likely to be representative/comparative to the place where budding occurred (Vangenderen et al., 1994). In a variety of viruses, the lipid composition is distinct from that of the host cell plasma membrane from which they are derived. This led to the assumption that viruses might bud from specific microdomains in the plasma membrane (Scheiffele et al., 1999). In CyHV-3, this view is supported by the observation that the sequestration of cholesterol from the cell membrane hindered the egress of CyHV-3 from infected cells (current communication). In addition, annexin A2, a cellular lipid rafts-associated protein not encoded by the CyHV-3 genome (Aoki et al., 2007), was identified in the CyHV-3 virion (Michel et al., 2010), suggesting that this protein was derived from the cell and not produced by the virus. The lipid profile of CyHV-3 (rich in triglycerides, free fatty acids, cholesterol, monoacylglycerol and phosphatidylcholine) appears similar to the lipid raft fraction isolated from the cell membranes of CCB cells as well as from several carp tissues (Brogden et al., 2014) and differs from that of the DSM fraction. In particular when the lipid content was displayed as polar and non-polar lipids, the composition of the lipid raft fraction is significantly different when compared to the DSM fraction. Interestingly, there is no significant difference between the polar:non-polar ratio of the virus and the lipid rafts. Viruses acquire their lipid envelope directly from their immediate surrounding area, therefore the similarity between the lipid raft and virus lipid composition suggests that lipid rafts are involved in entry, trafficking and/or budding of CyHV-3. The observations on the composition of the

lipid envelope, the presence of Annexin 2a in the CyHV-3 as well as the reduced release of virus particles from cells after cholesterol sequestration from the cell membrane would support the hypothesis that the CyHV-3 uses lipid rafts as a portal of exit during egress.

In summary, this is the first study that demonstrates an implication of cholesterol in the replication cycle of a piscine herpesvirus. As CyHV-3 is a member of the family *Alloherpesviridae*, which is an ancient phylogenetic diversification of herpesviruses, our data suggests that this method is probably conserved amongst all members of the larger clade of the *Herpesvirales* and might represent an ancient pathway. These results might have fundamental connotations for further research on the innate immune responses influencing cholesterol-rich lipid rafts, in particular studies on the antiviral effect of type I interferon stimulated proteins such as viperin, which in mammals was shown to stunt virus egress by disrupting lipid rafts in the cell membrane of infected cells (Wang et al., 2007). Furthermore the cholesterol pathway could be a good target for effective treatment or prevention of viral infections in fish.

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