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Ultrastructural analysis of sequential Cyprinid herpesvirus 3 morphogenesis *in vitro*

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23 **Abstract**

24 Cyprinid herpesvirus 3 (CyHV-3) is an alloherpesvirus, and the aetiological agent of koi
25 herpesvirus disease. Although the complex morphogenic stages of the replication cycle of
26 CyHV-3 were shown to resemble that of other members of the *Herpesvirales*, detailed
27 analysis of the sequence and timing of these events was not definitively determined. This
28 study describes these features through a time course using cyprinid cell cultures (KF-1 and
29 CCB) infected with CyHV-3 (KHV isolate, H361) and analysed by transmission electron
30 microscopy. Rapid viral entry was noted, with high levels of intracellular virus within 1-4
31 hours post-infection (hpi). Intra-nuclear capsid assembly, paracrystalline array formation and
32 primary envelopment of capsids occurred within 4 hpi. Between 1-3 days post infection (dpi),
33 intra-cytoplasmic secondary envelopment occurred, as well as budding of infectious virions
34 at the plasma membrane. At 5-7 dpi, the cytoplasm contained cytopathic vacuoles, enveloped
35 virions within vesicles, and abundant non-enveloped capsids; also there was frequent nuclear
36 deformation. Several morphological features are suggestive of inefficient viral assembly, with
37 production of non-infectious particles, particularly in KF-1 cells. The timing of this
38 alloherpesvirus morphogenesis is similar to other members of the *Herpesvirales* but there
39 may be possible implications of using different cell lines for CyHV-3 propagation.

40 **Keywords:** Cyprinid herpesvirus 3, CyHV-3, Koi herpesvirus (KHV), sequential
41 morphogenesis, transmission electron microscopy (TEM)

42 **Introduction**

43 Cyprinid herpesvirus 3 (CyHV-3) is the official taxonomical classification of koi herpesvirus
44 (KHV) (Waltzek *et al.* 2005), the highly virulent and economically important aetiological
45 agent of koi herpesvirus disease (KHVD) (Hedrick *et al.* 2000; 2005). The virus has had a
46 devastating impact on to the global koi and carp (*Cyprinus carpio* Linnaeus, 1758)
47 aquaculture industries since outbreaks were first reported in Israel and the U.S. in 1998
48 (Hedrick *et al.* 2000; Perelberg *et al.* 2003). CyHV-3 is a member of the recently formed
49 family *Alloherpesviridae* (Waltzek *et al.* 2005) in the order *Herpesvirales* (Davison *et al.*
50 2009). This classification has been based on the close phylogenetic relationship of the virus
51 with CyHV-1 (Aoki *et al.* 2007; Waltzek *et al.* 2009), the causative agent of carp pox (Sano
52 *et al.* 1991; 1992; Pääk *et al.* 2011), CyHV-2; goldfish haematopoietic necrosis virus
53 (GHNV) of goldfish (*Carassius auratus* Linnaeus, 1758) (Goodwin *et al.* 2006; 2009; Lovy
54 & Friend 2014) and AngHV-1, causing herpesvirus disease in European eels (*Anguilla*
55 *anguilla* Linnaeus, 1758) (Van Beurden *et al.* 2011; Armitage *et al.* 2014). Morphogenic
56 stages of CyHV-3 have been shown to resemble those of other herpesviruses, both *in vitro*
57 (Miwa *et al.* 2007) during cytopathic effects of CyHV-3 inoculated cells and *in vivo*
58 (Miyazaki *et al.* 2008) during clinically diseased, experimentally challenged fish, expressing
59 KHVD, supporting the designation of the virus as a herpesvirus (Hedrick *et al.* 2000).
60 However, little is known with regards to the timing of the various stages of *Alloherpesviridae*
61 virion maturation. As with other herpesviruses, CyHV-3 displays differential infection
62 phases, including fatal lytic infection and potential latent infection (Gilad *et al.* 2003; 2004;
63 St-Hilaire *et al.* 2005; Eide *et al.* 2011; Reed *et al.* 2014; Sunarto *et al.* 2014), which can be
64 influenced by temperature (Ronen *et al.* 2003; St-Hilaire *et al.* 2005; Sunarto *et al.* 2014).
65 These different infection states can impact serological and molecular detection sensitivities,

66 as limited antibodies or viral DNA copy numbers are produced during acute and latent
67 infections, respectively (Bergmann *et al.* 2010; Matras *et al.* 2012; Monaghan *et al.* 2015).
68 Despite the development of tools for CyHV-3 detection and progress made in understanding
69 viral replication and stages of infection, most studies related to this virus have been based on
70 molecular and antibody-based methodologies (Gilad *et al.* 2004; Pikarsky *et al.* 2004; Ilouze
71 *et al.* 2012a; b; Monaghan *et al.* 2016). A potential pitfall of these approaches is that viral
72 DNA concentrations and expressed antigen may not directly correlate with the number of
73 infectious CyHV-3 particles, as virion particle formation may be incomplete. An example of
74 this is the prototype of herpesviral replication, herpes simplex virus type 1 (HSV-1), where
75 only around 25% of viral DNA and protein is considered to be assembled into virions
76 (Ginsberg 1988). The kinetics of actual virion morphogenesis during replication is currently
77 unknown for CyHV-3, and information relating to this cannot be obtained by using the
78 techniques mentioned above.

79 Morphogenic stages of herpesviruses are complex, occurring partly in the nucleus (i.e. capsid
80 assembly, packaging of replicated genome, primary envelopment and nuclear egress/de-
81 envelopment) and in the cytoplasm (i.e. tegumentation, secondary envelopment and budding
82 of infectious virions at the plasma membrane) (reviewed in Mettenleiter, 2002; Mettenleiter
83 *et al.*, 2009). Although the morphology of CyHV-3 has previously been shown to resemble
84 that of other members of the *Herpesvirales*, detailed analysis of the sequence and timing of
85 the events involved in virion production have not been fully determined for the
86 *Alloherpesviridae*. The stages of CyHV-3 morphogenesis have been described in detail by
87 Miwa *et al.* (2007) after 7 dpi including the formation of three capsid types during assembly
88 in the nucleus and maturation and egress from the nucleus through two distinct envelopment
89 events.

90 In the current study the stages of CyHV-3 morphogenesis were investigated from 1 hpi to 7
91 days post inoculation in two of the most commonly used cyprinid cell lines for CyHV-3 virus
92 propagation, common carp brain (CCB) and koi fin (KF-1). The goal of the study was to
93 evaluate inconsistencies that may occur between the cell lines with respect to virus
94 maturation and cell pathology, as these can have potential implications for successful
95 propagation of infectious CyHV-3 virus particles.

96 **Materials and Methods**

97 **Cell culture**

98 The KF-1 cells used in the study were developed from epidermal tissue of koi (Hedrick *et al.*
99 2000), and were kindly provided by Dr. Keith Way (Centre for Environment, Fisheries and
100 Aquaculture Science (CEFAS), Weymouth, UK). The CCB cells were kindly provided by Dr.
101 Matthias Lenk (Friedrich Loeffler Institut (FLI), Greifswald, Germany), and were developed
102 from brain tissue of common carp (Neukirch *et al.* 1999). Both cell lines were cultured in
103 Eagle's Minimum Essential Medium (EMEM) containing Eagles's salts (Invitrogen), 10 %
104 foetal bovine serum (FBS), 1 % Non-Essential amino acids (NEAA, Invitrogen) and 2 mM L-
105 glutamine at 22-25°C with 4 % CO₂. During all stages of the study, including optimisation of
106 the KHV infection and harvest protocol for TEM analysis, the KF-1 cells were maintained
107 between a subculture passage of 108–144 and CCB cells between passage 69-84.

108 **Virus culture**

109 The isolate of CyHV-3 (genotype U, KHV, H361) used in this study originated from an adult
110 koi with clinical KHVD in Eastern USA in 1998 (Hedrick *et al.* 2000). Cell lines were sub-
111 cultured and maintained at 22°C for a period of 24-36 h, until a monolayer of 50% or 70-80%
112 confluence was obtained for KF-1 and CCB cells, respectively. At this point, the culture
113 medium was removed and the monolayers were carefully washed with Dulbecco's phosphate

114 buffered saline (DPBS) prior to inoculating the cells with the virus. The KF-1 cells were
115 inoculated with CyHV-3 at a multiplicity of infection (MOI) of 0.01 and CCB cells with an
116 MOI of 0.02 from a virus stock of KHV $10^{4.4}$ tissue culture infectious dose (virus infection of
117 50 % inoculated cells (TCID₅₀)) mL⁻¹. An adsorption period of 1-2 h at 20°C was performed
118 before re-supplementing the cultures with fresh EMEM (+2 % FBS). After a cytopathic effect
119 (CPE) of 90–100 % was obtained, the virus was harvested by exposing the monolayer to two
120 cycles of freeze-thawing at -70°C, and then centrifuging the lysed cell suspension at 3,800 x
121 g (Eppendorf 5804 R). The clarified supernatant was collected and stored as aliquots at -70°C
122 until used.

123 **Virus quantitation**

124 KF-1 and CCB cells were cultured overnight at 22°C in 24 or 96 well tissue culture plates
125 (Nunc, Denmark) to form a monolayer. After the monolayers were 50-60 % confluent, the
126 culture medium was removed and the cells were inoculated with 100 µL of a 5-fold serial
127 dilution of the virus, diluted in Hank's buffered salt solution (HBSS), 2 % FBS. Mock
128 infected cells were also included, which received only culture medium without virus. Virus
129 was absorbed onto the cells for 1-2 h at 20°C, before re-supplementing the cells with fresh
130 EMEM medium containing 2 % FBS. Cells were checked for the development of a CPE after
131 7 and 14 dpi. For calculating the initial virus inoculation dose the TCID₅₀ was determined
132 according to the Spearman-Kärber method (Kärber 1931). Multiplicity of infection (MOI)
133 was determined as described by Voronin *et al.* (2009).

134 **Time course infection of cell lines**

135 Nine tissue culture flasks (75cm²) per cell line were used for time course analysis of CyHV-3
136 morphogenesis. KF-1 cultures were seeded at 5×10^6 cells flask⁻¹ and CCB cultures were
137 seeded at 2×10^6 cells flask⁻¹. Cells were cultured for 24 h at 22°C prior to initiating the trial.

138 Two non-infected flasks per cell line were used as negative controls, which were sampled at 1
139 dpi and 7 dpi. For the seven test flasks, monolayers were inoculated with 3 mL KHV as
140 described above, and sampled at 1, 4 and 8 hpi, and 1, 3, 5, and 7 dpi. Samples were taken by
141 washing the monolayers twice with 10 mL DPBS, and then fixing the cells *in-situ* with 6 mL
142 2.5% glutaraldehyde (Sigma-Aldrich, UK) in 0.1M sodium cacodylate buffer, pH 7.3. Cells
143 were then scraped into suspension using a rubber policeman and 6 mL (3 mL x 2) of the
144 suspension were centrifuged at 2000 x g for 10 min at 4°C to form a pellet (slow speed
145 centrifugation was used to avoid cell rupture). Pellets were post fixed with fresh 2.5%
146 glutaraldehyde for 2-4 h or overnight at 4°C. The fixative was removed and 2 mL 0.1M
147 sodium cacodylate buffer was added to the pellets, which were detached from the tube wall
148 with a wooden applicator and stored at 4°C until processed.

149 **Transmission Electron Microscope (TEM) processing and visualisation**

150 Glutaraldehyde fixed cell pellets were post-fixed in 1 % osmium in 0.1M sodium cacodylate
151 buffer in closed vials for 1 h at 22°C. The pellets were then washed for 3 x 10 min in distilled
152 H₂O to remove the cacodylate buffer. This enabled 'En-bloc' staining of pellets with 2 %
153 uranyl acetate in 30 % acetone in the dark for 1 h. This was followed by dehydration: 60 %
154 acetone for 30 min, 90 % for 30 min, 100 % for 30 min then incubation in fresh 100 %
155 acetone for 1 h. Pellets were then infiltrated with agar low viscosity resin (ALVR) on a
156 rotator (Taab, UK). The pellets were first incubated with ALVR diluted 1:1 in acetone for 45
157 min followed by 100% ALVR for 1 h and then into fresh ALVR for another 1 h. The pellets
158 were finally embedded in block moulds and polymerised in an oven at 60°C overnight. One
159 hundred micron ultra-thin sections were prepared from the resin blocks using a microtome
160 (Reichert Ultracut E, Leica, UK) with a diamond knife (Diatome, US) and placed on 200 µm
161 mesh Formvar-coated copper grids. These were first stained with 4 % uranyl acetate in 50 %
162 ethanol for 4 min, followed by Reynold's lead citrate for 7 min. The sections were examined

163 under an FEI Tecnai Spirit G2 Bio Twin Transmission Electron Microscope. Measurements
164 of virions were made using FEI Tecnai software.

165

166 **Results**

167 **TEM analysis of CyHV-3 morphogenesis in infected cells**

168 Virus particle sizes differed depending on the stage of morphogenesis, the measured
169 immature capsids ($n=48$) = 97.56 nm (SD \pm 8.78), nucleocapsids ($n=16$) = 114.12 nm (SD \pm
170 12.13), primary enveloped virions ($n=5$) = 138.32 nm (SD \pm 18.43) and secondary enveloped
171 mature virions ($n=18$) = 167.97 nm (SD \pm 31.38), were all within the size range of CyHV-3
172 particles reported in the literature (Hedrick *et al.* 2005; Miwa *et al.* 2007; Miyazai *et al.*
173 2008). No discernible difference was noted in the sizes of virions obtained from the CCB
174 cells or the KF-1 cells.

175 **KHV in infected cells during the first 24 hpi**

176 Many cells were devoid of virions and their ultrastructure was normal and similar to the
177 control cells at this stage (Fig. 1 A). A small number of both CCB and KF-1 cells contained
178 intranuclear paracrystalline capsid arrays at 4 hpi. This was associated with reduction of
179 heterochromatin/euchromatin ratio and chromatin margination (Fig. 1 B and C). The capsids
180 observed were predominantly devoid of electron dense cores, and were occasionally toroid
181 (Fig. 1 B-D). The capsids observed within the paracrystalline arrays were predominantly
182 toroid in appearance (Fig. 1 C). Lamellar bodies, reminiscent of lipofuscin, were occasionally
183 observed regardless of the infection status (Fig. 1 B).

184 Between 4 hpi – 1 dpi, virus capsids were observed throughout the nucleus at various stages
185 of maturation in a large number of the infected cells. Capsids were often located below the

186 inner nuclear envelope, occasionally featuring an envelope – primary envelopment (Fig. 1 E).
187 Capsids with electron dense cores were more frequent at this stage.

188 By 1 dpi, naked nucleocapsids (without a secondary envelope) had assembled in the
189 cytoplasm of infected cells, some contained an electron dense core, while others were empty
190 (Fig. 1 F). At this stage, intracytoplasmic secondary enveloped particles within vesicles were
191 already present, with nuclei harbouring large numbers of capsids as described above (Fig 1
192 F). There were no discernable differences in virion formation or cell pathology between KF-1
193 or CCB cells at this stage.

194 *Figure 1 positioned here*

195 **CyHV-3 virus in infected cells at 3 dpi**

196 At 3 dpi, although the frequency of infected cells had increased, the cells contained relatively
197 few virus particles and many of the cells remained uninfected (Fig. 2 A). Mature virions
198 could be observed in intracytoplasmic vesicles of CCB cells however, which also featured
199 large, clear intracytoplasmic cytopathic vacuoles (Fig. 2 B-C). Extracellular virions were
200 observed at this stage in KF-1, but not in CCB cells (Fig. 2 D). Rare abnormalities were
201 apparent within the nucleus, with intra-nuclear vesicles in CCB cells suggestive of primary
202 envelopment of empty capsids (Fig. 2 E). Capsids at various stages of maturation, including
203 intranuclear paracrystalline arrays were common observations in both CCB and KF-1 cells at
204 this time (Fig. 2 F).

205 *Figure 2 positioned here*

206 **CyHV-3 virus in infected cells at 5 and 7 dpi**

207 At 5 dpi there was a greater abundance of virus particles in both cell lines, at various stages
208 of morphogenesis. Primary envelopment was clearly seen where sometimes \geq three

209 nucleocapsids were contained within the perinuclear cisterna at one time (Fig. 3 A-B). At 5
210 dpi the occurrence of cytopathic vacuoles increased with some containing internalised mature
211 enveloped virus particles, while other virions had budded into cytoplasmic vesicles,
212 especially in CCB cells (Fig. 3 C). Mature virions were often observed associated with, and
213 budding within, vesicles from the Golgi apparatus (Fig. 3 D) or from the cell membrane,
214 which were sometimes in clumped, membrane bound extracellular aggregates (Fig. 3 E-F).
215 While the majority of intracellular enveloped virus particles were observed in CCB cells,
216 there were greater numbers of extracellular mature virions observed in TEM micrographs of
217 infected KF-1 cells.

218 *Figure 3 positioned here*

219 Clusters of naked nucleocapsids were often found in close proximity to cytopathic vacuoles
220 (Fig. 4 A), sometimes with protruding cores (Fig. 4B) and secondary enveloped mature
221 virions within intracytoplasmic vesicles (Fig. 4 C). By this stage the compartments of the
222 mature herpesvirus virion were clearly defined, including the projections of the glycoprotein
223 envelope, amorphous tegument layer, capsid and electron-dense core (Fig. 4 D).

224 *Figure 4 positioned here*

225 **Virion morphogenesis and egress at membranous compartments throughout the course** 226 **of infection**

227 As the infection of cells was not synchronised, it is not possible to determine the precise
228 timing of various morphogenesis events at the cellular level. However, by 3 dpi all stages of
229 morphogenesis had been observed, including docking of nucleocapsids at the nuclear pore
230 (Fig. 5 A), primary envelopment resulting in both viable virions with electron dense cores
231 (Fig. 5 B) and likely aberrant capsidless intracisternal L-particles (Fig. 5 C). Budding of

232 tegumented capsids within Golgi-derived vesicles could be observed within the cytosol -
233 secondary envelopment, often associated with deformation and fragmentation of these
234 organelles (Fig. 5 D). Increased numbers of mature infectious virions were observed budding-
235 off from the cell membrane from 3 dpi onwards, resulting in numerous extracellular mature
236 virions (Fig. 5 E–F). No extracellular virions were observed during the first day of infection,
237 suggesting that extracellular virions noted later were the result of viral replication in both
238 CCB and KF-1 cells and not from inoculated virus.

239 *Figure 5 positioned here*

240 **Cytopathologies / abnormalities in cells at late stages of CyHV-3 infection**

241 The nuclei were often deformed at late infection stages with intra-nuclear vesicles and
242 cytoplasmic invaginations (Fig. 6 A–B). Proliferating intra-nuclear membranes exhibiting
243 thickening and folds were also noted in some cells, extending inwards from the inner nuclear
244 leaflet (Fig. 6 C–D). Occasionally, these nuclear envelope changes were associated with
245 extreme levels of immature capsid primary envelopment (Fig. 6 D). The nuclear membrane in
246 these cells exhibited re-duplication as well as thickening of the inner leaflet (Fig. 6 A & D).
247 On rare occasions apparently disrupted nuclear membranes were observed resulting in loose
248 folds surrounded by putative nucleocapsids or capsid-like structures at various stages of
249 maturation (Fig. 6 E–F). Both CCB and KF-1 cells exhibited nuclear deformations, but
250 greater numbers of capsids were observed in the affected CCB cells.

251 *Figure 6 positioned here*

252 **Discussion**

253 The morphogenesis of CyHV-3 has been described in some detail in cultured cyprinid cells
254 NGF-2 (epithelial-like cells from the fins of coloured carp) (Miwa *et al.* 2007), and in

255 infected carp (Miyazaki *et al.* 2008), however, analysis was only undertaken after 7 dpi. In
256 the current study the sequence of morphological development of the CyHV-3 virion from 1
257 hour through to 7 days post inoculation was examined, together with the cell changes
258 associated with CyHV-3 infection in CCB and KF-1 cells.

259 One of the most notable findings of the investigation, not previously reported, was the
260 presence of capsids within the cell nucleus at various stages of maturation within the first 4
261 hpi. DNA replication of other herpesviruses, as measured using molecular methods, is
262 initiated as early as 3 hpi (Ben-Porat & Veach 1980), and Dishon *et al.* (2007) and Ilouze *et*
263 *al.* (2012b) demonstrated that CyHV-3 DNA synthesis occurs between 4-8 hpi in CyHV-3-
264 infected CCB cells. Capsid assembly does not occur until late mRNAs have been translated
265 and the structural proteins incorporated into the nucleus. Pseudorabies virus (PrV) and
266 channel catfish virus (CCV) capsids, for example, were not detected in the nucleus of
267 infected cells until 4 hpi (Wolf & Darlington 1971; Granzow *et al.* 1997). Transcripts of
268 genes coding proteins involved in CyHV-3 maturation and assembly were not observed by
269 Ilouze *et al.* (2012a) until 4-8 hpi. It has also been shown that there is no expression of
270 structural proteins encoded by ORF149 or 84 (an envelope glycoprotein and capsid-
271 associated protein, respectively) at this stage of the infection (Monaghan *et al.* 2016).
272 Monoclonal antibodies and polyclonal anti-sera to specific CyHV-3 antigens have now been
273 produced (Rosenkranz *et al.* 2008; Aoki *et al.* 2011; Dong *et al.* 2011; Fuchs *et al.* 2014),
274 which may facilitate further studies on CyHV-3 virion replication and maturation in cultured
275 cells. For example it would be possible to confirm the timing of production of capsids using
276 immunofluorescence or immuno-gold TEM by detecting capsid-associated proteins, e.g. with
277 antibodies recognising antigens expressed by ORF84 or ORF92 (Dong *et al.* 2011;
278 Monaghan *et al.* 2016).

279 The characteristics of CyHV-3 capsid assembly have been previously described by Miwa *et*
280 *al.* (2007), including the most abundant type of virion consisting of two concentric circles
281 (the inner containing heterogenous material, thought to be capsomers and scaffolding protein
282 in PrV (Granzow *et al.* 1997)), a second type with an electron dense core and a third type that
283 is empty. By harvesting infected cell cultures during the first day of inoculation it was
284 possible to determine the earlier capsid formation type in our study which was similar to that
285 described for the third type described by Miwa *et al.* (2007). These were similar to those
286 previously described for avian and mammalian herpesviruses by Nii (1991) being mostly
287 empty with no electron dense core or toroid in appearance (), and thus were likely to lack
288 DNA at this stage. This is supported by the absence of more mature virions in the cells at this
289 early stage of infection.

290 Due to the low MOI used in the study, i.e. 0.01–0.02, many cells were uninfected at this early
291 stage (≤ 4 hpi). However, where cells were infected, virus particles were predominantly
292 observed within the nucleus. Due to the short window for virus replication post-inoculation in
293 cells harvested at 4 hpi, this study confirms that these phases of virion formation, also
294 reported by Miwa *et al.* (2007), occurred within those 4 hours of infection. Therefore, like
295 other herpesviruses, absorption of infectious virus particles to the cell, translocation of
296 capsids to the nucleus and subsequent initiation of capsid assembly of CyHV-3 appears to be
297 rapid. In mammalian herpesvirus infection experiments, shifting of PrV infected cells from
298 non-permissive to permissive temperatures resulted in virion attachment to the cell membrane
299 within 1 min and intracellular importation of virions after only 5 min (Granzow *et al.* 1997).
300 Imported PrV nucleocapsids are found in close proximity to microtubules, and had
301 sometimes already docked at the nuclear pore within 30 min (Granzow *et al.* 1997; Kaelin *et*
302 *al.* 2000). This was not observed in the current study thus may have been missed as cells

303 were only harvested from 1 hpi or could possibly be due to the low MOI, which limited the
304 infection to a relatively small proportion of cells, although electron dense capsids were found
305 at the nuclear pore of some cells later in the experiment. Although viropexis via coated pits
306 has been observed in the cell membrane in early infection stages of PrV (Granzow *et al.*
307 1997), Brogden *et al.* (2015) recently provided evidence to suggest that CyHV-3 infection of
308 CCB cells is facilitated via lipid rafts. To determine this at the ultrastructural level,
309 temperature manipulation of CyHV-3 inoculated cells would have to be performed using a
310 higher MOI, similar to the studies carried out for PrV and HSV-1 (Granzow *et al.* 1997;
311 Klupp *et al.* 2000; Nicola *et al.* 2003; Abaitua *et al.* 2012). Nonetheless, coated pits were
312 observed in infected cells in the current study in close proximity to possible naked
313 nucleocapsids (not shown). These may have been migrating towards the nuclear pores,
314 although no microtubules were observed near these. Interestingly, electron dense and electron
315 lucent virus-like particles were observed in linear arrays, some in close proximity to the
316 nuclear envelope within the first 4 hpi. These resembled capsids and were observed at later
317 stages of infection in cells with disrupted nuclei (7 dpi), but the lack of electron density of
318 some of these structures suggests that no DNA was present to be released at the nuclear pore,
319 or perhaps had already been released.

320 As mentioned above, the capsids observed within the nucleus during the first day of infection
321 exhibited all 3 stages of maturation, similar to findings for other herpesviruses (Nii *et al.*
322 1968; Wolf & Darlington 1971; Nii 1991; Granzow *et al.* 1997). In addition to this, primary
323 envelopment of nucleocapsids was also observed during the first day of infection, with no
324 envelopment observed within the cytoplasm. Despite using a non-synchronised infection
325 model in the current study, all infected cells analysed during the first day after inoculation
326 were infected for < 1 dpi, suggesting that an eclipse stage of the infection was still ensuing, as

327 mature extracellular infectious enveloped virions were absent until > 1 dpi (i.e. there was no
328 production of infectious particles evident (Flint *et al.* 2009)). However, secondary
329 envelopment was reported from 12-14 hpi with mammalian herpesviruses PrV and HSV-1
330 (Mettenleiter, 2004), and nucleocapsids could be seen budding within intracytoplasmic
331 vesicles by 1 dpi with CyHV-3 in the current study.

332 Within the first day of infection cytopathic changes were observed including nuclear
333 hypertrophy and margination of chromatin. This is similar, although not as rapid as the
334 chromatin margination and initiation of syncytia reported after only 2 hpi in cells infected
335 with another member of the *Alloherpesviridae*, CCV (Wolf & Darlington 1971). This is not
336 surprising as infectious progeny virus can be isolated from CCV infected catfish after only 1
337 dpi (Kancharla & Hanson 1996) compared to the lag time of CyHV-3 infected carp (i.e. >
338 3dpi from blood leukocytes, Matras *et al.*, 2012). This may correspond to differences in viral
339 replication kinetics between different alloherpesviruses, which unlike members of the
340 *Herpesviridae*, express different optimal temperature ranges between fish species (Hansen *et*
341 *al.* 2011).

342 The formation of paracrystalline-like arrays of intra-nuclear capsids had previously been
343 reported within the nucleus of infected carp gill epithelial cells (Hedrick *et al.* 2000), and in
344 the cytoplasm of a more recently developed koi caudal fin cell line (KCF-1) (Dong *et al.*
345 2011), and this study revealed that this occurs within just 4 hpi in both CCB and KF-1 cells..
346 These arrays are typical of herpesvirus infected cells (Nii *et al.* 1968; Granzow *et al.* 1997).
347 These have been described as pseudocrystals in PrV infected cells, which are hypothesised to
348 dissolve during replication and release individual capsids as they are not found in necrotic
349 cells following replication (Granzow *et al.* 1997). The current study supports this as these

350 capsid formations were no longer observed after 3 dpi, despite being found in a relatively
351 large number of cells prior to this.

352 In contrast to the rapid production of progeny virus of the alloherpesvirus, CCV within 10-12
353 hpi (Wolf & Darlington 1971), release of extracellular infectious virions appears much
354 slower for CyHV-3 and other herpesviruses (i.e. 3-5 dpi) as shown from their growth curves
355 (Ahlqvist *et al.* 2005; Dishon *et al.* 2007; Costes *et al.* 2008; 2009; Dong *et al.* 2011).
356 Although infectious titre (TCID₅₀) was not measured at each time point in the current study,
357 an increase in production of infectious secondary enveloped virions was observed after 3 dpi
358 by ultrastructural analysis. Dishon *et al.* (2007) also reported that 3-7 days are required for
359 progeny virus to be released from CyHV-3 infected CCB cells at the permissive temperature
360 as measured by qPCR. Later in the infection all stages of virus morphogenesis could be
361 observed and the size of capsids, nucleocapsids, primary enveloped and secondary enveloped
362 virions were in agreement with other TEM studies on CyHV-3 and other alloherpesviruses
363 (Wolf & Darlington 1971; Hedrick *et al.* 2000; 2005; Miwa *et al.* 2007; Miyazaki *et al.*
364 2008). In a recent study, there was elevated expression and abundance of capsid-associated
365 protein after 1 dpi compared to envelope glycoprotein (Monaghan *et al.* 2016). This
366 corresponds with fewer secondary enveloped (mature) virions, i.e. containing envelope
367 glycoproteins, during early infection stages, compared to abundant non-enveloped capsids
368 and nucleocapsids observed throughout the infection. With other herpesviruses, aberrant
369 particles are able to leave the cell through exocytosis (Granzow *et al.* 1997), thus they may
370 increase the production of non-infectious particles. Production of infectious CyHV-3 particles
371 in cell culture may be similar to other herpesviruses for which only ~100 virus particles may
372 be infectious out of a total of ~10⁴-10⁵ particles (Ginsberg 1988). More extracellular virions
373 appeared to be present in the KF-1 cells compared to the CCB cells, however, the presence of

374 enveloped virions within intracytoplasmic vesicles or budding off from the trans-golgi
375 network (TGN) appeared more prominent in CCB cells. These differences may have
376 implications with regards to the production of high titre virus stocks, as KF-1 cells appeared
377 more prone to lysis (Pers obs.) and thus reduced their potential for producing mature virus
378 particles. For example, after 5-7 dpi, aggregates of extracellular mature enveloped virus
379 particles were apparent in the KF-1 cells, which were likely defective. Further studies using
380 immuno-labelling methods for tegument proteins such as ORF62 (Aoki *et al.* 2011) or
381 membrane proteins ORF81 and ORF149 (Rosenkranz *et al.* 2008; Fuchs *et al.* 2014) could
382 elucidate more definitively the tegumentation and envelopment / de-envelopment processes
383 of CyHV-3 morphogenesis.

384

385 Lamellar bodies were observed in the cytoplasm of both infected and control cells,
386 reminiscent of lipofuscin, a change associated with cell aging. These aging cells are likely to
387 have been passaged on to the subcultured monolayers inoculated in the trial, and the lamellar
388 bodies should therefore not be considered as a pathology related with herpesvirus infection.
389 However, a number of nuclear deformations were observed after 5–7 dpi, that were not
390 observed in non-infected control cells, thus were likely to be associated with elevated virus
391 production and infection and not the senescence of old cells.

392 Miwa *et al.* (2007) also commented on the finding of compartment-like structures in CyHV-3
393 infected NGF-2 cell nuclei after 7 dpi, but without specific details. A high competition
394 between nucleocapsids for budding, via the perinuclear envelope and intracytoplasmic
395 vesicles of the TGN observed in the current study, may have contributed not only to these
396 irregular formations found within the nuclear envelope, but also other deformed organelles
397 and the formation of syncytia. Miyazaki *et al.* (2008) reported on the degeneration of

398 organelles during later stages of CyHV-3 infection in carp cells. The re-duplication of the
399 nuclear envelope, intra-nuclear folds and incorporated vesicles may occur in herpesvirus
400 infected cells through the accumulation of virus-derived antigens within the cisternae, partly
401 due to virions acquiring the inner nuclear envelope during primary envelopment (Miyazaki *et*
402 *al.* 2008). Similar formations are found in alphaherpesviruses, where primary enveloped
403 virions accumulate in the perinuclear region in the absence of proteins that are required for
404 successful egress and further maturation (Granzow *et al.* 2004). This can result in thickening
405 (Ghadially 1997) leading to nuclear envelope proliferations, fusions and subsequent abnormal
406 concentric lamellar structures (Nii *et al.* 1968). These are characteristic cytopathologies
407 observed in CyHV-3 and other herpesviruses (Nii *et al.* 1968; Wolf & Darlington 1971; Nii
408 1991; Ghadially 1997; Miwa *et al.* 2007). Disrupted nuclei in the current study contained not
409 only nuclear envelope proliferations in both cell lines, but also occasionally CCB cells
410 contained intra-nuclear vesicles, which were more pronounced at later stages of infection and
411 sometimes resembled those reported in CyHV-3 infected carp cells by Miyazaki *et al.* (2008).
412 In contrast to the results reported by Miwa *et al.* (2007), primary envelopment within the
413 nuclear envelope was observed more often at the later stages of infection. Furthermore, the
414 production of capsidless particles in the perinuclear envelope, possibly intracisternal L-
415 particles, as previously reported for alphaherpesviruses by Granzow *et al.* (2001), may lead to
416 inefficient viral assembly, and also contribute to the production of non-infectious particles
417 following increased viral infection pressure. Formation of syncytia on the other hand, is
418 thought to result from mutations in glycoprotein genes (Pereira 1994), with an extensive
419 production of intracellular mature and immature virus particles, which with CyHV-3 occurred
420 more often in CCB cells than KF-1 cells, probably due to the latter being more prone to lysis.
421 Syncytial formation has previously been described in CyHV-3 infected CCB cells (Adamek
422 *et al.* 2012), which also occurred in the current study with viral particles possibly released

423 gradually through budding instead of cell lysis. As a result of cell lysis there may have been a
424 greater loss of virus from KF-1 cells as non-infectious particles, although budding events at
425 the cell plasma membrane of KF-1 cells was observed. The greater loss of virus particles
426 from KF-1 cells by 7 dpi may also explain why only few particles were observed in the KF-1
427 cell line in the study by Miwa *et al.* (2007).

428 Cytopathic vacuoles have been noted in KHV infected cells after 7 dpi (Miwa *et al.*, 2007),
429 however, by analysing infected cells at different times post-inoculation an increased
430 abundance of these vacuoles was observed between 5–7 dpi compared with earlier time
431 points. These cytopathic vacuoles contained infectious virus particles in CCB cells to a
432 greater extent than KF-1 cells. These may be associated with vacuolation (*i.e.* CPE) at this
433 later stage of the infection, as reported in other studies (Dishon *et al.* 2007), which is possibly
434 as a result of competitive budding processes occurring with increased infectious virus
435 progeny. This can be explained by either fusion of a large number of secretory vesicles, or
436 many virions budding through limited golgi-derived vesicles (Granzow *et al.* 1997), which
437 increases over the course of infection in the presence of greater numbers of mature infectious
438 virions, later resulting in fragmentation and damage in the cytoplasm. However, synchronised
439 infection experiments using higher MOI would be required to determine the actual time at
440 which these formations occur and whether they're due to competitive budding.

441 In conclusion, sequential ultrastructural analysis of CyHV-3 morphogenesis within the first
442 day post-inoculation revealed rapid formation of capsids, including paracrystalline array
443 formation, within the first 4 hpi. Assessment of morphogenic stages from 1 – 7 dpi indicated
444 that by 1 dpi CyHV-3 virions undergo primary and secondary envelopment and virion
445 maturation is complete, but it is not until 3-5 dpi that abundant mature infectious virions are
446 produced. These mature infectious particles bud off via the cell plasma membrane, sometimes

447 in defective aggregates, but often resulting in accumulated infection levels in adjacent cells.
448 Such high infection levels may result in deformations in the cell, such as nuclear envelope
449 reduplication and vast vacuolation and subsequent production of non-infectious, as well as
450 infectious virus particles. In the current study this was evident at the ultrastructural level as
451 abundant non-enveloped nucleocapsids and capsids compared to enveloped particles. KF-1
452 cells appear more prone to lysis, possibly releasing immature particles and non-infectious
453 particles, whereas more virus particles are retained in CCB cells for complete maturation and
454 budding at the cell plasma membrane. This should be taken into account when propagating
455 CyHV-3 in CCB and KF-1 cells for the production of infectious virus.

456

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462

463 **References**

464 Abaitua F., Hollinshead M., Bolstad M., Crump C.M. & O'Hare P. (2012) A nuclear
465 localization signal in the herpesvirus protein VP1-2 is essential for infection via capsid
466 routing to the nuclear pore. *Journal of Virology* **86**(17), 8998-9014.

467

468 Adamek M., Rakus K.L., Chyb J., Brogden G., Huebner A., Irnazarow I. & Steinhagen D.
469 (2012) Interferon type I responses to virus infections in carp cells: *In vitro* studies on
470 *Cyprinid herpesvirus 3* and *Rhabdovirus carpio* infections. *Fish and Shellfish Immunology*
471 **33**, 482-493.

472

473 Ahlqvist J., Fotheringham J., Akhyani N., Yao K., Fogdell-Hahn A. & Jacobson S. (2005)
474 Differential tropism of human herpesvirus 6 (HHV-6) variants and induction of latency by
475 HHV-6A in oligodendrocytes. *Journal of Neurovirology* **11**, 384-394.
476

477 Aoki T., Hirono I., Kurokawa K., Fukuda H., Nahary R., Eldar A., Davison A.J., Waltzek
478 T.B., Bercovier H. & Hedrick R.P. (2007) Genome sequences of three koi herpesvirus
479 isolates representing the expanding distribution of an emerging disease threatening koi and
480 common carp worldwide. *Journal of Virology* **81**, 5058-5065.
481

482 Aoki T., Takano T., Unajak S., Takagi M., Kim Y.R., Park S.B., Kondo H., Hirono I., Saito-
483 Taki T., Hikima J.-I. & Jung T.S. (2011) Generation of monoclonal antibodies specific for
484 ORF68 of koi herpesvirus. *Comparative Immunology, Microbiology and Infectious Diseases*
485 **34**, 209-216.
486

487 Armitage J., Hewlett N.R., Twigg M., Lewin N.C., Reading A.G., Williams C.F.,
488 Aprahamian M., Way K., Feist W. & Peeler E.J. (2014) Detection of *herpesvirus anguillae*
489 during two mortality investigations of wild European eel in England: implications for fishery
490 management. *Fisheries Management and Ecology* **21**(1), 1-12.
491

492 Ben-Porat T. & Veach R.A. (1980) Origin of replication of the DNA of a herpesvirus
493 (pseudorabies). *Proceedings of the National Academy of Sciences* **77**, 172-175.
494

495 Bergmann S.M., Riechardt M., Fichtner D., Lee P. & Kempter J. (2010) Investigation on the
496 diagnostic sensitivity of molecular tools used for detection of Koi herpesvirus. *Journal of*
497 *Virological Methods* **163**(2), 229-233.
498

499 Brogden G., Adamek M., Proepsting M.J., Ulrich R., Naim H.Y. & Steinhagen D. (2015)
500 Cholesterol-rich lipid rafts play an important role in the Cyprinid herpesvirus 3 replication
501 cycle. *Veterinary Microbiology* **179**, 204-212.
502

503 Costes B., Fournier G., Michel B., Delforge C., Raj V.S., Dewals B., Gillet L., Drion P.,
504 Body A., Schynts F., Lieffrig F. & Vanderplasschen A. (2008) Cloning of the Koi
505 herpesvirus genome as an infectious bacterial artificial chromosome demonstrates that
506 disruption of the thymidine kinase locus induces partial attenuation in *Cyprinus carpio koi*.
507 *Journal of Virology* **82**(10), 4955-4964.
508

509 Costes B., Raj V.S., Michel B., Fournier G., Thirion M., Gillet L., Mast J., Lieffrig F.,
510 Bremont M. & Vanderplasschen A. (2009). The major portal of entry of koi herpesvirus in
511 *Cyprinus carpio* is the skin. *Journal of Virology* **83**(7), 2819-30.
512

513 Davison A.J., Eberle R., Ehlers B., Hayward G.S., McGeoch D.J., Minson A.C., Pellet P.E.,
514 Roizman B., Studdert M.J. & Thiry E. (2009) The order Herpesvirales. *Archives of Virology*
515 **154**, 171-177.

516
517 Dishon A., Davidovich M., Ilouze M. & Kotler M. (2007) Persistence of *Cyprinid*
518 *herpesvirus 3* in infected cultured carp cells. *Journal of Virology* **81**(9), 4828-4836.
519
520 Dong C., Weng S., Li W., Li X., Yi Y., Liang Q. & He J. (2011) Characterisation of a new
521 cell line from caudal fin of koi, *Cyprinus carpio koi*, and first isolation of cyprinid
522 herpesvirus 3 in China. *Virus Research* **161**, 140-149.
523
524 Eide K.E., Miller-Morgan T., Heidel J.R., Kent M.L., Bildfell R.J., LaPatra S., Watson G. &
525 Jin L. (2011) Investigation of koi herpesvirus latency in koi. *Journal of Virology* **85**(10),
526 4954-4962.
527
528 Flint S.J., Enquist L.W., Racaniello V.R. & Skalka A.M. (2009). Principles of Virology,
529 Volume II: Pathogenesis and Control, Chapter 4: Immune Defenses, 3rd Edition, ASM Press,
530 pp. 87-132.
531
532 Fuchs W., Granzow H., Dauber M., Fichtner D. & Mettenleiter T.C. (2014). Identification of
533 structural proteins of koi herpesvirus. *Archives of Virology* **159**(12), 3257-3268.
534
535 Fuchs W., Veits J., Helferich D., Granzow H., Teifke J.P. & Mettenleiter T.C. (2007)
536 Molecular biology of avian infectious laryngotracheitis virus. *Veterinary Research* **38**, 261-
537 279.
538
539 Ghadially F. (1997). Mitochondria. *In* Ultrastructural pathology of the cell and matrix, Fourth
540 Edition, (Ghadially F. Ed.) Butterworth-Heinemann **1**: 195-328.
541
542 Gilad O., Yun S., Adkison M.A., Way K., Willits N.H., Bercovier H. & Hedrick R.P. (2003)
543 Molecular comparison of isolates of an emerging fish pathogen, koi herpesvirus, and the
544 effect of water temperature on mortality of experimentally infected koi. *Journal of General*
545 *Virology* **84**, 2661-2668.
546
547 Gilad O., Yun S., Zagmutt-Vergara F.J., Leutenegger C.M., Bercovier H. & Hedrick R.P.
548 (2004) Concentrations of a Koi herpesvirus (KHV) in tissues of experimentally infected
549 *Cyprinus carpio koi* as assessed by real-time TaqMan PCR. *Diseases of Aquatic Organisms*
550 **60**, 179-187.
551
552 Ginsberg H.S. (1988) Herpesviruses. *In* Virology, (Dulbecco R. and Ginsberg H.S. Eds.),
553 Second Edition, J. B. Lippincott Co. Chapter 53, pp. 161-177.
554
555 Goodwin A.E., Merry G.E. & Sadler J. (2006) Detection of the herpesviral hematopoietic
556 necrosis disease agent (Cyprinid herpesvirus 2) in moribund and healthy goldfish: validation
557 of a quantitative PCR diagnostic method. *Diseases of Aquatic Organisms* **69**(2-3), 137-143.
558

559 Goodwin A.E., Sadler J., Merry G.E. & Marecaux E.N. (2009) Herpesviral hematopoietic
560 necrosis virus (CyHV-2) infection: case studies from commercial goldfish farms. *Journal of*
561 *Fish Diseases* **32**(3), 271-278.

562

563 Granzow H., Klupp B.G., Fuchs W., Veits J., Osterrieder N. & Mettenleiter T.C. (2001)
564 Egress of alphaherpesviruses: Comparative ultrastructural study. *Journal of Virology* **75**(8),
565 3675-3684.

566

567 Granzow H., Klupp B.G. & Mettenleiter T.C. (2004) The pseudorabies virus US3 protein is a
568 component of primary and of mature virions. *Journal of Virology* **78**, 1314-1323.

569

570 Granzow H., Weiland F., Jöns A., Klupp B.G., Karger A. & Mettenleiter T.C. (1997)
571 Ultrastructural analysis of the replication cycle of Pseudorabies virus in cell culture: A
572 reassessment. *Journal of Virology* **71**(3), 2072-2082.

573

574 Hansen L., Dishon A. & Kotler M. (2011) Herpesviruses that infected fish. *Viruses* **3**, 2160-
575 2191.

576

577 Hedrick R.P., Gilad O., Yun S.C., McDowell T.S., Waltzek T.B., Kelley G.O. & Adkison
578 M.A. (2005) Initial isolation and characterization of a herpes-like virus (KHV) from koi and
579 common carp. *Bulletin of the Fisheries Research Agency* **2**, 1-7.

580

581 Hedrick R.P., Gilad O., Yun S., Spangenberg J.V., Marty G.D., Nordhausen R.W., Kebus
582 M.J., Bercovier H. & Eldar A. (2000) A herpesvirus associated with mass mortality of
583 juvenile and adult koi, a strain of common carp. *Journal of Aquatic Animal Health* **12**, 44-57.

584

585 Ilouze M., Dishon A. & Kotler M. (2012a) Coordinated and sequential transcription of the
586 cyprinid herpesvirus-3 annotated genes. *Virus Research* **169**, 98-106.

587

588 Ilouze M., Dishon A. & Kotler M. (2012b) Down-regulation of the cyprinid herpesvirus-3
589 annotated genes in cultured cells maintained at restrictive high temperature. *Virus Research*
590 **169**, 289-295.

591

592 Kaelin K., Dezélee S., Masse M.J., Bras F. & Flamand A. (2000) The UL25 protein of
593 Pseudorabies virus associates with capsids and localises to the nucleus and to microtubules.
594 *Journal of Virology* **74**(1), 474-482.

595

596 Kancharla S.R. & Hanson L. (1996) Production and shedding of channel catfish virus (CCV)
597 and thymidine kinase negative CCV in immersion exposed channel catfish fingerlings.
598 *Diseases of Aquatic Organisms* **27**, 25-34.

599

600 Kärber G. (1931) Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche.
601 *Naunyn-Schmiedeberg's Archives of Pharmacology* **162**(4), 480-483.

602
603 Klupp B.G., Granzow H. & Mettenleiter T.C. (2000) Primary envelopment of pseudorabies
604 virus at the nuclear membrane requires the UL34 gene product. *Journal of Virology* **74**,
605 10063-10073.
606
607 Klupp B.G., Granzow H., Mundt E. & Mettenleiter T.C. (2001) Pseudorabies virus UL37
608 gene product is involved in secondary envelopment. *Journal of Virology* **75**(19), 8927-8936.
609
610 Lovy J. & Friend S.E. (2014) Cyprinid herpesvirus-2 causing mass mortality in goldfish:
611 applying electron microscopy to histological samples for diagnostic virology. *Diseases of*
612 *Aquatic Organisms* **108**(1), 1-9.
613
614 Matras M., Antychowicz J., Castric J. & Bergmann S.M. (2012) CyHV-3 infection dynamics
615 in common carp (*Cyprinus carpio*) – evaluation of diagnostic methods. *Bulletin of the*
616 *Veterinary Institute in Pulawy* **56**, 127-132.
617
618 Mettenleiter T.C. (2002) Herpesvirus assembly and egress. *Journal of Virology* **76**(4), 1537-
619 1547.
620
621 Mettenleiter T.C. (2004) Budding events in herpesvirus morphogenesis. *Virus Research*
622 **106**(2), 167-180.
623
624 Mettenleiter T.C., Klupp B.G. & Granzow H. (2009) Herpesvirus assembly: An update. *Virus*
625 *Research* **143**(2), 222-234.
626
627 Miwa S., Ito T. & Sano M. (2007) Morphogenesis of koi herpesvirus observed by electron
628 microscopy. *Journal of Fish Diseases* **30**, 715-722.
629
630 Miyazaki T., Kuzuya Y., Yasumoto S., Yasuda M. & Kobayashi T. (2008) Histopathological
631 and ultrastructural features of koi herpesvirus (KHV)-infected carp *Cyprinus carpio*, and the
632 morphology and morphogenesis of KHV. *Diseases of Aquatic Organisms* **80**(1), 1-11.
633
634 Monaghan S.J., Thompson K.D., Adams A. & Bergmann S.M. (2015) Sensitivity of seven
635 PCRs for early detection of koi herpesvirus in experimentally infected carp (*Cyprinus carpio*
636 L.) by lethal and non-lethal sampling methods. *Journal of Fish Diseases* **38**(3), 303-319.
637
638 Monaghan S.J., Thompson K.D., Bron J.E., Bergmann S.M., Jung T.S., Aoki T., Muir K.F.,
639 Dauber M., Reiche S., Chee D., Chong S.M., Chen J. & Adams A. (2016) Expression of
640 immunogenic structural proteins of Cyprinid herpesvirus 3 *in vitro* assessed using
641 immunofluorescence. *Veterinary Research* **47**(1), 8.
642
643 Neukirch M., Böttcher K. & Bunnajirakul S. (1999) Isolation of a virus from koi with altered
644 gills. *Bulletin of the European Association of Fish Pathologists* **19**(5), 221-224.

645
646 Nicola A.V., McEvoy A.M. & Straus S.E. (2003) Roles for endocytosis and low pH in herpes
647 simplex virus entry into HeLa and Chinese hamster ovary cells. *Journal of Virology* **77**,
648 5324-5332.
649
650 Nii S. (1991) Electron microscopic study on the development of herpesviruses. *Journal of*
651 *Electron Microscopy* **41**, 414-423.
652
653 Nii S., Morgan C. & Rose H.M. (1968) Electron microscopy of herpes simplex virus: II.
654 Sequence of development. *Journal of Virology* **2**, 517-536.
655
656 Pääk P., Hussar P., Järveots T. & Paaver T. (2011) Club cells active role in epidermal
657 regeneration after skin hyperplasia of koi carp *Cyprinus carpio*. *AAFL Bioflux* **4**(4), 455-462.
658
659 Pereira L. (1994) Function of glycoprotein B homologues of the family Herpesviridae.
660 *Infectious Agents and Disease* **3**, 9-28.
661
662 Perelberg A., Smirnov M., Hutoran M., Diamant A., Bejerano Y. & Kotler M. (2003)
663 Epidemiological description of a new viral disease afflicting cultured *Cyprinus carpio* in
664 Israel. *The Israeli Journal of Aquaculture – Bamidgeh* **55**(1), 5-12.
665
666 Reed A.N., Izume S., Dolan B.P., LaPatra S., Kent M., Dong J. & Jin L. (2014) Identification
667 of B cells as a major site for Cyprinid herpesvirus 3 latency. *Journal of Virology* **88**(16),
668 9297-9309.
669
670 Ronen A., Perelberg A., Abramowitz J., Hutoran M., Tinman S., Bejerano I., Steinitz M. &
671 Kotler M. (2003) Efficient vaccine against the virus causing a lethal disease in cultured
672 *Cyprinus carpio*. *Vaccine* **21**, 4677-4684.
673
674 Rosenkranz D., Klupp B.G., Teifke J.P., Granzow H., Fichtner D., Mettenleiter T.C. & Fuchs
675 W. (2008). Identification of envelope protein ORF81 of koi herpesvirus. *Journal of General*
676 *Virology* **89**, 896-900.
677
678 Sano N., Sano M., Sano T. & Hondo R. (1992). *Herpesvirus cyprini*: detection of the viral
679 genome by in situ hybridisation. *Journal of Fish Diseases* **15**, 153-162.
680
681 Sano T., Morita N., Shima N. & Akimoto M. (1991) *Herpesvirus cyprini*: lethality and
682 oncogenicity. *Journal of Fish Diseases* **14**, 533-543.
683
684 St-Hilaire S., Beevers N., Way K., Le Deuff R.M., Martin P. & Joiner C. (2005) Reactivation
685 of koi herpesvirus infections in common carp *Cyprinus carpio*. *Diseases of Aquatic*
686 *Organisms* **67**, 15-23.
687

688 Sunarto A., McColl K.A., Crane M.S.J., Schat K.A., Slobedman B., Barnes A.C. & Walker
689 P.J. (2014) Characteristics of Cyprinid herpesvirus 3 in different phases of infection:
690 Implications for disease transmission and control. *Virus Research* **188**, 45-53.
691

692 Tu C., Lu Y.P., Hsieh C.Y., Huang S.M., Chang S.K. & Chen M.M. (2014) Production of
693 monoclonal antibody against ORF72 of koi herpesvirus isolated in Taiwan. *Folia*
694 *Microbiologia (Praha)* **59**(2), 159-165.
695

696 Van Beurden S.J., Leroy B., Wattiez R., Haenen O.L.M., Boeren S., Vervoort J.M., Peeters
697 B.P.H., Rottier P.J.M., Engelsma M.Y. & Vanderplasshen A.F. (2011). Identification and
698 localization of the structural proteins of anguillid herpesvirus 1. *Veterinary Research* **42**, 105-
699 120.
700

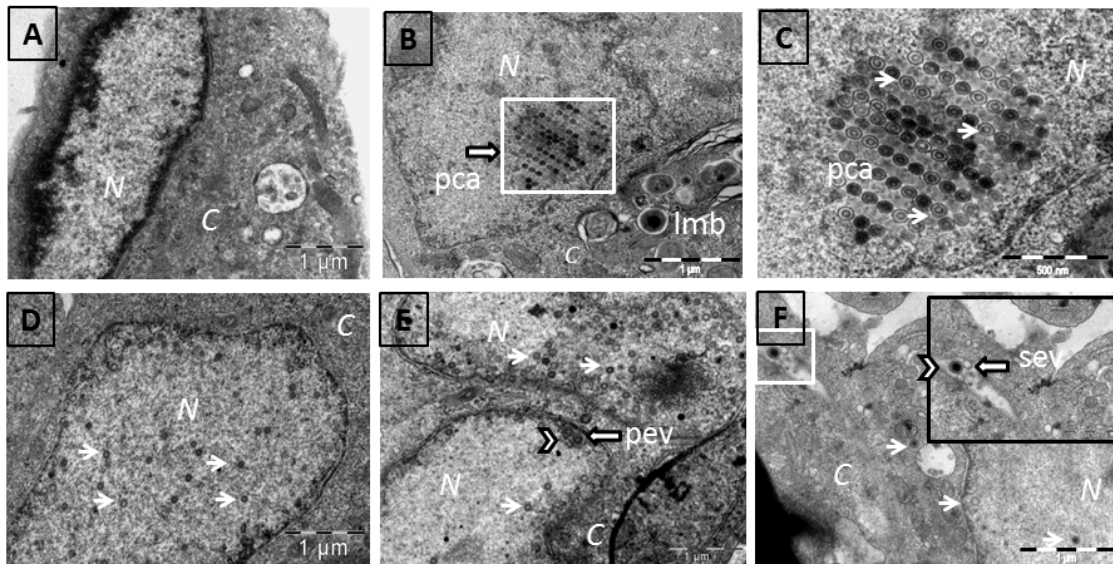
701 Voronin Y., Holte S., Overbaugh J. & Emerman M. (2009) Genetic drift of HIV populations
702 in culture. *PloS Genetics* **5**(3), e1000431.
703

704 Vrancken R., Boutier M., Ronsmans M., Reschner A., Leclipteux T., Lieffrig F., Collard A.,
705 Mélard C., Wera S., Neyts J., Goris N. & Vanderplasschen A. (2013) Laboratory validation
706 of a lateral flow device for the detection of CyHV-3 antigens in gill swabs. *Journal of*
707 *Virological Methods* **193**, 679-682.
708

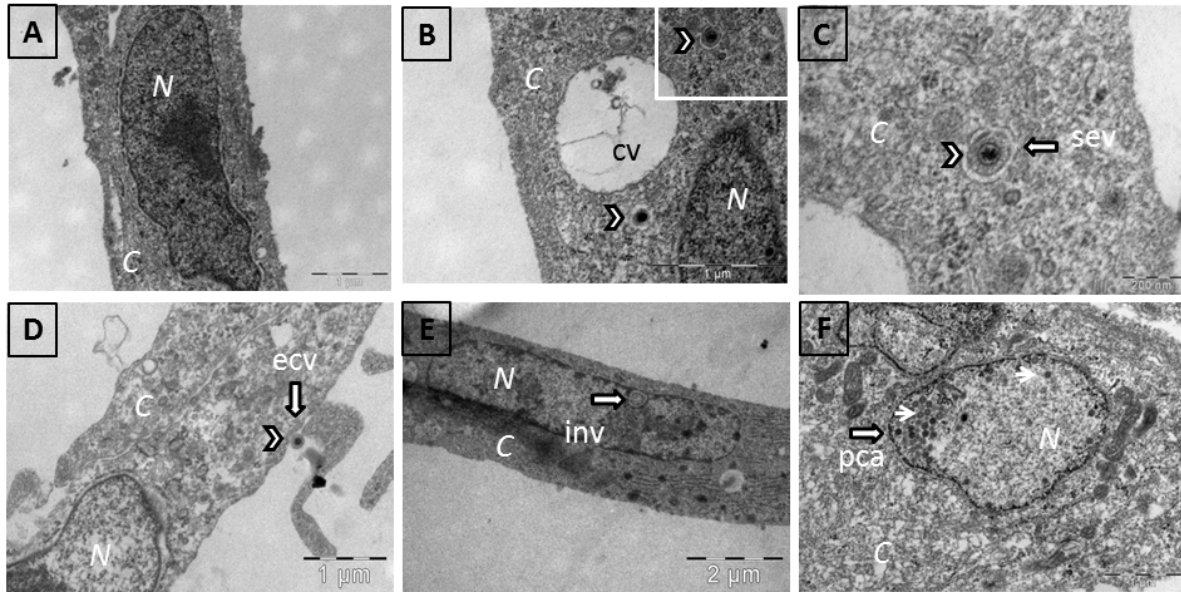
709 Waltzek T.B., Kelley G.O., Alfaro M.E., Kurobe T., Davison A.J. & Hedrick R.P. (2009)
710 Phylogenetic relationships in the family Alloherpesviridae. *Diseases of Aquatic Organisms*
711 **84**, 179-194.
712

713 Waltzek T.B., Kelley G.O., Stone D.M., Way K., Hanson L., Fukuda H., Hirano I., Aoki T.,
714 Davison A.J. & Hedrick R.P. (2005) Koi herpesvirus represents a third cyprinid herpesvirus
715 (CyHV-3) in the family Herpesviridae. *Journal of General Virology* **86**, 1659-1667.
716

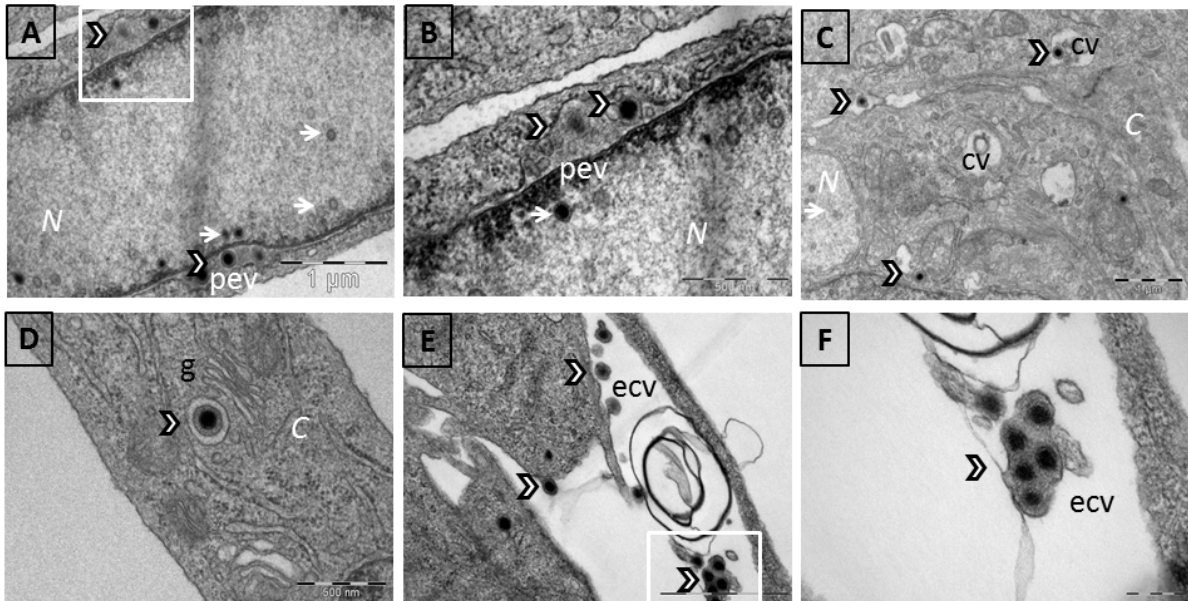
717 Wolf K. & Darlington R.W. (1971) Channel catfish virus: a new herpesvirus of ictalurid fish.
718 *Journal of Virology* **8**, 525-533.
719



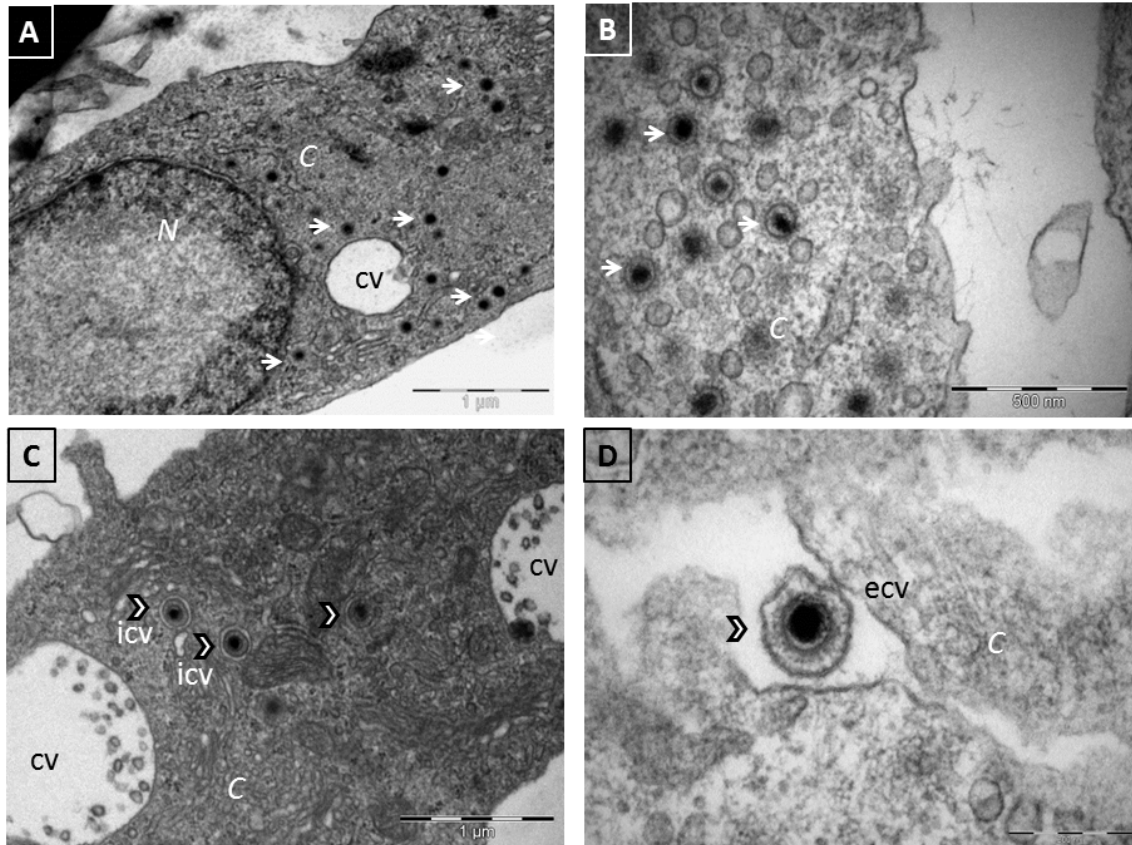
721
 722 **Figure 1. TEM micrographs of CCB and KF-1 cells infected with Koi herpesvirus 1–24 hpi.** (A)
 723 Uninfected CCB cell; (B) Infected KF-1 cells 4 hpi with paracrystalline formation of capsids in the
 724 nucleus; (C) High mag. of capsids shown in square of B; (D) Nucleus of infected CCB cells; (E) 2
 725 infected CCB cells in close proximity 4 hpi. Note the accumulation of capsids towards the periphery
 726 of the diffuse cell nucleus with varying degree of maturation. Primary enveloped virions can also be
 727 observed; (F) Infected CCB cells after 1 dpi showing the formation of capsids within the nucleus and
 728 cytoplasm and mature virions that have acquired a secondary envelope in the cytoplasm
 729 (Magnified in box). *N* = Nucleus; *C* = Cytoplasm; *pca* = Paracrystalline array; *lmb* = lamellar bodies;
 730 Arrow = capsids; Arrow heads = Enveloped virions; *pev* = Primary enveloped virions; *sev* = Secondary
 731 enveloped virions.



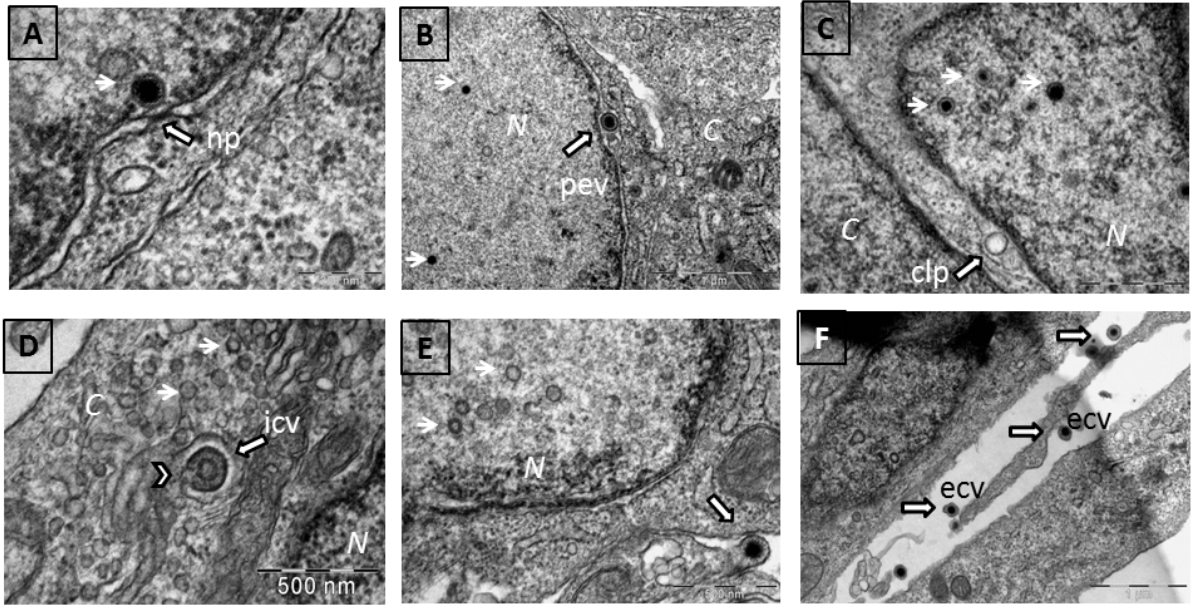
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 733 **Figure 2. TEM micrographs of CCB and KF-1 cells infected with Koi herpesvirus 1–3 dpi.** (A)
 734 CCB cells inoculated with KHV but uninfected; (B) Infected CCB cells with mature secondary
 735 enveloped virions; (C) High magnification of mature secondary enveloped virion (shown in
 736 square of B) within a vesicle in the cytoplasm; (D) Severely damaged KF-1 cells with budding
 737 infectious enveloped mature virion on cell membrane; (E) CCB cells with KHV showing
 738 intranuclear vesicles; (F) Infected KF-1 cell with paracrystalline array of capsids formed
 739 within the nucleus. *N* = Nucleus; *C* = Cytoplasm; *pca* = Paracrystalline array; Arrow = capsids;
 740 Arrow heads = Enveloped virions; *cv* = Cytopathic vacuole; *inv* = Intranuclear vesicle; *ecv* =
 741 Extracellular virion.



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 743 **Figure 3. TEM micrographs of CCB and KF-1 cells infected with Koi herpesvirus 5–7 dpi.** (A)
 744 Infected CCB cells containing capsids at various maturational stages in the nucleus and
 745 nucleocapsids budding through the nuclear envelope and acquiring a primary envelope. (B)
 746 High magnification of square in D showing 3 primary enveloped virions within the nuclear
 747 envelope while smaller immature and mature capsids remain in the nucleus. (C) Low
 748 magnification of infected CCB cells, 5 dpi, with a number of cytopathic vacuoles and
 749 secondary enveloped mature virions budding from various membranous organelles. (D)
 750 Mature secondary enveloped virion within the cell cytoplasm, budding from golgi apparatus
 751 derived vesicle in CCB cells, 7 dpi. (E) Infected KF-1 cells, 5 dpi with many mature secondary
 752 enveloped virions budding through the cell membrane. (F) High mag. of square in C showing
 753 aggregates of extracellular, mature, infectious secondary enveloped virions. *N* = Nucleus; *C* =
 754 Cytoplasm; Arrow = capsids; Arrow heads = Enveloped virions; pev = Primary enveloped
 755 virion; cv = Cytopathic vacuole; g = Golgi body; ecv = Extracellular virion.



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 757 **Figure 4. TEM micrographs of CCB and KF-1 cells infected with Koi herpesvirus 5–7 dpi.** (A)
 758 CCB cells with KHV – Many cytoplasmic nucleocapsids in close vicinity to vacuoles; (B)
 759 Clusters of naked/unenveloped capsids in the cytoplasm of infected CCB cells after 7 dpi.
 760 Note the protruding core into the cytoplasm; (C) Infected KF-1 cells, 7 dpi, containing
 761 secondary enveloped mature virions within cytoplasmic vesicles. Large cytopathic vacuoles
 762 are also evident containing cell debris; (D) High magnification of mature secondary
 763 enveloped virion in infected CCB cells, 5 dpi – note the defined layers: glycoprotein envelope
 764 with surface projections, tegument layer, capsid and electron dense core. *N* = Nucleus; *C* =
 765 Cytoplasm; Arrow = capsids; Arrow heads = Enveloped virions; cv = Cytopathic vacuole; icv =
 766 Intracytoplasmic vesicle; g = Golgi body; ecv = Extracellular virion.



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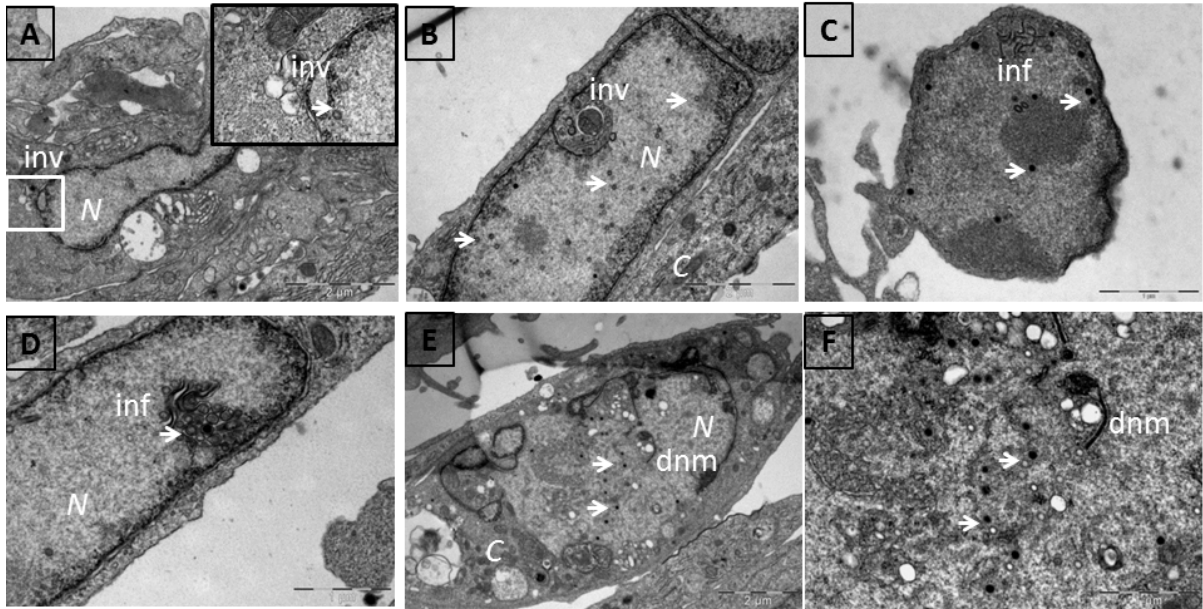
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Figure 5. TEM micrographs of CyHV-3 virion morphogenesis and egress at membranous compartments. (A) Infected CCB cells showing a nucleocapsid at a nuclear pore; (B) Infected CCB cells with KHV with electron dense nucleocapsid within the nuclear envelope; (C) Infected CCB cell with electron-lucent nucleocapsid (intracisternal L-particles) within the nuclear envelope; (D) Infected CCB cell containing a tegumented nucleocapsid budding in an intracytoplasmic vesicle; (E) Infected CCB cell showing enveloped virion in the process of budding-off from the cell membrane; (F) Infected CCB cells with many secondary enveloped mature infectious virions in the extracellular space at later stages of infection. *N* = Nucleus; *C* = Cytoplasm; Arrow = capsids; Arrow heads = Enveloped virions; np = nuclear pore; pev = primary enveloped virion; clp = capsidless intracisternal L-particle; icv = intracytoplasmic vesicle; ecv = Extracellular virion.



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780 **Figure 6. TEM micrographs of CCB and KF-1 cells infected with Koi herpesvirus 5–7 dpi**
 781 **showing various cytopathologies.**

782 (A) Infected KF-1 cells containing large vacuoles and large vesicle within the nuclear
 783 membrane; (B) CCB cells exhibiting a large intranuclear vesicle protruding inwards from the
 784 nuclear membrane containing putative disrupted virus particles after 7 dpi; (C) Infected KF-1
 785 cells containing intranuclear folds; (D) Infected CCB cell after 7 dpi exhibiting proliferation of
 786 the inner membrane of the nuclear envelope surrounded by immature virus particles at
 787 various stages of maturation; (E) Disrupted nucleus of infected CCB cell exhibiting loose
 788 disrupted nuclear membrane with electron dense and electron lucent capsid-like structures
 789 present; (F) Higher mag. of E showing naked electron dense and empty capsid-like structures
 790 released from the disrupted nucleus. *N* = Nucleus; *C* = Cytoplasm; Arrow = capsids; *inv* =
 791 Intranuclear vesicle; *inf* = Intranuclear folds; *dnm* = Disrupted nuclear membrane.

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