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Cyprinid herpesvirus-3 (CyHV-3) disturbs osmotic balance in carp (*Cyprinus carpio* L.)—A potential cause of mortality

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ABSTRACT

Cyprinid herpesvirus-3 (CyHV-3) causes a fatal disease in carp (*Cyprinus carpio*) and its ornamental koi varieties which seriously affects production and trade of this fish species globally. Up to now, the pathophysiology of this disease remains unclear. Affected individuals develop most prominent lesions in gills, skin and kidney, in tissues which are involved in the osmotic regulation of freshwater teleosts. Therefore, here serum and urine electrolyte levels were examined during the course of an experimental infection of carp with CyHV-3. In infected carp an interstitial nephritis with a progressive deterioration of nephric tubules developed, which was paralleled by elevated electrolyte losses, mainly Na^+ in the urine. The urine/plasma ratio for Na^+ increased from 0.03 in uninfected carp to 0.43–0.83 in carp under CyHV-3 infection, while concentration of divalent ions were not significantly changed. These electrolyte losses could not be compensated since plasma osmolality and Na^+ concentration dropped significantly in CyHV-3 infected carp. This was most probably caused by the progressive deterioration of the branchial epithelium, which in teleosts plays a prominent role in osmoregulation, and which was seen concomitantly with decreasing electrolyte levels in the serum of carp under CyHV-3 infection.

Immediately after infection with CyHV-3, by day 2 post exposure, affected carp showed severe anaemia and prominent leucocytosis indicating the development of an acute inflammation, which could intensify the observed hydro-mineral imbalances.

The data presented here show that an infection with CyHV-3 induces an acute inflammation and a severe dysfunction of osmoregulation in affected carp or koi, which may lead to death in particular in the case of acute disease progression.

1. Introduction

The *Cyprinid herpesvirus*-3 (CyHV-3), also known as koi herpes virus (KHV) (Waltzek et al., 2005), is considered to be one of the most dangerous carp pathogens. CyHV-3 infection in common carp *Cyprinus carpio* L. and its ornamental koi varieties, can induce a severe disease known as koi herpesvirus disease (KHVD), which is associated with morbidity and mass mortality in farm and wild populations of carp and has become a serious threat to carp aquaculture (Bretzinger et al., 1999; Walster, 1999; Hedrick et al., 2000; Garver et al., 2010).

During a KHVD outbreak, clinical symptoms such as apathy, skin and gill necrosis can be observed (Hedrick et al., 2000). A histopathological analysis of the diseased gills showed hyperplasia, destruction and fusion of secondary branchial lamella (Hedrick et al., 2000; Pikarsky et al., 2004). In internal organs the histopathological signs

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of the disease included interstitial nephritis, spleenitis and enteritis (Hedrick et al., 2000; Miyazaki et al., 2008; Pikarsky et al., 2004).

The pathophysiology of this disease, however, remains largely unclear. Most prominent lesions occur in gills, skin and the kidney. In particular gills and kidney are involved in osmotic processes in fish (Evans et al., 2005). The gills of fishes with their large surface area, thin branchial epithelium and high vascularisation are not only specialised for gas exchange, but also remain an excellent site for osmotic movement of ions and water (Evans et al., 2005; Evans, 2011). When diffusion of oxygen and carbon dioxide across gills increases during exercise or other challenges, ion and water diffusion increases as well and specialised ionocytes in the fish gills are involved in active processes of osmotic, ionic and nitrogenous waste balances (Evans et al., 2005; Gonzalez, 2011). Due to the steep osmotic and ionic gradients between body fluids and the freshwater environment, freshwater teleosts like carp are continuously subject to water gain and ion loss by passive diffusion across the gill tissue. In order to cope with living in a dilute environment, the kidneys of freshwater teleosts produce plentiful amounts of dilute urine. This involves high rates of ultrafiltration, ion reabsorption from the primary urine in the nephric duct and active extraction of salts from external water across the gill epithelium (Hwang, 2011).

It has been hypothesised whether tissue damage seen in gills, gut or kidney of carp suffering from KHVD, may lead to an impairment of osmoregulation in these individuals, resulting in lower serum osmolality and this might reinforce the clinical signs of the disease (Hedrick et al., 2000; Miwa et al., 2014). The present study therefore focuses on electrolyte levels in carp serum and urine, on haematological parameters as well as on histological changes of gills and kidney in order to support this hypothesis.

2. Materials and methods

Animal experiments for this study were approved by the Lower Saxony State Office for Consumer Protection and Food Safety (reference number 04856) and performed using internationally accepted veterinary standards and federal guidelines.

2.1. Fish

Clinically healthy, parasite and virus free sibling common carp, *C. carpio* L., from a single crossing (R8 × R3, Wageningen University, Netherlands) were used throughout the study. Carp were raised and kept in aerated and filtered recirculated tap water at 20–23 °C. The water quality was kept stable by using aquarium recirculation filters (Eheim Germany) and partial water exchange every 3–4 days. For this study, approximately nine month old carp (70.4 ± 34.2 g, 14.0 ± 2.0 cm) were used. Carp were anaesthetized and killed by immersion into a solution of MS 222 (Sigma-Aldrich, Germany) at a concentration of 0.15 mg l^{-1} and 0.5 mg l^{-1}, respectively.

2.2. Cells and virus

For the infection experiment, tissue culture derived CyHV-3 suspension of the isolate KHV-I was used, which was originally isolated by Hedrick et al. (2000) and was kindly provided by the Friedrich-Loeffler Institute, Insel Riems, Germany in a culture at passage no. 6. The KHV-I isolate was propagated on *C. carpio* brain (CCB) cells (Neukirch et al., 1999). CCB cells were cultured in minimum essential medium (MEM) with Earle's salts (Applichem, Germany) supplemented with 2 mMol l^{-1} glutamine (PAA, Germany), 0.35% D(+)-glucose (Applichem, Germany), 10% fetal calf serum (FCS; Biochrom, Germany) and 7.96% Non-Essential Amino Acids, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (PAA; Germany). The cells were incubated at 25 °C in a humid atmosphere containing 2% CO_2. For virus infection, one day old CCB monolayers were inoculated with CyHV-3 virus and observed daily for the occurrence of cytopathic effects (CPE). Infected CCB cultures were incubated until day 5 pi, when a CPE was clearly visible. Then the cells were mechanically lysed, the medium was harvested from infected cells including the virus and used for infection of the fish. The infectivity of the virus was determined in terms of 50% of the tissue culture infective dose ($TCID_{50}$ assay), which was performed in 96 well plates with CCB monolayers according to the method described by Bonin (1973). For the virus used in the infection experiment, the tissue culture infective dose was estimated at 5000 $TCID_{50}$ ml^{-1}.

2.3. Virus infection

Carp ($n = 56$) were infected with the CyHV-3 virus by peroral application of 0.5 ml of a virus suspension, containing a dose of 5000 $TCID_{50}$ ml^{-1} in 7 consecutive experiments (Table 1). For infection, carp were anaesthetized with MS 222 as described above and the virus solution was applied orally using a buttoned cannula fitted to a 2 ml disposable syringe (Omnilab, Germany). As controls, in each experiment, two fish (in total 14 individuals) were given the same amount of phosphate buffered solution (PBS). After application of the virus or PBS and recovery from anaesthesia, carp were kept in groups of 4 in sterilised glass aquariums (20 l) in tap water at 23 °C for 2–14 days. Control carp were kept in separate aquaria isolated from the CyHV-3 infected carp to avoid

Table 1
Overview of CyHV-3 infection experiments and time points of sample collection.

Infection experiment	Day 2[a]	Day 5[a]	Day 8[a]	Day 13[a]
1	2		2	1 (+2 died)
2	2	1	2	(+2 died)
3	3		2	(+2 died)
4		5	2	2
5	3			1
6		4	2	
7		4	4	6
Total	10	14	14	10 (+6 died)

[a] Days post peroral treatment with CyHV-3.