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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. Viruses

Viruses are genetic elements or nucleoprotein particles (Rosypal, 1997) that can exist in two forms. These forms are distinguished according to their presence inside or outside cells. The first form is an extracellular form called as virion. A virion cannot replicate itself, but it can exist independently outside of the cells. A virion possesses one or more molecules of nucleic acid (DNA or RNA) and protein capsid surrounding nucleic acid. Some viruses can possess also outer envelope as a rest of cytoplasmatic membrane from host cell. These viruses are named as "enveloped viruses"(Madigan and Martinko, 2006; Carter and Saunders, 2013).

Second form is an intracellular form, which is typical for virus existence. Although the extracellular particle (virion) is metabolically inert, the activity of intracellular particle is strongly dependent on structural and metabolic components of host cell because the virus genome has not any genes for rRNA (ribosomal RNA) and tRNA (transfer RNA) and it also has not any structural genes encoding ribosomal proteins (Rosypal, 1997; Madigan and Martinko, 2006; Carter and Saunders, 2013). The host cells have specific receptors for certain strain of virus which are sensitive and specific for any proteins of viral capsid. If not, the cell cannot be naturally infected by virus (Carter and Saunders, 2013).

1.2. Loss of natural resistance of fish

In the past 100 years, the aquaculture has significantly developed and it is now very important for the economies of many countries. Displacement of aquatic animals from their natural environment, their breeding in high density, exposure to environmental stress, using unnatural feeds, extensive global trade, over-exploitation of fisheries and anthropogenic stress on aquatic ecosystems lead to pressure on wild fish populations (Walker and Winton, 2010; OIE, 2019). For example, Salonius and Iwama (1993) found that some species of wild fish became more susceptible to disease (infection by *Vibrio anguillarum*) after their transferring to hatchery. Further interesting observation was also the fact, that wild fish had higher numbers of antibody-producing cells compared to cultured fish (Salonius and Iwama, 1993). In addition, in recent time, there is a danger that climate change and global warming may facilitate outbreaks of existing and new pathogens and parasites (Sae-Lim et al., 2017).

Subsequently, all these factors may also lead to emergence and spread of new fish diseases, such as viral diseases. Due to both potential risk of diseases spread in environment and threat of commercial trade in finfish, many of the viral diseases must be monitored (Walker and Winton, 2010; OIE, 2019).

Viral diseases triggered by highly infective viral agents, e. g. KHVD (Koi herpesvirus disease), IHN (Infectious haematopoietic necrosis), VHS (Viral haemorrhagic septicemia) etc. are considered as serious (Official Journal of the European Union–CS, 2018). For this reason, such diseases are placed in the List of notifiable diseases by the World Organization for Animal Health (OIE, 2019; http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2019/) as well as in the EU list of notifiable diseases (Council Directive 2006/88/EC; https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32006L0088). Moreover, it is necessary to keep in mind possibility of transmission of viral agents by various vectors which don't show any signs of disease and which need to be monitored too. Such information is mentioned by Animal Health Law of European Union (More et al., 2017; Official Journal of the European Union–CS, 2018).

All established rules of treatment are declarated with the relevant applicable law for each country of European Union (Official Journal of the European Union–CS, 2016; More et al., 2017; Official Journal of the European Union–CS, 2018).

1.3. Koi herpesvirus – Cyprinid herpesvirus 3

In 1997 in Germany, it was the first time when unknown disease connected with high mortality in carp was described (Bretzinger et al. 1999). Next outbreaks of similar disease were noticed also in Israel and the USA in 1998 (Hedrick et al., 2000). By usage of modern biological-molecular techniques, there was retrospectively found that the unknown viral agent was present in Europe (UK) aquaculture already in 1996 (Aoki et al., 2007).

Detailed investigation of the unknown viral agent revealed its similarity with herpesviruses, but, on the other hand, there was a relatively big difference in size of viral particles and, therefore, the agent started to be called as "herpes-like virus" (McColl, 2013).

1.3.1. Classification and morphology

Because of the fact that "herpes-like virus" was in disease manifestation mainly associated with common carp (*Cyprinus carpio carpio*) and its ornamental color variety–koi (*Cyprinus carpio koi*) (Perelberg et al. 2003), this causative agent started to be unofficially called as koi herpesvirus–KHV (McColl, 2013).

Due to most characteristic signs – pale and irregularly colored gills (Hedrick et al., 2000; Body et al., 2000) and nephritis (Ronen et al., 2003), this virus has been given also the name "carp nephritis and gill necrosis virus" (CNGV) which was the preferred name by some authors such as Pikarsky et al. (2004); Siwicki et al. (2006); Siwicki et al. (2012).

Waltzek et al. (2005) revealed the genetic relationship of KHV to Cyprinid herpesvirus-1 (CyHV-1), also known as "carp pox herpesvirus"; Cyprinid herpesvirus-2 (CyHV-2; "hematopoietic necrosis herpes-virus of goldfish"), and Ictalurid herpesvirus 1 (IcHV-1). Currently, on the basis of these results, koi herpesvirus has been classified as Cyprinid herpesvirus-3 (CyHV-3) (Waltzek et al. 2005). CyHV-3 is the member of the new family *Alloherpesviridae* since 2009 (including also some fish and frog herpes viruses) (Davison et al., 2009). According to the study of Waltzek et al. (2005), the CyHV-3 is most related to three fish viruses belonging to the order *Herpesvirales*. Ilouze et al. (2006) found that CyHV-3 bears at least four genes encoding proteins similar to those expressed by pox viruses: thymidilate monophosphate kinase (TmpK), ribonucleotide reductase (RNR), and thymidine kinase (TK). These proteins are significant for synthesis dNTPs (deoxynucleotide triphosphates) required for DNA synthesis (Ilouze et al., 2006). These findings were also supported by Aoki et al. (2007), who found that 15 of CyHV-3 genes had obvious homologous in IcHV-1. Not long ago, Engelsma et al. (2013) identified at least three novel groups of CyHV-3-like viruses during routine screening of common carp and koi without clinical signs of disease. As Engelsma et al. (2013) mentioned, these CyHV-3 variants share 95–98 % of nucleotide identity with CyHV-3 strains and they might represent low- or non-pathogenic variants of CyHV-3 (Engelsma et al., 2013).

In contrast to common properties of herpesviruses, the genome of CyHV-3 is larger. The size of genome is 295 kbps (kilo base pairs) containing a terminal direct repeat of a size 22 kbps. Nucleotid composition consists of guanine and cytosine of more than 59 % (an average of three KHV strains from Japan, the United States and Israel) (Aoki et al., 2007). Hutoran et al. (2005) reported the size of the double-stranded DNA molecule of 270 to 290 kbps. Aoki et al. (2007) found out that CyHV-3 bears only small fragments with lengths from 16 to 45 bp that are similar to the genomes of several DNA viruses (IcHV-1, CyHV-1 and CyHV-2) or even to human virus HCMV as mention Hutoran et al. (2005). CyHV-3 possesses 156 protein-coding genes (protein-coding open reading frames – pORFs) and 8 of them are duplicated in the terminal repeat (Aoki et al., 2007; Rosenkranz et al., 2008).

Thanks to the size of CyHV-3 as well as its round or hexagonal shape of envelope, lots of viral structures are most frequently observed in the cell nucleus resulting in margination of cell chromatin. At the beginning of knowledge of CyHV-3 (when first outbreaks occurred), Bretzinger et al. (1999) revealed "virus-like"particles in the nuclei of respiratory epithelial cells of gills and in the cytoplasm of these cells. They also reported the size of virus-like agent from 80 to 100 nm in diameter. The mature virion has a size in diameter between 170 to 200 nm and can be seen in cytoplasmic vesicles or outside the cell (Miwa et al., 2007). Miwa´s report is supported by the investigation of Body et al. (2000), who observed viral particles of the size from 150 to 200 nm on the surface of epithelioma papulosum cyprinid (EPC) cells. The sizes of viral particles detected by Hedrick et al. (2005) and Hutoran et al. (2005) were very similar: 100–110 nm in diameter and mature virion had an overall diameter of 170–230 nm.

Michel et al. (2010) found that CyHV-3 possesses 40 structural proteins, which were divided as capsid (including three), envelope (including thirteen), tegument (including two) and unclassified (including 22) structural proteins. It is assumed that the envelope proteins could be glycosylated, which was predicted by bioinformatic analysis (Michel et al., 2010). Aoki et al. (2007) revealed 27 membrane proteins. Fifteen of them have a putative function of glycoprotein and one of them is related to poxvirus B22R protein which is involved to immune evasion of pox virus (McFadden and Nazarian, 2006). Rosenkranz et al. (2008) revealed that predicted membrane protein type III of protein-coding open frames (ORFs) has immunogenic character, but according to Western blot analyses, the protein pORF81 did not appear to be glycosylated. These analyses also revealed that post-translational adjustments of pORF81 are missing. KHV encodes also protein pORF134, which is clearly related to interleukin 10, like in human herpesvirus-5 (HHV-5), which is according to Chang et al. (2004) its homologue. Rosenkranz et al. (2008) also mentioned that pORF134 modulates the immune response of the host cell.

1.3.2. Temperature and its influence on virus

Cultivation of CyHV-3 on koi fin cells (KF-1) showed that the best replication of the virus in laboratory conditions conducted at the temperature between 15 °C and 25 °C (Gilad et al., 2003). Siwicki et al. (2006) used the optimal temperature conditions that led to cytopathic effect (vacuolization and consequent destruction of cells at cell lines) at 22 °C.

In environmental conditions, Antychowicz et al. (2005) reported mortality of carp at water temperature between 18 °C and 20 °C and Siwicki et al. (2006) reported permissive water temperature in ponds between 18 °C and 28 °C. However, the optimal temperature for viral activity is 23 °C–25 °C. This suggests that most outbreaks appear in spring and summer season, which correlates with permissive water temperature (Hedrick et al., 2000; Perelberg et al., 2003; OIE, 2019).

When the water temperature is 16 °C, the exposure of clinical signs is lower and the onset of mortality is relatively slow. At the higher edge of the temperature range (28 °C), clinical signs are expressed in the same speed as at the optimal temperature but the cumulative mortality is low (Gilad et al., 2004).

The water temperature influences also the speed and amount release of virus from infected fish. Yuasa et al. (2008) confirmed that the virus is released for 34 days (from 7th to 40th dpi) at 16 °C. At 23 °C, the virus is released for 14 days (from 1st to 14th dpi) and at 28 °C, release of virus is for 12 days (from 3rd to 14th dpi). Moreover, if the water temperature decreases to below 16 °C, infected fish may release the virus even 14 days earlier than the first clinical signs occur (Yuasa et al., 2008).

On the other hand, higher water temperatures (above 28 °C up to 30 °C) might be used for natural immunization of fish because the virus stops its replication (Ronen et al., 2003). Interestingly, such "natural immunization mechanism" is used also by carps instinctively. Rakus et al. (2013) found that CyHV-3-infected carps can spontaneously choose water with the highest temperature (24 °C, 28 °C, 34 °C) to wait for survival of disease. This mechanism was called as "behavioral fever" (Rakus et al., 2017).

Extreme temperatures such as 37 °C, high temperatures such as 30 °C or in opposite, low temperatures such as 4 °C do not lead to onset of disease, because 28 °C is the maximal water temperature tolerated by the virus (Gilad et al., 2003). The temperature which can effectively inactivate virus is 50 °C (exposition for 1 min) (Kasai et al., 2005).

1.3.3. Surviving of virus in the environment

It was found, that if CyHV-3 is appropriately protected against inappropriate conditions, it may persist in the aquatic environment for a long time. Main factors influencing the length of viral activity in water environment are e. g. water temperature (see 1.3.2.), presence of ultra-violet radiation, presence of benthic organisms in the sediment, "anti-CyHV-3" bacteria and presence of susceptible host fish species (Perelberg et al., 2003; Kasai et al., 2005; Ilouze et al., 2006; Shimizu et al., 2006; Minamoto et al., 2009).

Perelberg et al. (2003) but also Ilouze et al. (2006) found virus in the water of 22 °C, remaining active in the water for four hours and during this period CyHV-3 was still able to induce disease. Minamoto et al. (2009) revealed the presence of CyHV-3 at high levels in the Yura river (Japan) not only during the mass mortality caused by the disease, but also for at least 3 months after the end of the mass mortality. Further examination of distribution and the amount of virus (in a Japanese river system) revealed that detection of CyHV-3 DNA is not the same as detection of infectious virus. Furthermore, Minamoto et al. (2009) hypothesized that CyHV-3 might have been introduced into the river in the spring or winter when the temperature was low, and as the water temperature increased in spring, the virus became more active, and then, in summer, surviving fish became carriers. As the water temperature decreased in the autumn (from higher, permissive temperatures), the virus was inactivated again. So, virus was found in environmental water before, during and after an outbreak of the disease (Minamoto et al., 2009; McColl, 2013).

Exposition to ultra-violet radiation could be used naturally during summer time. When ponds are empty of water, the bottom of ponds with all material which might be potential reservoir of virus is exposed to UV. It was found that CyHV-3 is more resistant to sunshine and UV than other viruses (Piackova et al., 2015). The appropriate dose of UV leading to completely inactivation of virus was 4×10^3 $\mu WS.cm^{-2}$ (Kasai et al., 2005).

Smith et al. (1978) found that sediment may help viral particles to survive for long time especially if the sediment surface is polluted (for example by faeces). These conditions may prolong survival of viruses adsorbed to sediment (Smith et al., 1978). Honjo et al. (2012) found an amount of CyHV-3 in sediment, which was 46–1238 times higher than that in water from the same localities examined a few years ago (Honjo et al., 2010).

Besides sediment, also droppings of infected fish could be a source of active virus (Dishon et al., 2005). They found viral DNA and active virus, which can be infective. Due to this fact CyHV-3 can be detected without need of taking biopsy or killing the fish. CyHV-3 excreted by infected fish in the faeces can diffuse from stools into the water and can induce disease of fish depending on epizootological conditions. Viral DNA could be detected in faeces of intraperitoneal infected fish as early as 4–8 days post infection (Dishon et al., 2005; Fournier et al., 2012; McColl, 2013).

Shimizu et al. (2006) found a significant correlation between presence of some strains of bacteria and the reduction of amounts of CyHV-3 in water and sediment in environmental water. They registered a reduction of infectious titer of CyHV-3 within 3 days in intact environmental water and sediment. In contrast to previously autoclaved or filtered water where the presence of infective viral particles was observed for more than 7 days (Shimizu et al., 2006). Thus it is conceivable that in the absence of hosts, bacteria present in environmental water can directly inactivate CyHV-3, and so it could be possible to use these antiviral bacteria in natural environments as an effective additional control strategy for fish viral diseases (Shimizu et al., 2006).

Minamoto et al. (2011) identified that CyHV-3 could be present in plankton in waters where an outbreak occurs. Examination of zooplankton and phytoplankton revealed a significant positive correlation between CyHV-3 in the plankton and the numbers of rotifers (species of zooplankton) (Minamoto et al., 2011). The way how infected zooplankton may get into carp is either directly (when carp is stirring-up the muddy bottom during looking for food) or indirectly (by eating bivalves that have concentrated the plankton and virus) (Minamoto et al., 2011). Bivalves feed with organic matter including plankton by filtration (Landry, 1983; Lees, 2000), therefore it can lead to concentration of the virus in the digestive tract (Lees, 2000). All these facts clearly suggest that CyHV-3 might be easily bioconcentrated in the food chain of carp. On the other hand, all those "CyHV-3-findings" do not clarify whether the virus penetrates these animals actively, or whether they were fomites (Kielpinski et al., 2010).

1.4. Koi herpesvirus disease (KHVD)

1.4.1. History of KHVD

KHVD is known since its first major outbreaks in Israel and in the United States in 1998 (Hedrick et al., 2000). After these outbreaks, some other cases of new disease had also occurred in Europe. The first cases of KHVD were confirmed in 2001 in Netherlands, in 2002 in Italy and Denmark and then in 2003 in France, Austria and Switzerland (Engelsma and Haenen, 2005). In 2005, sick fish with clinical signs typical for KHVD was observed in Ireland (McCleary et al., 2011). Retrospective investigation revealed presence of CyHV-3 in five locations of the Czech Republic in 2005 (Pokorova et al., 2007). The first positive detection of CyHV-3 in Poland was carried out in 2006 (Bergmann et al., 2006). Over the time, symptoms of the disease began to be compared with earlier findings of "symptom-similar" diseases. After that, retrospective analysis revealed the presence of the viral origin of KHVD even in cases in Great Britain in 1996, (Walster, 1999; Haenen et al., 2004) and in 1997 in Germany (Bretzinger, 1999).

According to the last available resources for Europe, CyHV-3 presence was reported in 2007 again from Ireland and, consequently, from Luxembourg (More et al., 2017). After that, in 2008, there was information about CyHV-3 from Germany, Netherlands, Slovenia, United Kingdom, Denmark, and Sweden (More et al., 2017; OIE, 2019). In 2011, there was a report about this virus from France, and in 2012 also from Spain (More et al., 2017).

The situation in Asia was as follows: in 2007, there was a report from Singapore (More et al., 2017). Then, in 2008, there was found evidence for presence of CyHV-3 in Hong Kong, Indonesia, Malaysia, and Thailand. After that, virus was detected in 2011 in South Korea (Gomez et al., 2011). But in Japan, the last known CyHV-3 capture was in 2014 (More et al., 2017; OIE, 2019).

Elsewhere, it is the CyHV-3 occurence as follows: in 2008, CyHV-3 was reported from Israel, Canada and Maldives. Last available report of CyHV-3 occurence from the USA was in 2015 (Garver et al., 2010; More et al., 2017; OIE 2019).

1.4.2. Clinical signs

Affected carp and koi usually exhibit irregular, uncoordinated movement; fish breathe badly and they are apathetic. Fish become lethargic, separate from the shoal and gather at the water inlet or at the surface of the water (Hedrick et al., 2000; Waltzek et al., 2005). Some fish may suffer from a loss of equilibrium and disorientation but they may also show signs of hyperactivity (OIE, 2019).

1.4.3. Pathology and histopathology

External signs can be as follows: focal or complete loss of the epidermis with pale discoloration and only little reddening of the lesion´s margins and also erosions of fins (Bretzinger et al., 1999, Hedrick et al., 2000). The bases of fins are congested; also findings of skin ulcers or hemorrhages at the caudal fin as well as at epidermis use to be observed (Hedrick et al., 2000; Garver et al., 2010).

Bretzinger et al. (1999) also described transient appearance of the skin (due to excessive or insufficient production of mucus), increased production of gill mucus (beginning of the disease) and sunken eyes (enophthalmus).

Pathological examination revealed increased production of gills mucus at the surface of respiratory epithelium of gill (beginning of the disease) succumbing to progressive necrosis (in more protracted course of the disease) (Neukirch et al., 1999; Hedrick et al., 2000; 2005). Histological examination of gills showed fusion in the lamellae due to hyperplasia of cells of respiration epithelium and hemorrhagic patches on the tips of lamellae (Mohi El-Din, 2011).

Internal organs are mostly pale and anterior kidneys are commonly enlarged (Bretzinger et al., 1999; Hedrick 2005). Next severely affected organs are posterior kidneys. They show peritubular inflammation, congestion of veins and degeneration of tubular epithelium which leads to failure of kidney function (Miyazaki et al., 2008). Detailed histological examination of kidney tissue revealed interstitial infiltration with lymphocytes and a lot of large polygonal to round cells with abundant basophilic cytoplasm and large pale basophilic nuclei with marginated chromatin and intranuclear inclusions (Mohi El-Din, 2011).

Dishon et al. (2005) demonstrated presence of virus in the intestine of infected fish. In intestinal villi was found hyperplasia of epithelial cells which were lost and sloughed inside the lumen (Mohi El-Din, 2011). Histopathological examination of early stage of infection (from two to six days post infection performed by bath) revealed severe inflammation of gills and kidney, minimal focal inflammation in liver and brain (Pikarsky et al., 2004).

1.4.4. Way of infection

The way of the infection is only horizontal, usually by the water. However, the specific entry of the virus to the organism may be various and it determines the speed of the onset of the disease, severity of infection and, also the manifestation.

The amount of virus (concentration) which could cause onset of the disese could be very low. Consequent outbreak of disease significantly correlates both with concentration of virus and optimal temperature conditions of water simultaneously (Gilad et al., 2003; McColl, 2013). As Gilad et al. (2003) found, if the concentration of virus is 1.2 $TCID_{50}$/mL and the water temperature is 18 °C, the mean number of days to death (MDTD) were higher than at higher viral concentration at the same temperature (Gilad et al., 2003).

Transmission of CyHV-3 in relation to the reproductive cycle of common carp can be also observed. Spring is the most abundant period when it might happen, due to beginning of

fish spawning (and also water temperature increase) (Uchii et al., 2011). These authors also found (on the basis of seroprevalence survey) that the infections occur mostly in mating fish in breeding habitats. Since fish rubbe on each other and on the objects, this leads to removal of skin mucus and decrease of immunity of common carp or koi during mating time (Raj et al. 2011; Uchii et al., 2011). In addition, decrease of immunity is caused also by suppression influenced by sex hormones (Watanuki et al., 2002), where the high testosterone levels in common carp are in the spring (Uchii et al., 2011). This corresponds with findings of Hedrick et al. (2000) or Minamoto et al. (2009), who reported outbreaks in spring.

Skin mucus plays a very significant role as the mechanical, chemical and immunological barrier against many pathogens. Skin mucus contains antibodies and anti-bacterial substances (immunoglobulins, enzymes and lytic agents able to neutralize microorganisms) and thanks to these compounds, skin mucus can reduce pathogen access to epithelial cells (Evans, 1998; Ellis, 2001; Subramanian et al., 2007; Subramanian et al., 2008). Skin mucus exhibits anti-viral neutralizing activity and it is very efficient for reduction of CyHV-3 infectivity (Raj et al., 2011; Adamek et al., 2013). For this reason, if the skin mucus is removed, or if skin epidermal lesions are on the surface of the fish body, entry of the virus into carp is drastically enhanced (Raj et al., 2011).

The skin is probably the major gate of entry of CyHV-3 (Costes et al., 2009). These authors revealed and consequently confirmed presence of CyHV-3 by usage of bioluminescence as well as by the examination of the skin epithelium. There was showed presence of viral particles in the nuclei and in the cytosol of epithelium cells. Therefore, gills alone are not preferred for viral entry (OIE, 2019) as it was mentioned in earlier studies (Dishon et al., 2005; Gilad et al., 2004; Pikarsky et al., 2004), but it is true that gills in conjucunction with skin represent the portal, from which is then a systemic spread of the virus to the internal organs (Gotesman et al., 2013; OIE, 2019). So, if the loss of the natural mucosal barrier occurs, it leads not only to increased susceptibility of fish to bacterial infections (Lemaître et al., 1996; Hellio et al., 2002), but in the case of CyHV-3, this loss also enhances the binding of CyHV-3 to epidermal cells (Raj et al. 2011). Moreover, during KHVD, the expression of mentioned antimicrobial molecules is diminished and in the case of claudin marked downregulation of expression was observed as well (Adamek et al., 2013; Adamek et al., 2014). All in all, if the mucus-free epidermis of fish is injured, entry of CyHV-3 through the skin is possible (Raj et al., 2011). On the other hand, it remains possible that viral DNA found in the skin might originate from infected white blood cells (granulocytes) in the bloodstream (Miyazaki et al. 2008).

Fish may be also infected via infected food. In this case, the virus infiltrates the host through the pharyngeal periodontal mucosa after oral contamination, which may also occur probably during mastication when food or pharyngeal teeth can cause removal of mucus or micro lesions induced in protruding foliaceous papillae (Fournier et al., 2012). Consequent inflammatory responses in carp intestine as well as TK (thymidine kinase) mRNAs are detectable 3 days post immersion with CyHV-3 (Gotesman et al., 2013; Syakuri et al., 2013). In case of claudin genes (claudin–2,–3,–11, and –23) participating in the maintenance of tight junctions in epithelial cells are modulated in the gut during CyHV-3 infection (Syakuri et al., 2013).

1.4.5. Molecular tools of CyHV-3 helping its infection

Regarding to general properties of herpesviruses, there was discovered also inhibition of the host MHC class I antigen processing and presentation pathway, thereby reducing the presentation of virus-derived epitopes on the surface of the infected cell (Griffin et al., 2010). This process includes shut-down of MHC class I molecule synthesis, blockage of proteasome-mediated peptide generation and prevention of TAP-mediated peptide transport (transporter

associated with antigen processing) (Griffin et al., 2010). Moreover, herpesvirus proteins can retain MHC class I molecules in the endoplasmic reticulum, or influence their retrograde translocation from the endoplasmic reticulum or endocytosis from the plasma membrane, with subsequent degradation. This all mentioned here results in down-regulation of cell surface MHC class I peptide complexes reducing the ability of cytotoxic T-lymphocytes to recognize and eliminate virus-infected cells (Griffin et al., 2010). This situation is known from studies in mammals that a limited number of major genes, including the MHC, are involved in the regulation of the immune response (Van Muiswinkel et al., 1999). The observed absence of linkage between class I and class II of MHC in fish would allow for an independent segregation of immunological traits associated with cytotoxic responses to virus infection (class I) or humoral responses to bacteria (class II) (Van Muiswinkel et al., 1999; Rakus et al., 2012).

1.4.6. Latency of CyHV-3

Bergmann et al. (2010a) identified CyHV-3 in leucocytes from koi infected by cohabitation. They also found DNA of viral agent in "uncharacterized" cells of the granular layer of mesencephalon. Nevertheless, Bergmann et al. (2010a) mentioned that CyHV-3 seems to be rather lymphotropic than neurothrophic virus. Eide et al. (2011a) revealed portions of major capsid gene DNA that were amplified from white blood cells from two of ten infected koi. They compared these results with DNA sequence alignments of archive CyHV-3 – U (strain of virus from United States) and they found that DNA sequences from white blood cells (WBCs) of these two fish had 100% homology. Their findings lead to the conclusion that CyHV-3 may be latent in the peripheral WBCs. In a later study, Eide et al. (2011b) demonstrated that CyHV-3 can be latent similar to mammalian herpesviruses in peripheral white blood cells of various tissues.

Other study of CyHV-3 potential latency in wild common carp in two geographically distinct populations without the history of CyHV-3 outbreak in Oregon, USA, revealed latent KHV infection in white blood cells from each of these populations (Xu et al., 2013). Preliminary results show that lymphocytes B (or other non-T cells) are the major latency sites of KHV, but investigations are still carried out about concrete type of cells (Eide et al., 2011b).

In addition, investigation of influence of temperature stress on induction of CyHV-3 latency suggests that CyHV-3 may become latent in leukocytes and other tissues, that it may be reactivated from latency thanks to temperature stress (Eide et al., 2011b). This fact is supported by Reed et al. (2014), who discovered expression of open reading frame ORF6 of the viral genome in IgM+ WBC during latency (Reed et al. 2014). Fish which were exposed to temperature stress condition contained CyHV-3 DNA in brain, spleen, gills, heart, eye, intestine, kidney, liver, and pancreas in euthanized koi 1 month after temperature stress (Eide et al., 2011b). On the other hand, it is not clear if presence of viral DNA cannot be caused by spreading of infected leucocytes into these tissues (McColl, 2013).

1.4.7. Susceptible species, breeds and hybrids

Natural susceptibility to CyHV-3 was found only in common carp (*Cyprinus carpio carpio*) and its ornamental variety koi (Hedrick et al., 2006).

Some laboratories tested also the susceptibility of hybrids of common carp or koi with other species of cyprinid fish to CyHV-3. Tested hybrids were as follows: carp × goldfish (*Carassius auratus*, family: *Cyprinidae*, order: *Cypriniformes*) and carp × crucian carp (*Carassius carassius*, family: *Cyprinidae*, order: *Cypriniformes*) (Hedrick et al., 2006; Bergmann et al., 2010b; More et al., 2017).

Some of these hybrids were found susceptible such as hybrids of koi and crucian carp, which showed the highest cumulative mortality (91 %) (Bergmann et al., 2010b). Next, hybrids of koi and Prussian carp showed cumulative mortality only 35 % (Bergmann et al., 2010b). The best surviving rate showed hybrids of common carp and *Carassius auratus auratus* which cumulative mortality was only 5 % (Hedrick et al., 2006).

Therefore, as it is mentioned above, the World Organization for Animal Health claims as species susceptible to CyHV-3: common carp, koi and its hybrids with *Carassius auratus auratus* and *Carrassius carassius* (More et al., 2017; OIE, 2019).

1.4.8. Influence of fish age on susceptibility to CyHV-3

The disease is not restricted on certain age of fish, but young fish (approximate weight of 2.5 g and 6 g) and fingerlings seemed to be more sensitive for this infection than adult fish (weight 230 g) (Perelberg et al., 2003; Siwicki et al., 2006). Perelberg et al. (2003) demonstrated that more than 90% of 2.5 g and 6 g fish were killed following exposure to infected fish, but only 56% of 230 g fish were killed. Therefore, Perelberg et al. (2003) claimed that young fish are more susceptible to disease than adults, but other authors stated different values of mortality, such as 70–100% (Ishioka et al., 2005) and 80–95% (Sunarto et al., 2005).

Consequent studies about usceptibility of larval forms of carp and koi did not prove it (Ito et al., 2007), but a few years ago, the presence of CyHV-3 was confirmed by bioluminisence method in embryonal (immediately after fertilization) as well as in larval stadium (Ronsmans et al., 2014). These new results suggest that all ages of fish might be susceptible, but some of them show better resistance against CyHV-3.

1.4.9. Transmission of virus to other fish species

It is natural to assume that other species of fish that share water with affected fish might be also infected. Perelberg et al. (2003) investigated transmission of CyHV-3 to other cyprinid and non-cyprinid species, namely Nile tilapia (*Oreochromis niloticus*), silver perch (*Bidyanus bidyanus*), silver carp (*Hypophthalmichthys molitrix*), goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon idella*) and also to common carp (*Cyprinus carpio*) as a positive control. Results of this cohabitation test revealed that 72% of common carps died whereas all fish of other species survived. Surviving fish were consequently cohabited with healthy naive carps and only common carp was able to transfer the virus to healthy carps.

Despite of results mentioned above, the viral DNA was found in numerous other fishes after cohabitation with infected fish, but without obvious symptomatic reactions. These species are listed in table 1.

Table 1. *Fish species cohabited with demonstrably infected carp and tested by PCRs.*

	PCR positive for presence of CyHV-3 DNA	
Species	**Tissues**	**References**
Goldfish (*Carassius auratus)*	Gills[1,2,3,4], spleen[2,4], kidney[2,4], intestine[1,2], brain[1,3] leucocytes [1,2]	(1) Bergmann et al., 2009; (2) Bergmann et al., 2010a; (3) El-Matbouli and Soliman, 2011; (4) Radosavljević et al., 2012
Grass carp (*Ctenopharyngodon idella)*	Pools of kidney[1] and gills[1]; leucocytes[1]; gills[2], kidney[2] and spleen[2]	(1) Bergmann et al., 2009; (2) Radosavljević et al., 2012
Silver carp (*Hypophthalmichthys molitrix)*	Gills[1,2], kidney[1,2], spleen[1,2]	(1) Kempter et al., 2012; (2) Radosavljević et al., 2012
Common bream (*Abramis brama)*	Gills	Kempter et al., 2012
Tench (*Tinca tinca)*	Gills[2,3], kidney[1,2,3], spleen[1,2,3], kidney[1], intestine[1], brain[1]	(1) Fabian et al., 2012; (2) Kempter et al., 2012; (3) Radosavljević et al., 2012
Rudd (*Scardinius erythrophtalmus)*	Kidney, liver intestine, brain	Fabian et al., 2012
Ruffe (*Gymnocephalus cernua)*	Gills, kidney, spleen	Kempter et al., 2012
Spined loach *(Cobitis taenia)*	Gills, kidney, spleen	Kempter et al., 2012
European perch (*Perca fluviatilis)*	Gills[2], kidney[1,2], liver[1], spleen[1,2], intestine[1], brain[1]	(1) Fabian et al., 2012; (2) Kempter et al., 2012
Atlantic sturgeon *(Acipenser oxyrinchus)*	Pool of gills, gut, heart, spleen and kidney	Kempter et al., 2009
Bushymouth catfish (*Ancistrus* sp.)	Pools of kidney and gills	Bergmann et al., 2009
Brown bullhead (*Ameiurus nebulosus*)	Kidney, liver intestine, brain	Fabian et al., 2012
Three spined stickleback (*Gasterosteus aculeatus*)	Kidney, liver intestine, brain	Fabian et al., 2012
Northern pike (*Esox lucius*)	Kidney, liver, intestine, brain	Fabian et al., 2012

Some species were even able to transmit CyHV-3 to naive common carp, for example goldfish (Bergmann et al., 2010a; El-Matbouli and Soliman, 2011; Radosavljevic et al., 2012), grass carp, silver carp (Kempter et al., 2012; Radosavljevic et al., 2012), common bream (Kempter et al., 2012), tench (Fabian et al., 2012; Kempter et al., 2012; Radosavljevic et al., 2012), ruffe (Kempter et al., 2012) and european perch (Kempter et al., 2012). Sadler et al. (2008) found similar results about the presence of CyHV-3 in goldfish. Their study demonstrated that goldfish exposed to CyHV-3 during epizootics in koi are carriers of the genome of CyHV-3, which may persist in this species of cyprinids for long period after an outbreak. However, their results could also be explained that goldfish were mechanical vectors of the virus, because there was no evidence for replication of CyHV-3 in goldfish (McColl, 2013).

El-Matbouli et al. (2007) published that goldfish, after being exposed to infected koi, were consequently able to transmit the virus to naive koi. Also Bergmann et al. (2010a) confirmed that goldfish are indeed asymptomatically susceptible to infection and they also suggested that goldfish can spread the virus to naive koi carp. In this case, leucocytes were found as the place of the virus in both koi and goldfish (Bergmann et al., 2010a).

Furthermore, Kempter et al. (2009) reported that sturgeons could act as carriers of CyHV-3 but thanks to small amount of affected fish, these sturgeons could simply be acting as fomites (McColl, 2013). Moreover, Kempter et al. (2009) revealed presence of CyHV-3 DNA in tissues of farmed and wild species such as: goldfish (*Carassius auratus*), crucian carp (*Carassius carassius*), grass carp (*Ctenopharyngodon idella*), bighead carp (*Hypophthalmichthys nobilis*), silver carp (*Hypophthalmichthys molitrix)*, tench (*Tinca tinca*), wels catfish (*Silurus glanis*), vimba (*Vimba vimba*) and Prussian carp (*Carassius gibelio*). Other potential species, that could be asymptomatically susceptible to CyHV-3, are mentioned in chapter two and chapter three.

1.5. Diagnostics of CyHV-3

1.5.1. Cultivation on cell cultures

At the beginning of awareness about KHVD, isolation on cell cultures was the first method used for the detection of its causal agent. Infected tissues were harvested and a mixture of them was applied on suitable (susceptible) cell cultures which were consequently affected of first signs of CyHV-3 replication. Tissues infected by CyHV-3 exhibit cythopathic effect (CPE) characterized by vacuolization of cells followed by development of giant syncytial formation (Neukirch et al., 1999; Body et al., 2000). For detection of CyHV-3, special cell lines developed from koi fin (KF-1) were used (Pokorova et al., 2005; OIE, 2019). Other possibility is cultivation of the virus on CCB (brain cell lines) and CCG (gill cell lines), in which development of syncitial formation begins 5 and 6 days post inoculation at 20 ºC respectively (Neukirch et al., 1999). Cell culture isolation of CyHV-3 is currently not considered to be as sensitive as other, molecular–biological methods for detecting CyHV-3 DNA. The virus is isolated in only a limited number of cell lines and these cells can be difficult to handle (Haenen et al., 2004; OIE, 2019).

1.5.2. PCR (Polymerase Chain Reaction)

Currently, the polymerase chain reaction is the main recommended and in national reference laboratories performed method to reliably detect CyHV-3 in tissues of infected fish and in cell lines. Types of used PCRs are as follows: i) convential PCR ("single-round" PCR), ii) nested PCR, also called as "two-round" PCR, iii) real-time PCR, known also as qPCR (quantitative PCR) and iv) reverse-transcription PCR.

The pilot study for realization of PCR assay for detection CyHV-3 was published by Gilad et al. (2002). They developed this assay with sequences obtained from one restriction fragment of CyHV-3 DNA (*Kpn*I/*Sac*I fragment), which leads to amplification of the specific 484 bp fragment. The detection limit is in this case 1 pg of viral DNA.

Gray et al. (2002) published their own design of primers for PCR. They designed two sets of primers. The first set has been targeted on amplification of a 365 bp product. The second set has been targeted on amplification of a 290 bp product. The PCR test was sensitive enough to detect 100 femtograms or approximately 600 DNA copies of CyHV-3 genomic DNA (Gray et al. 2002). Hutoran et al. (2005) published primer sets that provide band signal of 517 bp.

Bercovier et al. (2005) developed another, very sensitive primers that has been targeted on fragment of viral DNA encoding thymidine kinase gene (TK). This PCR assay based on primers selected from the defined DNA sequence of the TK gene allows result of a 409 bp amplified fragment. Nowadays, it is the most frequently used and favorite assay. Moreover, it is even recommended by the OIE organization (Office International des Epizooties). This assay is able to detect as little as 10 femtograms of CyHV-3 DNA corresponding to 30 virions (Bercovier et al., 2005). However, there is a concern about using the convential TK-PCR method, because as Engelsma et al. (2013) claimed, this CyHV-3 sensitive molecular tool may not be able to detect other CyHV-3-related strains that were found recently (Engelsma et al., 2013). The probable reason of this failure was a mismatch of the primers for the TK gene of the variants strains, and therefore, the potential usage of generic primers (focused on partial sequences of the DNA polymerase and and the Major Capsid Protein genes) capable of detecting a wide range of cyprinid herpesviruses should be also keep in mind, especially during screening (Engelsma et al., 2013).

Inclusion of second round into PCR assay leads to improvement of sensitivity of single-round assay significantly. Such type of PCR is called nested PCR. It uses two sets of primers (outer set for the first round and internal set for the second round) that allow to reach higher specificity of amplification of required sequence (Porter-Jordan et al., 1990; Elnifro et al., 2000). Pokorova et al. (2010) conducted comparison of the results of the two round PCR methods. They revealed that Bercovier´s nested PCR assay and Gilad´s nested PCR assay showed 62 % agreement of the results. In addition, Bercovier's nested PCR proved to be the most appropriate method for detection of small numbers of CyHV-3 DNA copies in tissue homogenate samples. An internal primer set additional to first round of Bercovier´s PCR allows amplicon of 348 bp. Bergmann et al. (2006) used their own designed internal primers added to single-round PCR of Gilad et al. (2002). Internal primers for second round of PCR were targeted on final PCR product of 392 bp. This method showed positive findings of viral DNA also in other studies, e. g. Bergmann et al., 2009; Kempter et al., 2009; Kielpinski et al., 2010.

Real-time PCR allows direct quantification of amplification product in real time (during reaction). This process uses added dye, which fluoresces after binding with dsDNA. The fluorescence signal is increasing with increasing amount of PCR product. It is also possible to use fluorescence hybridized probes (Rosypal et al., 2002). Here, in case of CyHV-3, the TaqMan (called according to Thermus aquaticus Polymerase and PacMan PC game) real-time PCR is very sensitive, specific assay with a rapid cycling time and it is quantitative. It is not necessary to use electrophoresis and if standard is available; it is possible to use qPCR for quantification both DNA and RNA (Leutenegger et al., 1999; Gilad et al., 2004). This technique may detect very low amount of viral particles (or its gene), so it can be used for testing of potential fish-carriers (Bergmann et al., 2010c; Gaede et al., 2017).

Yuasa et al. (2012) had developed reverse-transcription PCR specific for mRNA for detection of replication of CyHV-3. However, in this assay, it is important to use right primer set (Yuasa et al., 2012) as well as annealing temperature (Yuasa et al., 2005). Usage of less specific primer sets (Yuasa et al., 2012) and thanks to genetic relationship of CyHV-3 to CyHV-1 and CyHV-2 (similar nucleotide sequences) (Waltzek et al., 2005), there is a risk of false positive signals. Moreover, the unstable character of mRNA in fish tissues is also a disadvantage for using this technique for diagnosis (Yuasa et al., 2012). On the other hand, this method is promissing mainly as a tool for the determination whether CyHV-3 is replicating in organs, eventually if the virus is in latency because this assay can detect viral replication stage already at 24 hours post exposure (hpe), (Yuasa et al., 2012).

1.5.3. Immunofluorescence

Immunofluorescence is a powerful technique that utilizes fluorescent-labeled antibodies to detect specific target antigens (Odell and Cook, 2013). Labeled antibodies bind (directly or indirectly) to the antigen of interest which allows for antigen detection through fluorescence techniques. Direct immunofluorescence uses the antibody chemically conjugated to a fluorescent dye against the molecule of interest. This method includes shorter sample staining times and simpler dual and triple labeling procedures. But this method has lots of disadvantages: lower signal, generally higher cost and even less flexibility (Robinson et al., 2009).

1.5.4. ELISA (Enzyme-linked immunosorbent assay)

ELISA is a simple, sensitive, rapid, reliable, and versatile assay for the quantification of antigens and antibodies (Rees, 1994). This technique is useful for identification of viral diseases (such as CyHV-3) in fish that had prior exposure to this virus or in fish that can transmit viral agent (St-Hilaire et al., 2009) or in fish that bear latently hidden virus (St-Hilaire et al., 2005).

ELISA is a solid-phase technique which can be divided into two main types: i) competitive assay using either antigen-enzyme conjugate or antibody-enzyme conjugate and ii) noncompetitive assay using two antibodies. The second of them has an indicator enzyme conjugated to it. Direct ELISA uses immobilized antibodies on the bottom of 96-well microtiter plates that are adsorbed through noncovalent interactions. Immobilized antibody is incubated with a test solution containing the assumed antigen. Enzyme conjugated antibody binding to remaining antigenic sites on the antigen are added to those compounds. During all process, phases of incubation (binding of antigen at room temperature) as well as washing out of unbound antigen are carried out. The amount of antigen is visualized by the addition of a chromogenic or fluorogenic substrate, which leads to color visualization of positive detection. Moreover, quantification of coloured samples is necessary quantified by spectrophotometer (Rees, 1994).

This assay is suitable for detection of CyHV-3 without need to sacrifice the fish (Dishon et al., 2005), the ELISA is also easy to package in a kit format that may be used commercially at the farm (Li et al., 2017) and, moreover, it can identify positive serum after a long time of exposure (Azila et al., 2012). For example, Adkinson et al. (2005) found presence of anti-CyHV-3 antibodies in koi that had high levels of antibodies even after 1 year in virus-free water. Uchii et al. (2009) found also viral antibodies in wild common carp which survived viral exposition in Lake Biwa from year 2004.

On the other hand, ELISA assay is limited mainly because of low sensitivity, which is suitable only for detection of high levels of antibodies assumed on basis of high levels of CyHV-3 found in clinically diseased fish tissue. Therefore, it is not useful for pursuance of surveillance of CyHV-3 in healthy populations or to detect viral agent in potential fish-carriers containing low level of viral particles (OIE, 2019). However, there are some authors (Li et al., 2017), who use indirect ELISA as an additional information to evaluate the potential risks of virus transmission when PCR or other virological methods fail to reveal evidence for KHV latent or persistent infection (Li et al., 2017).

1.5.5. The other methods

Loop-mediated isothermal amplification of DNA (LAMP) was developed by Eiken Chemical Co., Japan. It is a detection assay, which amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions that lead to autocyclic displacement reaction during constant temperature and with usage a DNA polymerase (Notomi et al., 2000; Ushikubo, 2004). The detection limit was reported similar to PCR or equal. Detection of amplicons is possible to do by naked eye and it is not needed to use gel electrophoresis (Gunimaladevi et al., 2004; Haenen and Hedrick, 2006).

Yoshino et al. (2009) used this method for detection of CyHV-3. They modified LAMP by monitoring amplification in real-time on basis observation of increasing turbidity with magnesium pyrophosphate as the by-product. Soliman and El-Matbouli (2010) used for visual detection of the LAMP products nucleic acid lateral flow assay which relies on DNA hybridization technology and antigen-antibody reaction in combination with LAMP. The DNA complex could be visualized as a purple band at the strip test line within 5 minutes of sample exposure. This visualization is more rapid than agarose gel electrophoresis, does not require specialized equipment such as thermocycler and it requires very little time to training (Soliman and El-Matbouli, 2010).

Currently, there is also a new emerging method for the isothermal amplification of nuclei acid combined with lateral flow detection called as Recombinase polymerase amplification (RPA) acid (Piepenburg et al. 2006). First studies presented positive results of CyHV-3 DNA findings in latently infected koi during 20 minutes claimed this method as very effective, cheap and suitable mainly in case of latency of virus (Prescott et al., 2016).

1.6. Methods of prevention

Methods of prevention of KHVD should be mainly focused on avoiding exposure to the virus connected with good hygiene and biosecurity practices (OIE, 2019). Then, it is important to prevent introduction of infected fish to breeding stock (installation of quarantine). In addition, it is also necessary to keep a general procedure such as disinfection of eggs, regular disinfection of ponds, chemical disinfection of farm equipment, careful handling of fish to avoid stress and safe disposal of dead fish (Piackova et al., 2015; Bergmann et al., 2017; OIE, 2019). For future, methods as stocking with resistant strains and vaccination are very promising, but in case of vaccination, there are few disadvantages that may complicate its usage in praxis (OIE, 2019).

1.6.1. Vaccination

Viruses as intracellular pathogens are vulnerable to the humoral immune system during the initial entry into the body and while spreading from one cell to another, but they are more effectively controlled by the cell-mediated immune system (York, 1996). From this reason, virus-specific antibodies are important at the beginning of infection, because they block viral spreading among cells before entry of viruses into the cell (Peters and Sperber, 1999).

Fishes from the order Teleostei (common carp and koi carp) have an excellent immunological memory, so this fish may be vaccinated against diseases. Unlike terrestrial animals, fish can be immunized not only by injection or perorally but also by putting antigen into the water in which they live (Evans, 1998).

Induction of antibodies that are directed against antigen depends closely on condition of carp themselves and their age, way of infection and mainly on the temperature of water,

because growth of pathogens as well as immune response of fish are temperature-dependent (Ahne, 1986; Evans, 1998; Ahne et al., 2002).

Ronen et al. (2003) conducted the first attempt for preparing an efficient vaccine. They isolated attenuated non-pathogenic virus that rendered virus-vaccinated common carps resistant to CyHV-3. They obtained this live attenuated virus from "naturally resistant" fish that were exposed to the virus at 23 °C for 2–3 days and then transferred to the high (non-permissive) temperature of 30 °C. This challenge led to resistance to other viral challenges, because they created own high serum levels of antiviral antibodies induced by attenuated virus (Ronen et al., 2003). On the other hand, the fact that this virus was attenuated does not exclude possible virulence of CyHV-3.

Perelberg et al. (2005) reported that immunization of carp against CyHV-3 must be performed with pathogenic or attenuated virus under permissive temperature. If infection occurs in non-permissive temperature, fish are not rendered resistant to the disease (Perelberg et al., 2005). In connection with this finding, Perelberg et al. (2008) tested immunization of common carp with wild type and attenuated virus for inducing an immune response that renders the fish resistant to further virus challenges. On the basis of challenge of naive koi inoculated with attenuated virus or with wild type virus at permissive conditions, which were previously kept at various temperature conditions (14 °C, 24 °C, and 31 °C), they found typical temperature dependency both on the presence of antibodies and on anti-virus resistance. The results showed that maintenance of fish at the lowest temperature resulted in an antibody titer about 4–5 times lower in comparison to that at the highest temperature (Perelberg et al., 2008). All those findings led to a licensed usage of vaccine (duration of protection was at least eight months) in carp farms in Israel (Ilouze et al., 2011; OIE, 2019). On the other hand, there is still a question of transmissibility of virus by such immunized fish (Gotesman et al., 2013) and cases of outbrakes of KHVD in naïve carp after cohabitation with immunized carp exist (Pretto et al., 2013). Therefore, this Israeli vaccine (made by Cavoy, KoVax, Ltd) authorized in USA in 2012 was withdrawn after a year (Boutier et al., 2015).

Nonetheless, some authors (e. g. O´Connor et al., 2014) continued on this topic and some of them, in USA, even examined efficacy and safety of the vaccine (Weber et al., 2014). They found it safe and efficacious for fish of weight more than 87 g (Weber et al., 2014).

In Japan, usage of oral administration of a liposome-based vaccine containing inactivated CyHV-3 was effective in protection of carp against CyHV-3 infection (Yasumoto et al., 2006) and the newest study showed that possible way how to protect koi against CyHV-3 is to use the DNA vaccine pIRES-ORF25 (Zhou et al., 2014).

In Indonesia, Aonullah et al. (2016) used $1.3.10^8$ $CFU.ml^{-1}$ (colony forming unit) of heat-killed *Escherichia coli* carrying DNA vaccine encoding glycoprotein-25. Their vaccine used for two densities of one month old carp stock (800 and 1200 fish in one cubic meter) led to production of specific anti-CyHV-3 antibody on 28 and 36 days post vaccination in 800 and 1200 fish in one cubic meter, respectively (Aonullah et al., 2016). Although vaccination has advantages such as increasing of resistance of husbandry important fish, simple delivery and low-dose requirements (Gudding et al., 1999), there is a risk represented by reversion of virus to potential virulence (Ronen et al., 2003). Therefore, vaccination against CyHV-3 is not allowed within the EU, as the disease is included in Annex IV, Part II of the European Directive 2006/88/EC (Pretto et al., 2013).

On the other hand, Schröder et al. (2018) exhibited efforts to obtain safe and efficacious live vaccines. They performed adjustment of nonessential genes of CyHV-3 encoding two enzymes of nucleotide metabolism, thymidine kinase (TK, ORF55) and deoxyuridine-triphosphatase (DUT, ORF123). They made single-deletion mutants based on a CyHV-3 isolate (abbreviated here as KHV) from Israel (KHV-I), which exhibited only partial attenuation (Fuchs et al., 2011)

and a corresponding double mutant which was generated and tested *in vivo*, and shown to be almost avirulent but still protective (Schröder et al., 2018). They also performed single and double TK and DUT deletions into a cell culture originated from CyHV-3 strain from Taiwan (KHV-T) due to overcome the low *in vitro* virus titres of KHV-I ($\leq 10^5$ p.f.u. ml^{-1}) (Schröder et al., 2018). Their conclusion was that the deletions did not affect *in vitro* virus replication, KHV-T mutants exhibited wild-type-like plaque sizes and titres exceeding 10^7 p.f.u. ml^{-1} (prerequisite for economic vaccine production) and compared to wild-type viruses, the single-deletion mutants of KHV-T were significantly attenuated *in vivo*, and immersion of juvenile carp in water with such mutant caused almost no fatalities. Nevertheless, the "deletion mutants" induced similar levels of CyHV-3-specific serum antibodies to the parental wild-type virus, and seems to be a solid protection against disease after challenge with wild-type CyHV-3 (Schröder et al., 2018).

1.6.2. Resistant strains and hybrids

Since strains of carp may differ in susceptibility to CyHV-3, there was a strategy of cross-breeding of natural resistant strains with to CyHV-3 susceptible strains.

One way of reduction of the risk of a CyHV-3 outbreak could be also the breeding of naturally resistant strains of fish and their hybrids. Shapira et al. (2005) tested the resistance of Israeli domesticated strains of common carp (Dor 70 and Nasice) and their hybrids with wild Sassan (Amur wild carp). Hybrids with Sassan showed significantly higher survival in comparison with pure strains in laboratory as well as in field conditions.

Piačková et al. (2013) evaluated the resistance to CyHV-3 of various local strains of common carp and their hybrids. In accordance with Shapira et al. (2005), they found that strains and hybrids consisted of or derived from *Cyprinus carpio haematopterus* (Amur wild carp; Sassan) are more resistant then those consisted of or derived from *Cyprinus carpio carpio*. Ropsha scaly carp (95% of survivors) and Amur mirror carp (73.3% of survivors) seemed to be very promising for establishment of CyHV-3-resistant stocks.

The analysis of resistance on the molecular level revealed connection to polymorphism of genes playing a role in immune response, such as major histocompatibility complex (MHC), genes of class IIB (Rakus et al., 2012) and gene of interleukin 10 (IL 10) (Kongchum et al., 2011). Therefore, these studies indicate great utilization in selective breeding researches focused just on resistance (Palaiokostas et al., 2018).

Studies on a population of hybrid male goldfish × female common carp revealed them to be resistant to KHVD (Hedrick et al., 2006). These hybrids showed rapid growth and had a morphological appearance most similar to their maternal parent. Unfortunately, CyHV-3 DNA was detected by PCR in surviving hybrids suggesting that they are potential virus carriers (Hedrick et al., 2006).

Finally, all these findings indicate that there are genetic markers for resistance to KHVD (Woo and Cipriano, 2017), which is a promising future solution of CyHV-3 infection mainly due to enhance selective breeding (Palaiokostas et al., 2018).

1.6.3. Stocking with resistant species

Interestingly, no natural outbreaks of KHVD were reported in commonly farmed herbivorous cyprinid species, including silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*), and bighead carp (*Aristichthys nobilis*). Herbivorous carp species are bred in polyculture with common carp, but no signs of disease or mortalities were observed in these species (under normal polyculture conditions, under experimental

cohabitation with infected fish and direct exposure to the virus) (Schlotfeldt et al., 2004; Sunarto et al., 2005; Takashima et al., 2005; OIE, 2019).

1.6.4. Quarantine

Biosecurity measures should include ensuring that new introductions of fish are from disease-free sources and installation of a quarantine system where new fish can be held with sentinel fish at permissive temperatures for KHVD. The fish are then quarantined for a minimum of 4 weeks to 2 months before transfer to the main site and mixing with naïve fish. Hygiene measures on site should be similar to those recommended for other viral diseases (e.g. spring viremia of carp–SVC) and include disinfection of eggs, regular disinfection of ponds, chemical disinfection of farm equipment, careful handling of fish to avoid stress and safe disposal of dead fish (OIE, 2019).

1.6.4. Disinfection

Little information is available on the disinfection of water intended for fish containment; daily usage of Huwa-San (a disinfectant used in food and water industries, concentration was 60 $mg.l^{-1}$) prevented KHV infection of carp in an experiment of Bergmann et al. (2017). Their results showed that water treatment with a disinfectant may prevent transmission of infectious KHV to naive carp cohabited with infected carp (Bergmann et al., 2017).

Disinfection of eggs can be achieved by iodophor treatment (OIE, 2019). CyHV-3 is consequently inactivated by iodophor at 200 $mg.l^{-1}$ for 30 seconds at 15 °C (Kasai et al., 2005). Iodophor (200 $mg.l^{-1}$), ethylalkohol (30%) and chlorine-based solutions (concentration of chlorine is 3 $mg.l^{-1}$) can be used for disinfection of tools, utilities and tanks as well (Piackova et al., 2015).

1.7. Objectives of the thesis

The aims of this dissertation thesis were:

- To find next cyprinid and non-cyprinid fish species which might be susceptible to CyHV-3.
- Next find out if such species might play a role as a potential fish carrier of CyHV-3 to naïve koi carp.
- Last point of thesis was to investigate potential risk of cohabitation of the potential fish carrier in one husbandry system with economically important common carp or koi.

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CHAPTER 2

SUSCEPTIBILITY OF STONE LOACH (*BARBATULA BARBATULA*), AND HYBRIDS BETWEEN STERLET (*ACIPENSER RUTHENUS*) AND BELUGA (*HUSO HUSO*) TO CYPRINID HERPESVIRUS-3

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My share on this work was about 60 %.

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Susceptibility of stone loach (*Barbatula barbatula*) and hybrids between sterlet (*Acipenser ruthenus*) and beluga (*Huso huso*) to cyprinid herpesvirus 3

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ABSTRACT: Cyprinid herpesvirus 3 also known as koi herpesvirus is a causative agent of highly contagious disease (koi herpesvirus disease) and can cause significant losses in fish stocks. The disease is restricted to koi and common carp, but recent investigations have shown that other cyprinids as well as non-cyprinid species are asymptomatically susceptible to this virus and can play either a role as a potential carrier or can contribute to biological conservation of this virus. The susceptibility of two non-target species, stone loach (*Barbatula barbatula*) and sterbel – a hybrid between sterlet *(Acipenser ruthenus)* and beluga *(Huso huso)* to cyprinid herpesvirus 3 was tested by means of their co-habitation together with naïve koi and intraperitoneally KHV-infected koi (primary challenge). On the 15 th day post-infection (dpi), a secondary challenge was started (a portion of the surviving stone loach and sterbel were transferred to tanks with other naïve koi). All dead as well as surviving fish were investigated for the presence of KHV DNA in pooled samples of tissue from individual fish by nested PCR. Sampling for PCR from surviving fish was performed on the 15th dpi and on the 30th dpi of the primary challenge, and on the 30th dpi of the secondary challenge. During the primary challenge (up to the 30th dpi), average cumulative mortality in duplicated experimental groups was as follows: koi 100%, sterbel and stone loach both 5%. In the primary challenge, no surviving stone loach or sterbel sampled on the 15th dpi or those that died previously were found to be positive for viral DNA. Results of PCR revealed the presence of KHV DNA in 95% of co-habited naïve koi samples. PCR analysis of tissues taken from surviving fish on the 30th dpi revealed the presence of viral DNA in 77.8% (7/9) of stone loach and in 22.2% (2/9) of sterbel. Cumulative mortality of fish in the secondary challenge was 100% for stone loach and for koi co-habitating with them, and 50% for koi co-habitating with sterbel, which all survived. Despite the high mortality of koi and stone loach in the secondary challenge (probably caused by malfunction of biofilters or bacterial infection), none of them, nor any of the sturgeon hybrids were considered to be positive for KHV DNA. In summary, the hybrid between sterlet and beluga and the stone loach seemed to be susceptible to cyprinid herpesvirus 3, but we could not prove that they can transfer this virus to naïve koi.

Keywords: CyHV-3; KHV; transmission; cohabitation

List of abbreviations

CyHV-3 = cyprinid herpesvirus 3, **CEFAS** = Center for Environment, Fisheries and Aquaculture Science, **dpi** = days post infection, **KHV** = koi herpesvirus, **PCR** = polymerase chain reaction

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Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), is a causative agent of a highly contagious and notifiable disease (Rakus et al. 2013). This disease was initially reported in 1998 in Israel and USA, where the outbreaks resulted in high mortality of koi and common carp (*Cyprinus carpio*) (Pokorova et al. 2005).

Fish affected by CyHV-3 show external signs of disease such as: focal or complete loss of the epidermis with discolouration, haemorrhages (particularly on the caudal fin), transient appearance of the skin (due to excessive or insufficient production of mucus), increased production of gill mucus (at the beginning of disease), severe branchial necrosis (in more protracted course of disease), and sunken eyes (enophthalmus) (Bretzinger et al. 1999; Hedrick et al. 2000; Hedrick et al. 2005). Histological examinations show necrotic gills, fusion of lamellae due to hyperplasia of respiration epithelium and even haemorrhagic patches on the tips of lamellae (Hedrick et al. 2000; Mohi El-Din 2011). The virus is present mainly in the intestine and kidney, but also in droppings of infected fish (Dishon et al. 2005). Moreover, Eide et al. (2011) detected KHV DNA in the brain, eye, spleen, gills and in the haematopoietic (anterior) kidney.

Despite the suggestion that CyHV-3 is restricted to common carp and koi (Perelberg et al. 2003), transmission of the virus to other cyprinid and non-cyprinid fish is also possible. The virus has been found in numerous other fishes after co-habitation with infected fish, but without obvious symptomatic reactions; these include other cyprinid species, e.g. goldfish *(Carassius auratus)* (Bergmann et al. 2009; Bergmann et al. 2010; El-Matbouli and Soliman 2011; Radosavljevic et al. 2012), grass carp *(Ctenopharyngodon idella)* (Bergmann et al. 2009), silver carp *(Hypophthalmichthys molitrix)* (Kempter et al. 2012; Radosavljevic et al. 2012), common bream *(Abramis brama)* (Kempter et al. 2012), tench *(Tinca tinca)* (Fabian et al. 2012; Kempter et al. 2012; Radosavljevic et al. 2012), some non-cyprinid species such as ruffe *(Gymnocephalus cernua),* spined loach *(Cobitis taenia)* (Kempter et al. 2012), European perch *(Perca fluviatilis)* (Fabian et al. 2012; Kempter et al. 2012), and even Atlantic sturgeon *(Acipenser oxyrinchus)* (Kempter et al. 2009). Some fishes were even able to transmit CyHV-3 to naïve common carp, for example goldfish (Bergmann et al. 2010; El-Matbouli et al. 2011; Radosavljevic et al. 2012), grass carp, silver carp (Kempter et al. 2012; Radosavljevic et al. 2012), common bream (Kempter et al. 2012), tench (Fabian et al. 2012; Kempter et al. 2012; Radosavljevic et al. 2012), ruffe (Kempter et al. 2012) and European perch (Kempter et al. 2012). For this reason, it is necessary to verify susceptibility to CyHV-3, as well as determining species which can act as carriers for susceptible species.

The aim of this study was to determine if two non-cyprinid species, stone loach *(Barbatula barbatula*, family Balitoridae, previously Cobitidae) and sterbel [sterlet (*Acipenser ruthenus*) × beluga *(Huso huso)*], family Acipenseridae), could be potential carriers of CyHV-3 and to establish the presence or absence of CyHV-3 DNA in their tissues after co-habitation using nested PCR.

MATERIAL AND METHODS

Fish. The origin of experimental fish was as follows: stone loach was provided by a private breeder. Hybrids of sterlet and beluga (so-called "sterbel") were obtained from a closed recirculation hatchery system of the Faculty of Fisheries and Protection of Waters. Koi were obtained from a private fish farm without any KHVD history. Before the start of the challenge test, all fish were treated in a long-term FMC bath (formalin, malachite green, methylene blue) for elimination of ectoparasites. After that, all fish were acclimated at 24 °C for three days in experimental aquaria.

Virus. Virus (US isolate F98/50) was provided by S. M. Bergmann, Friedrich-Loeffler-Institut, Germany. Virus was cultivated on CCB (*Cyprinus carpio* brain) cell lines incubated at 24 °C in the Veterinary Research Institute, Brno.

Design of experiment. The study consisted of two challenge experiments – a primary and a secondary one. The primary challenge was conducted to determine the susceptibility of stone loach and sterbel to CyHV-3 during co-habitation. The co-habitation method or communal stocking is based on infected fish and healthy fish sharing the same environment. The secondary challenge was applied to investigate whether fish (potential vectors) exposed to the infection by co-habitation with CyHV-3-infected fish are able to transfer infectious virus to healthy susceptible species (naïve koi).

Primary challenge. During this period, three experimental groups were established: experimental group-1 (E1), experimental group-2 (E2) and negative control group (NC). In each of these groups, koi

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(n = 10, average length 9.2 ± 0.4 cm), stone loach (n = 10, average length 10.1 ± 0.1 cm) and sterbel (n = 10, average length 20.1 ± 0.3 cm) were stocked in 175-L aquaria with three intraperitoneally infected koi. The duration of the primary challenge was 30 days. Fish were observed and fed twice a day and water quality (temperature, pH and concentration of nitrites and nitrates) was measured once a day.

Secondary challenge. Two experimental groups were established and placed in two new tanks – "transmission group 1" (T1) and "transmission group 2" (T2). Also, a new negative control group (NC2) was established. Each of these groups contained 10 healthy specimens of naïve koi (average length 10.6 ± 0.7 cm). When the cumulative mortality of koi in the primary challenge reached 60% in the E1 group and 100% in the E2 group (15th dpi), the secondary challenge was started: three stone loach each from experimental groups E1 and E2 (six specimens in total) were transferred to the T1 group and the same number of sterbel from both experimental groups were transferred to the T2 group. Before being transferred, all fish were rinsed with clean water so as to avoid contamination of the new experimental environment with infected water on the body surface of fish. At the same time (on the 15th dpi), two specimens of stone loach were sampled for PCR from both experimental groups E1 and E2 (four specimens in total) and the same number of sterbel were collected from both E1 and E2 groups. Experimental conditions for the secondary challenge were the same as in the primary challenge and the same parameters of water quality were checked daily.

Sampling for PCR assay. Pooled samples of gill, brain, hepatopancreas, kidney and intestine were taken from each deceased as well as surviving fish. Samples were diluted with ultra-pure deionised sterile water (1 : 5) and homogenised (QIAGEN homogeniser, Germany). After homogenisation, organ homogenates were centrifuged and supernatant was collected for total DNA extraction.

Extraction of DNA. The extraction of DNA was performed using the QIAamp DNA Mini Kit (QIAGEN, Germany) following the manufacturer's instructions. During the extraction process a positive control to verify the correctness of extraction (samples from intraperitoneally infected koi) was used.

PCR assay. The nested PCR uses primers based on the sequence of the thymidine kinase gene of CyHV-3 (Bercovier et al. 2005) supplemented with a nested primer set provided by D. Stone (Pokorova et al. 2010) which is still recommended for routine diagnostics. The first set of outer primers was used according to Bercovier et al. (2005) and give a final amplicon of 409 bp. The nested primer set was used according to D. Stone (described in the CyHV-3 protocol distributed by CEFAS for the ring test of reference laboratories), and gives a final amplicon of 348 bp. For preparation of PCR, PPP master mix (TopBio, Czech Republic) was used. The 25 µl master mix, both for conventional and nested PCR, consisted of 12.5 µl of PPP master mix, 9.5 µl of PCR water (TopBio, Czech Republic), 1 µl of DNA template, 1 µl of forward primer (0.1µM) and 1 µl of reverse primer (0.1µM). Cycle conditions for conventional and nested PCR were performed in accordance with Pokorova et al. (2010).

Detection of PCR products. PCR amplicons were detected using gel electrophoresis with 1.5% agarose gel prepared in TBE buffer, stained with GelRed Nucleic Acid Stain (Biotium, USA) and illuminated by UV light. TrackIt 1 Kb Plus DNA Ladder (Invitrogen, USA) was used to determine the size of nested PCR amplicons.

RESULTS

Results of co-habitation tests

During the primary challenge, the mortality rate of intraperitoneally infected koi and co-habited koi reached 100%. The mortality of stone loach reached 10% in E2, while no stone loaches died in E1. The mortality of sterbel reached 10% in E1 and was 0% in E2 (Figure 1). During the secondary challenge, the mortality of all fish in T1 (both stone loach and koi) was 100% on the 14th dpi (Figure 2). In T2, the mortality rate was 50% on the 18th dpi, but only koi died in this group; all sterbel from this group survived.

Results of nested PCR

In the primary challenge, nested PCR revealed that 100% (6/6) of intraperitoneally infected koi were positive for CyHV-3 DNA. 100% (10/10) of co-habited naïve koi from E1 and 90% (9/10) of cohabited koi from E2 were also positive for KHV DNA. Pooled samples obtained from sterbel that died on

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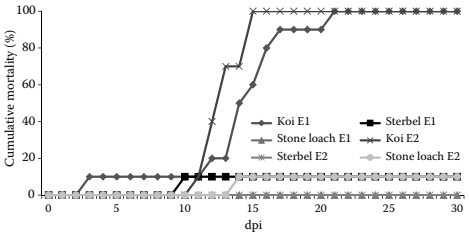

Figure 1. Cumulative mortality of fish during the primary challenge

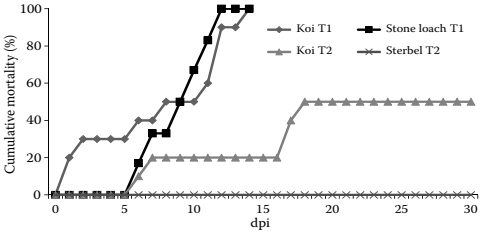

Figure 2. Cumulative mortality of fish during the secondary challenge

the 10th dpi in E1 and from stone loach that died on the 14th dpi in E2 were CyHV-3 DNA-negative as were four specimens of stone loach and sterbel sampled from E1 and E2 on the 15th dpi. PCR testing of tissues taken from surviving fish sampled on the 30th dpi revealed the presence of CyHV-3 DNA in 60% (3/5) of stone loach from E1 and 100% (4/4) of stone loach from E2. In the case of sterbel, 25% (1/4) from E1 and 20% (1/5) from E2 were CyHV-3 DNA-positive (Figure 3).

Even though the mortality was high during the secondary challenge, neither stone loach nor koi from T1 were found to be positive for viral DNA. Likewise in T2, none of the sterbel nor any of the dead or surviving koi were CyHV-3 DNA-positive. The total number of positive fish detected by nested PCR is listed in Table 1.

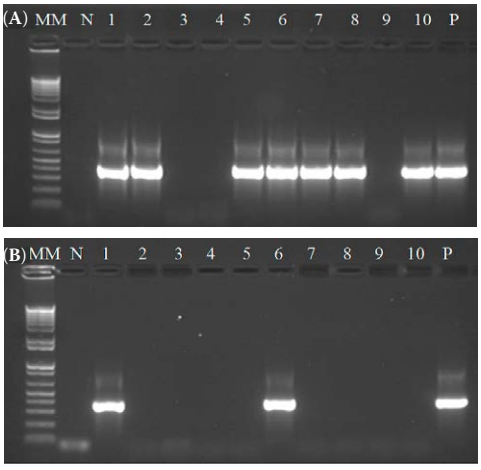

Figure 3. Electrophoresis of the final amplicon (348 bp) of nested PCR: **A** – stone loach (1, 2, 5: positive fish from E1; 3, 4: negative fish from E1; 6, 7, 8, 10: positive fish from E2; 9: negative fish – † 14 dpi). **B** – sterbel (1: positive fish from E1; 2, 3, 4, 5 – † 10 dpi: negative fish from E1; 6: positive fish from E2; 7, 8, 9, 10: negative fish from E2). N = negative control of PCR, P = positive control of PCR, MM = molecular marker

DISCUSSION

Kempter et al. (2009) reported that Atlantic sturgeon and Russian sturgeon are susceptible to CyHV-3; similarly, we found sterbel to be positive for CyHV-3 DNA. The susceptibility of stone loach (previously member of family Cobitidae, now Balitoridae) might suggest that species of the family Cobitidae could be generally susceptible to CyHV 3; for instance, Kempter et al. (2012) reported susceptibility of spined loach. Nevertheless, we did not find that those fish are able to transmit CyHV-3.

The fish which were transferred for secondary challenge (start of this challenge on the 15th dpi in the primary challenge) were found to be CyHV-3 DNA-negative as were fish that were sacrificed for PCR on the 15th dpi. One potential reason for these observations is that the fish were exposed to virus for too short a time. Sterbel from E1 and stone loach from E2 which died on the 10th dpi and 14th dpi, respectively, were also found to be negative for the presence of viral DNA.

Pooling effects should not play a role as a potential reason for the negative results of PCR testing of fish which died on the 10th and 14th dpi and also of those which were sacrificed for PCR on the 15th dpi. We pooled tissues, such as the intestine and kidney, that had previously been demonstrated to harbour viral particles (Dishon et al. 2005), and to contain viral DNA (brain, spleen and gill; Eide et al. 2011). Moreover, all samples satisfied weight parameters (minimum 25 mg of tissue samples) as

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Table 1. Ratio of positive fish as determined by the used primers. 1st round (single round PCR) using primer pair of Bercovier et al. (2005) and 2nd round (nested PCR) using the internal primer pair according to D. Stone (CEFAS protocol)

Final amplicon	1st round – 409 bp						2nd round – 348 bp					
Group	E1	E2	NC	T1	T2	NC2	E1	E2	NC	T1	T2	NC2
Fish	Number of positive fish from all fish tested											
Koi	10/10	9/10	0/10	0/10	0/10	0/10	10/10	9/10	0/10	0/10	0/10	0/10
I. p. infected koi	3/3	3/3	n.p.	n.p.	n.p.	n.p.	3/3	3/3	n.p.	n.p.	n.p.	n.p.
Stone loach												
Transfer time (15th dpi)	0/2	0/2	–	–	n.p.	–	0/2	0/2	–	–	n.p.	–
Final time (30th dpi)	3/5	3/5*	0/10	0/6	n.p.	0/10	3/5	4/5*	0/10	0/6	n.p.	0/10
Hybrid – sterbel												
Transfer time (15th dpi)	0/2	0/2	–	n.p.	–	–	0/2	0/2	–	n.p.	–	–
Final time (30th dpi)	1/5*	1/5	0/10	n.p.	0/6	0/10	1/5*	1/5	0/10	n.p.	0/6	0/10

*stone loach († 14 dpi) and sterbel († 10 dpi) included, n.p. = not present in this group

well as the dilution factor (acceptable ratio is 1 + 9 w/v) (EURL 2015).

The results of the nested PCR revealed the presence of viral DNA only in the stone loach and sterbel sacrificed and sampled at the end of the primary challenge (on the 30th dpi). Although the mortality of koi and stone loach reached 100% in T1 and 50% for koi in T2, CyHV-3 DNA was not found in any tissue samples. The most probable explanation for this mortality is impaired quality of the water in tanks caused by compromised function of biological filters which resulted in a higher concentration of nitrites (1.73 ± 1.65 mg/l for T1). Even if these parameters might lead to stressing and weakening of fish (Carballo and Munoz 1991) and, consequently, to a heightened sensitivity to bacterial (Decostere et al. 1999) or viral (Inendino et al. 2005) infections, nested PCR did not reveal the presence of viral DNA in samples taken during the secondary challenge. Therefore, even though stone loach and sterbel were found to be CyHV-3 DNA-positive at the end of the primary challenge (on the 30th dpi), the fact that fish that were removed from the infected tanks (E1 and E2) on the 15th dpi (sampled or transferred to the transmission tanks and sampled after a further 30 days) were CyHV-3-negative could suggest that 15 days of cohabitation with KHV-infected fish is not enough for infection of stone loach and sterbel.

Finally, for future investigations it might be useful and suitable to determine the positivity of remaining stone loach and sterbel survivors from experimental groups after the time of transfer and until the end of the primary challenge period. This would include PCR testing for the presence of viral DNA in tissues of target species over the course of the entire period until the end of the co-habitation test. In such a protocol, each day after the death of the last koi (or the majority of koi), samples of stone loach and sterbel would be taken for PCR or, more suitably, for qPCR examination, which could help us to explain not only the status of the virus after mass mortality of susceptible species (virus can be detected even three months subsequently (Minamoto et al. 2009), but also if the virus could still be infective for non-susceptible species. The use of various form of PCR for this purpose would be more suitable then serological assays such as iFAT or SNT. They are not considered to be reliable because CyHV-3 growth is slow and unpredictable even in susceptible cell cultures (OIE 2015). On the other hand, ELISA-based tests detecting antibodies in infected koi or common carp exist (Adkison et al. 2005; St-Hilaire et al. 2009), and ELISA focused on the direct detection of CyHV-3 antigen is under development (OIE 2015). However, no serological assays for determining the presence of CyHV-3 in non-susceptible fish species are currently available. Moreover, such techniques are still not accepted as a routine screening method mainly due to insufficient understanding of the serological responses of fish to viral infections (EURL 2015; OIE 2015).

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CHAPTER 3

SUSCEPTIBILITY OF THE TOPMOUTH GUDGEON (*PSEUDORASBORA PARVA*) TO CYHV-3 UNDER NO-STRESS AND STRESS CONDITIONS

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Susceptibility of the topmouth gudgeon (*Pseudorasbora parva*) to CyHV-3 under no-stress and stress conditions

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ABSTRACT: Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus, is the causative agent of the highly contagious koi herpesvirus disease, which is restricted to koi and common carp and causes significant losses in both fish stock. Some experimental investigations have shown that other cyprinid or non-cyprinid species may be asymptomatically susceptible to this virus and might play roles as potential carriers of CyHV-3 or might contribute to persistence of this virus in environment. Therefore, it seems important to verify not only the susceptibility of other cyprinid or non-cyprinid species, but also their ability to transmit CyHV-3 infection to susceptible species. Our previous investigation of the susceptibility of the topmouth gudgeon (*Pseudorasbora parva*) did not reveal the presence of CyHV-3 DNA in the tissues of this species after cohabitation with infected koi. Consequently, we changed the experimental conditions and applied two stress factors (removal of skin mucus and scaring) which would presumably mimic the stress most commonly encountered in the wild. Both experiments (without and with stress factors) consisted of primary and secondary challenges. In both the no-stress and stress experiments, the first challenge was focused only on testing the susceptibility of the topmouth gudgeon to the virus. With the secondary challenge, we investigated potential viral transmission from the topmouth gudgeon to healthy naive koi after exposure to stress factors. All fish (dead, surviving and sacrificed) were tested for the presence of CyHV-3 DNA using nested PCR (no-stress experiment) and real-time PCR (stress experiment). After the primary challenge of the no-stress experiment, PCR did not reveal the presence of CyHV-3 DNA in any specimen of cohabitated topmouth gudgeon, but all specimens of dead koi were CyHV-3 DNA-positive. PCR of fish tissues subjected to the secondary challenge did not show the transfer of virus to naive fish. After exposure to stress (removal of skin mucus), qPCR revealed four out of five samples (80%) of topmouth gudgeon to be positive for CyHV-3 DNA. Two out of five samples (40%) of topmouth gudgeon treated by scaring were found to be positive for the presence of viral DNA. Real-time PCR after the secondary challenge did not reveal any viral DNA positivity in specimens of topmouth gudgeon from groups previously exposed to stress. The stress experiments show that removal of skin mucus might potentially lead to susceptibility of topmouth gudgeon to CyHV-3 infection, but the transmission of the virus to koi carp was not observed.

Keywords: resistance; transmission; viral disease; KHV; carrier

Cyprinid herpesvirus (CyHV-3) or koi herpesvirus (KHV) is the causative agent of a highly contagious and lethal disease which is restricted to koi and common carp (Rakus et al. 2013). Morbidity of fish can reach 100%, and mortality can be as high as 78% but also 100% at water temperatures above

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20 °C (Bretzinger et al. 1999). Mortality rate can still reach 90% in some cases (Flamm et al. 2016), but nowadays, cases with much lower levels of mortality are more common, such as 3% at the Dremliny pond in the Czech Republic (Veronika Piackova, personal communication).

Manifestation of disease is as follows: focal or complete loss of the epidermis with pale discolouration, haemorrhages (particularly at the caudal fin), transient appearance of the skin (due to excessive or insufficient production of mucus), increased production of gill mucus (at the beginning of disease), severe branchial necrosis (in the more protracted course of disease) and sunken eyes (enophthalmos) (Bretzinger et al. 1999; Hedrick et al. 2000; Hedrick et al. 2005). Histological examinations show necrotic gills, fusion of lamellae due to hyperplasia of respiration epithelium and even haemorrhagic patches on the tips of lamellae (Hedrick et al. 2000; Mohi El-Din 2011). The presence of virulent virus was also observed and demonstrated in the faeces of infected fish, which means that the virus could be distributed also by droppings (Dishon et al. 2005). Antychowicz et al. (2005) demonstrated the presence of CyHV-3 in leukocytes using a co-cultivation method, and they also successfully transferred a viral isolate from blood cells into CCB cells for re-cultivation. Subsequently, Antychowicz et al. (2005) showed the cytopathic effects of typical icosahedral CyHV-3 virions in the CCB cell line using transmission electron microscopy. Infectious virus has not been isolated from blood plasma, but its DNA was detected using qPCR in peripheral blood leukocytes (Bergmann et al. 2009; Bergmann et al. 2010; Eide et al. 2011). Moreover, expression of open reading frame ORF6 of the viral genome was detected in IgM^+ WBC during latency (Reed et al. 2014). However, it is not clear if the detection of CyHV-3 DNA in tissues (spleen, gills, haematopoietic cells of the kidney, brain and eye) as found by Eide et al. (2011), indicates latent infection or if tissues contain latently infected WBC (McColl et al. 2013). However, distribution of the virus through various parts of the fish body (such as haematopoietic cells of the kidney, splenocytes, myocardial cells, nerve cells, hepatocytes, macrophages and granulocytes) occurs via bloodstream (Miyazaki et al. 2008).

The gill is the most damaged external organ and its inflammation as early as two days post infection suggests that the gills are the site of entry of the virus (Pikarsky et al. 2004). In addition, the gills are the site through which the virus enters the bloodstream through capillaries (Miyazaki et al. 2008), and, therefore, it is hard to clearly determine if CyHV-3 found in epidermal cells comes from skin due to infection by bath immersion. On the other hand, it was proven that disease leads to a decreased defensive capacity of skin mucus (Adamek et al. 2013) because the skin mucus loses the ability to inhibit binding of CyHV-3 to epidermal cells on the surface of the fish body (Raj et al. 2011). In addition, loss of or forced removal of mucus (e.g., because of rough handling with previously non-infected fish cohabitating with carps in fish farms or ponds) can distinctly increase the possibility of infection by CyHV-3 (Raj et al. 2011). It is also important to keep in mind that the risk of the spread of CyHV-3 infection is especially acute during spawning time (Raj et al. 2011; McColl et al. 2013), because carp immune functions are suppressed by sex hormones (McColl et al. 2013).

Transmission of virus occurs mainly through contaminated equipment (Flamm et al. 2016) and directly by infected susceptible species (Rakus et al. 2013; Flamm et al. 2016). All water organisms can probably act as mechanical vectors, because they can have the virus on their surface. Moreover, some other species of cyprinid or non-cyprinid fish were even described to transmit CyHV-3 to naive common carp under experimental conditions (Table 1). For this reason, it is important to verify susceptibility to CyHV-3 and also the transmission of CyHV-3 by other fish carriers to susceptible species and back again.

The topmouth gudgeon (*Pseudorasbora parva*) is a small and highly invasive cyprinid fish which is nowadays found in at least 32 countries outside its native range (Simon et al. 2011). The successful spread of the topmouth gudgeon is due to its wide ecological and physiological tolerance (Rossecchi et al. 2011). The species can be a vector of 84 different parasites (Margaritov and Kiritsis 2011), but no information about the transmission of viral and bacterial diseases by the topmouth gudgeon is available.

Our first investigations of the susceptibility of the topmouth gudgeon under standard (non-stress) conditions did not reveal the presence of CyHV-3 DNA in fish tissues. Therefore, we decided to expose experimental fish to stress factors. As Raj et al. (2011) found, removal of skin mucus can enhance the probability of infection. To determine if less destructive stress factors can also influence

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Table 1. Examples of cyprinid and non-cyprinid fish tested for the presence of CyHV-3 DNA after cohabitation with infected fish

Species	Tissues
Fish positive after cohabitation	
Goldfish	gills[1,2,3,7], spleen[2,7], kidney[2,7], intestine[1,2], brain[1,3] leucocytes [1,2]
Grass carp	pools of kidney[1] and gills[1]; leucocytes[1]; separated gills[7], kidney[7] and spleen[7]
Silver carp	gills[6,7], kidney[6,7], spleen[6,7]
Common bream	gills[6]
Tench	gills[6,7], kidney[4,6,7], spleen[4,6,7], kidney[4], intestine[4], brain[4]
Ruffe	gills[6], kidney[6], spleen[6]
Spined loach	gills[6], kidney[6], spleen[6]
European perch	gills[6], kidney[4,6], liver[4], spleen[4,6], intestine[4], brain[4]
Atlantic sturgeon	pool of gills[5], gut[5], heart[5], spleen[5] and kidney[5]
Ability to transmit CyHV-3 to naive common carp	
Goldfish	gills[2,3,7], kidney[2,7], spleen[2,7], intestine[3], brain[3], leucocytes[2]
Grass carp	gills[6,7], kidney[6,7], spleen[6,7]
Silver carp	gills[6,7], kidney[6,7], spleen[6,7]
Common bream	gills[6], kidney[6], spleen[6]
Tench	gills[6,7], kidney[4,6,7], spleen[4,6,7], liver[4], intestine[4], brain[4]
Ruffe	gills[6]
European perch	gills[6]

[1]Bergmann et al. (2009); [2]Bergmann et al. (2010); [3]El-Matbouli and Soliman (2011); [4]Fabian et al. (2012); [5]Kempter et al. (2009); [6]Kempter et al. (2012); [7]Radosavljevic et al. (2012)

the probability of infection, we frightened the fish in an aquarium using a method which we have developed ourselves. A further aim was to determine if the topmouth gudgeon can transfer CyHV-3 to susceptible species.

MATERIAL AND METHODS

Fish. Topmouth gudgeon were collected during the autumn harvesting of a pond. After transport to the aquarium, all fish were acclimated to a temperature of 24 °C and subjected to a long FMC (formalin, malachite green and methylene blue) bath to eliminate ectoparasites. Naive koi (*Cyprinus carpio*) were obtained from a fish farm with no CyHV-3 history and after transport to the aquarium they were treated in the same way as the topmouth gudgeon. After that, all fish were acclimated in experimental aquaria with an appropriate temperature (24 °C) for three days.

Virus. Virus (US isolate) F98/50 – Hedrick (KHV-I) was provided by S.M. Bergmann, Friedrich Loeffler Institut, Germany. Virus was cultured on CCB (*Cyprinus carpio* brain) (Neukirch et al. 1999) cells incubated at 24 °C at the Veterinary Research Institute in Brno. For intraperitoneal infection of fish (koi), a 0.25-ml volume of infected CCB cells containing 10^4 $TCID_{50}$/ml CyHV-3 was used.

Design of experiment. Both studies (under normal conditions and under stress conditions) consisted of two challenges – primary and secondary. The primary challenge was conducted to determine the susceptibility of topmouth gudgeon to CyHV-3 during cohabitation with infected koi. In the secondary challenge, we investigated whether fish exposed to infection by CyHV-3 (potential vectors) are able to transfer the virus to healthy susceptible species (koi or carp).

In the experiment performed under stress conditions we employed two stress factors. The goal of this part of our study was to, as much as possible, mimic the most common natural stress factors and conditions. Removal of skin mucus should mimic, e.g., the spawning of fish which leads to a decreased mucus layer and a loss of natural protection. This removal was performed according to the method published by Raj et al. (2011). The size of the area from which skin mucus was removed was appropriately adjusted to the overall small body size of the topmouth gudgeon.

The second stress factor was scaring by a net. This was carried out once daily (approximately 30 minutes for each experimental group) throughout the whole experimental period (30 days). This method was less destructive than the removal of mucus. While frightening the fish, we took care to avoid any contact of the net with fish so as not to remove skin mucus. The goal of this method was to apply a stress which might decrease immunity in the infective environment and thus increase the susceptibility of fish to infection.

Primary challenge in the no-stress experiment. Three experimental groups were established: experimental group 1 (E1), experimental group 2 (E2) and negative control group (NC). In each of these groups, koi (n = 10, average total length 10.5 ± 0.4 cm) and topmouth gudgeon (n = 20, average

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total length 9.6 ± 0.2 cm) were kept in 175-l aquaria. Three marked koi intraperitoneally infected with 0.25 ml of a mixture of infected CCB cells containing 10^4 $TCID_{50}$/ml of CyHV-3 were introduced to both E1 and E2 group (day 0). Fish were observed and dead fish were removed twice a day and water quality (temperature, pH and concentration of nitrites and nitrates) was measured once per day for 30 days. All tanks were strongly aerated.

Secondary challenge in the no-stress experiment. A new experimental group T (transmission) consisting of ten healthy specimens of naive koi (average length 9.8 ± 0.6 cm) was established and transferred to a new tank. On the 13th dpi of the primary challenge, when the virulence of CyHV-3 in E1 and E2 groups was sufficient (cumulative mortality of koi was 90% in E1 and 60% in E2), topmouth gudgeon from E1 (n = 10) and E2 (n = 10) were transferred into the new tank with healthy naïve koi (group T) and the secondary challenge began (time 0). All transferred topmouth gudgeon were rinsed with clean water to avoid the mechanical transfer of virus from the body surface of fish, which could lead to false positive results. The duration of the secondary challenge was also 30 days and all fish in these groups were treated in the same way as fish subjected to the primary challenge. The rest of the topmouth gudgeon in E1 and E2 were kept in their tanks up to the end of the primary challenge (30 dpi). Two days before the end of cohabitation (connected with consequent sampling), the remaining fish were netted to enhance the chance of detecting virus according to accepted diagnostic methods for CyHV-3 listed in the EURL for fish diseases (2015).

Primary challenge in the stress experiment. During this period, three experimental groups were established: experimental group 1 (E1) – fish were exposed to removal of skin mucus, experimental group 2 (E2) – fish were stressed by scaring by net and the negative control group (NC) which was not exposed to stress. In each of these groups, koi carp (n = 10, average length 10.2 ± 0.4 cm) and topmouth gudgeon (n = 20, average length 9.8 ± 0.1 cm) were stocked in 175-l aquaria (time 0) with two marked intraperitoneally infected koi carp. The duration of the primary challenge was determined by the cumulative mortality of koi in each group. The feeding of fish and removal of dead fish were carried out twice a day, while the testing of water quality parameters was performed once a day.

Secondary challenge in the stress experiment. When the cumulative mortality of koi in the primary challenge reached 90% in E1 (11 dpi) and 50% in E2 (15 dpi), stressed topmouth gudgeon from E1 (n = 10) were transferred to a new tank with ten naive koi (T1) and stressed topmouth gudgeon from E2 (n = 10) were transferred to another new tank with ten naive koi. As in the case of the no-stress experiment, all transferred topmouth gudgeon were rinsed in clean water to avoid the mechanical transfer of virus from fish body surfaces. A third new tank was stocked with ten naive koi together with surviving koi from E1 (n = 1) and E2 (n = 5) for the confirmation of virulence (positive control; PC). The size of naive koi used in the secondary challenge was similar as in the primary challenge (average length 10.5 ± 0.6 cm). During this challenge, fish were also observed and dead fish were removed during feeding twice a day and water quality was measured once per day. During the whole time of the experiment, all tanks were strongly aerated.

Sampling. In both experiments, dead fish were collected twice a day and immediately stored individually in plastic bags in the deep freezer (–80 °C). Pooled samples of gill, brain, hepatopancreas, kidney and intestine were taken from each fish. Because of the small size of body of topmouth gudgeon, tissues (mentioned above) from two fish were pooled in one sample. The final number of samples is given in Table 2. Samples were placed in Eppendorf tubes and consequently diluted with ultra-pure deionised sterile water (1 : 5) and homogenised (Tissue Lyzer II, QIAGEN homogenization machine, Germany). Homogenisation was performed with metal beads placed inside the Eppendorf tubes for 5 min at 30 shakes/s. After thorough homogenisation, organ homogenates were centrifuged and supernatants were collected for DNA extraction. All surviving fish were sacrificed and sampled after the end of the primary and secondary challenges (30 dpi). As mentioned above, surviving fish were netted two days before the end of cohabitation (sampling) to enhance the chance of virus detection (EURL for fish diseases 2015).

DNA extraction. Extraction of DNA from the supernatants of tissue homogenates was performed using the QIAamp DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. The protocol for extraction of DNA from blood or body fluids was used. Control measurements of the con-

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Table 2. Summary of the numbers of dead fish and viral DNA-positive fish as a proportion of the overall number of fish in the no-stress group

Type of experiment	No-stress experiment			
Cohabitation	primary			secondary
Group	E1	E2	NC	T
Results of cumulative mortality				
Koi	numbers of dead fish/all fish			
I.p. infected	3/3	3/3	n.p.	n.p.
To 13th dpi	9/10	6/10	0/10	0/10
From 14th to 30th dpi	1/10	4/10	0/10	0/10
Overall to 30th dpi (%)	100	100	0	0
Topmouth gudgeon	numbers of dead fish/all fish			
To 13th dpi	0/20	0/20	0/20	–
From 14th to 30th dpi	0/10	0/10	0/20	0/20
Overall to 30th dpi (%)	0	0	0	0
Results of nested PCR (first and second round)				
Koi	numbers of positive fish/all fish			
	1st round / 2nd round	1st round / 2nd round	1st round / 2nd round	1st round / 2nd round
Up to 13th dpi	9/10 / 9/10	6/10 / 6/10	0/10 / 0/10	– / –
From 14th to 30th dpi	1/10 / 1/10	4/10 / 4/10	0/10 / 0/10	0/10 / 0/10
Overall up to 30th dpi (%)	100 / 100	100 / 100	0 / 0	0 / 0
Topmouth gudgeon	numbers of positive samples/all samples (each sample pooled from two fish)			
	1st round / 2nd round	1st round / 2nd round	1st round / 2nd round	1st round / 2nd round
Up to 13th dpi	0/5 / 0/5	0/5 / 0/5	0/10 / 0/10	– / –
From 14th to 30th dpi	0/5 / 0/5	0/5 / 0/5	0/10 / 0/10	0/10 / 0/10
Overall up to 30th dpi (%)	0 / 0	0 / 0	0 / 0	0 / 0

E1 = experimental group 1, E2 = experimental group 2; I.p. = intraperitoneal; n.p. = not present in this group; NC = negative control group; T = transmission group

centration of DNA in samples was performed using a NanoDrop 2000 (Thermo Scientific).

Nested PCR and real-time PCR. A nested PCR assay using primers based on the sequence of the thymidine kinase gene of CyHV-3 (Bercovier et al. 2005) supplemented with internal primer set provided by D. Stone (mentioned in CyHV-3 PCR protocol of CEFAS) is still recommended for routine sensitive diagnostics. The first set of outer primers were used according to Bercovier et al. (2005) and resulted in a final amplicon of 409 bp. The internal primer set was used according to D. Stone (described in the CyHV-3 protocol distributed by CEFAS) and result in a final amplicon of 348 bp. The sequences of this primer set were according to Pokorova et al. (2010). For preparation of the PCR reaction mixture, PPP master mix (TopBio, Czech Republic) was used. Reaction mixtures (25 µl for both conventional and nested PCRs) consisted of 12.5 µl of PPP master mix, 9.5 µl of PCR water (TopBio, Czech Republic), 1 µl of DNA template, 1 µl of forward primer (0.1 µM) and 1 µl of reverse primer (0.1 µM). Cycling conditions for conventional and nested PCRs were according to Pokorova et al. (2010). The nested PCR described above was used only in the no-stress experiment. Final PCR amplicons were detected using gel electrophoresis with 1.5% agarose gels prepared in TBE buffer, stained with GelRed Nucleic Acid Stain (Biotium, USA) and illuminated by UV light. The TrackIt 1 Kb Plus DNA Ladder (Invitrogen, USA) was used to determine the size of targeted nested PCR amplicons.

Real-time PCR. Real-time TaqMan PCR was performed to amplify fragments of the CyHV-3 ORF 89 and ORF 90 genes (GenBank ID: AF411803) using KHV-86F and KHV-163R primers and the

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KHV-109P probe. Sequences of primers, probe and the thermal reaction conditions were according to Gilad et al. (2004): forward, KHV-86f GACGCCGGAGACCTTGTG; reverse, KHV-163r CGGGTTCTTATTTTTGTCCTTGTT. The probe used for detection was KHV-109p with the sequence CTTCCTCTGCTCGGCGAGCACG. The optimised conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The qPCR reaction mix contained 1× master mix (Maxima Probe qPCR Kit, Fermentas, Germany), 800 nM of each primer and 100 nM of fluorescent probe. The reaction was performed using a Stratagene Mx3005P thermocycler (Agilent, USA). A recombinant plasmid based on virus genome copy quantification was produced using PCR products amplified with the primers KHV-86F and KHV-163R (using Advantage 2 PCR kit, Clontech, USA) ligated into the pGEM-T Easy vector (Promega, USA) and propagated in JM109 competent *Escherichia coli* bacteria (Promega, USA). Plasmids were isolated with the GeneJETTM Plasmid Miniprep Kit (Fermentas, Germany); a standard curve from 10^0 to 10^7 gene copies was prepared and used for quantification of the copy numbers in each sample.

RESULTS

Cumulative mortality under no-stress conditions

In the primary challenge, only the mortality of koi was observed. One hundred per cent (3/3) of i.p. infected koi and 100% (10/10) of cohabited koi died in E1 (up to 14 dpi) and in E2 (up to 18 dpi). No mortality (0%) of topmouth gudgeon occurred in E1 and E2 groups and also every fish in the NC group survived (Figure 1). In the secondary challenge, all topmouth gudgeon transferred to the T groups as well as all the cohabitating naive koi survived.

Results of PCR in the no-stress experiment

Briefly, the results of the first round and the nested PCR in this experiment correspond with cumulative mortality, i.e., all fish that died during the challenge were found to be positive for CyHV-3 DNA both in the first round and by nested PCR (Figure 2). The results are listed in Table 2.

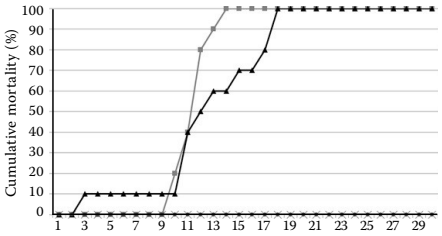

Figure 1. Graph of cumulative mortality of koi and topmouth gudgeon under no-stress conditions

= E1 – koi, = E1 – topmouth gudgeon, = E2 – koi, = E2 – topmouth gudgeon

Cumulative mortality under stress conditions

During the 30 days of the primary challenge, 90% (9/10) of koi died (up to 11 dpi) but no topmouth gudgeon in died in the E1 group during this period. In the E2 group, 50% (5/10) of koi but no topmouth gudgeon died (up to 15 dpi). All fish in the negative control group survived (Figure 3). During the secondary challenge, neither mortality (0%) nor clinical signs of disease were observed in any fish of T1 and T2. Only one koi survived in the positive control group, but it did not show any signs of disease.

Results of qPCR in the stress experiment

The results of qPCR assays showed that 9/9 (100%) of dead koi from the E1 group and 5/5 (100%) of dead koi from E2 from the primary

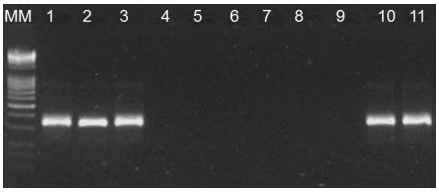

Figure 2. Electrophoresis of PCR products from the non-stress experiment (positive band is 348 bp)

1 = naive koi from E1; 2, 3 = naive koi from E2; 4, 5 = control koi group ; 6, 7 = *Pseudorasbora parva* from E1; 8, 9 = *P. parva* from E2; 10 = infected koi from E1; 11 = infected koi from E2; MM = molecular marker

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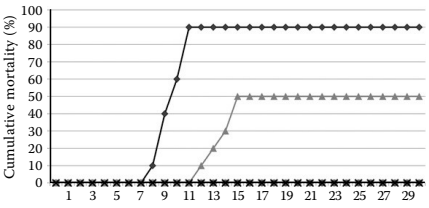

Figure 3. Graph of cumulative mortality of koi and topmouth gudgeon under stress conditions

—◆— = "Stress 1" koi, —●— = "Stress 1" topmouth gudgeon, —▲— = "Stress 2" koi, —✕— = "Stress 2" topmouth gudgeon

challenge were CyHV-3 DNA-positive. In E1, 4/5 pooled samples (80%) of topmouth gudgeon were found to be CyHV-3 DNA-positive by qPCR. In E2, 2/5 samples (40%) of topmouth gudgeon were found to be positive for the presence of viral DNA. No fish in the negative control group showed any signs of morbidity, and subsequent real-time PCR assays did not reveal any viral DNA in their tissues. After the end of secondary cohabitation (30 dpi), only one koi (10%) from transmission group one (T1) was found to be CyHV-3 DNA-positive, but all specimens of topmouth gudgeon were CyHV-3-negative. In transmission group two (T2), the

Table 3. Summary of the numbers of dead fish and viral DNA-positive fish as a proportion of the overall number of fish in the stress group

Type of experiment	Stress experiment					
Cohabitation	primary			secondary		
Group	E1	E2	NC	T1	T2	PC
Results of cumulative mortality						
Koi	numbers of dead fish/all fish					
I.p. infected koi	2/2	2/2	n.p.	n.p.	n.p.	n.p.
Transfer time (11th dpi)	9/10	–	0/10	0/10	–	0/10
Transfer time (15th dpi)	–	5/10	0/10	–	0/10	0/10
30th dpi	–	–	0/10	0/10	0/10	15/16
Overall up to 30th dpi (%)	90	50	0	0	0	94
Topmouth gudgeon	numbers of dead fish/all fish					
Transfer time (11th dpi)	0/20	–	0/20	0/10	–	n.p.
Transfer time (15th dpi)	–	0/20	0/20	–	0/10	n.p.
Final time (30th dpi)	0/10	0/10	0/20	0/10	0/10	n.p.
Overall up to 30th dpi (%)	0	0	0	0	0	0
Results of qPCR						
Group	E1	E2	NC	T1	T2	PC
Koi	numbers of positive fish/all fish (10 samples from 10 fish)					
Transfer time (11th dpi)	9/10	–	0/10	–	–	0/10
Transfer time (15th dpi)	–	5/10	0/10	–	–	0/10
Final time (30th dpi)	–	–	0/10	1/10	0/10	16/16
Overall up to 30th dpi (%)	90	50	0	10	0	100
Topmouth gudgeon	numbers of positive samples/all samples (each sample pooled from two fish)					
Transfer time (11th dpi)	0/5	–	0/10	0/5	–	n.p.
Transfer time (15th dpi)	–	0/5	0/10	–	0/5	n.p.
Final time (30th dpi)	4/5	2/5	0/10	0/5	0/5	n.p.
Overall up to 30th dpi (%)	80	40	0	0	0	n.p.

E1 = experimental group 1; E2 = experimental group 2; n.p. = not present in this group; NC = negative control group; T1 = transmission group 1; T2 = transmission group 2; PC = positive control group

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qPCR assay did not reveal any viral DNA positivity in samples of topmouth gudgeon. No positive koi (0%) were found in this group. In PC, 16/16 (100%) specimens of koi were found to be qPCR-positive. Real-time PCR results are summarised in Table 3.

DISCUSSION

Fish skin is composed of five layers: the mucus layer, epidermis, basement membrane, dermis and hypodermis (Raj et al. 2011; Roberts and Ellis 2012). The mucus layer is produced by goblet cells (Ringo et al. 2007) and mucus covering the epidermis provides mechanical, chemical and nonspecific immune protection against pathogenic microorganisms (Ellis 2001; Fontenont and Neiffer 2004). The main components of mucus are large filamentous, highly glycosylated glycoproteins which make a viscous matrix (McGuckin et al. 2011) composed of a mixture of ions, lipids and proteins (Thornton and Sheehan 2004; Bansil and Turner 2006). Antimicrobial peptides (AMPs) (Schauber and Gallo 2008; van der Marel et al. 2012), beta defensins, mucins and claudins (Adamek et al. 2013) are crucial elements of mucus. The expression of all of these molecules is diminished and in the case of claudin marked downregulation of expression was observed during CyHV-3 infection (Adamek et al. 2013; Adamek et al. 2014). Claudin is a major protein of epithelial tight junctions and plays an important role in the regulation of mucosal permeability (Adamek et al. 2013). Its decreased expression leads to a high loss of ions (Negenborn 2009), disintegration of skin (Negenborn 2009) and also to secondary microbial infections (Adamek et al. 2013). In addition, as Adamek et al. (2013) found in histological investigations, CyHV-3 infection decreases the number of goblet cells in the skin.

Taking into account that goblet cells are responsible for producing skin mucus (Ringo et al. 2007), the loss of the natural mucosal barrier leads not only to susceptibility of fish to bacterial infections (Lemaitre et al. 1996; Hellio et al. 2002), but in the case of CyHV-3 this loss also enhances the binding of CyHV-3 to epidermal cells (Raj et al. 2011). If the mucus-free epidermis of fish is injured, entry of CyHV-3 through the skin is possible (Raj e al. 2011). On the other hand, it remains possible that viral DNA found in the skin might originate from infected white blood cells (granulocytes) in the bloodstream (Miyazaki et al. 2008).

The topmouth gudgeon is an invasive species and its presence on farms is common. This species competes for food with farmed fish and it is also known to be a carrier of many different pathogenic agents (Margaritov and Kiritsis 2011). However, information about the transmission of viral diseases by this species is missing. In our first investigations of topmouth gudgeon as a carrier of viral disease we did not use an experimental setup which would mimic stress factors. Rather, we used a natural cohabitation method which, and the results did not suggest that the topmouth gudgeon could be a potential carrier of CyHV-3. Thus, in the experiments described here we aimed to enhance the susceptibility of topmouth gudgeon by weakening its immunity. It is generally accepted that a physical stress (such as rough handling, scaring by net or other injuries) which affects the skin mucus can increase fish susceptibility to infection by pathogens (Roberts and Ellis 2012). Therefore, in this study, we decided to apply two different stress factors which could mimic the most common stresses occurring on farms (not in the wild). Negenborn (2009), Raj et al. (2011), Adamek et al. (2013) and Adamek et al. (2014) suggested that the loss of skin mucus enhances the entry of pathogens into the fish body. With respect to these results, the loss of the skin mucus layer of the topmouth gudgeon seems to result in the efficient infection of this species with the virus.

Our results from the stress experiments showed that topmouth gudgeon stressed by removal of skin mucus exhibit slightly enhanced susceptibility to infection with CyHV-3 as found by Raj et al. (2011) in the case of common carp. We adapted the method of Raj et al. (2011) to the body shape of topmouth gudgeon. To determine if a less destructive method might also enhance susceptibility to infection, we used less stressful handling (scaring) which also might lead to a weakening of fish (Roberts and Ellis 2012; EURL for fish diseases 2015) and may enhance the possibility of infection as well as virus (viral DNA) detection.

Although qPCR is a highly sensitive assay for CyHV-3 detection (Monaghan et al. 2015), the rate of positive qPCR findings for CyHV-3 DNA in the tissues of topmouth gudgeon was relatively low (four samples in E1 and two samples in E2) and only one positive case of koi (T1) was detected. The

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most probable reason for such a low rate of positive findings is the negative pooling effect. This pooling method was used with respect to the small body size of topmouth gudgeon and reflected our efforts to maximise the chance of positive findings. On the other hand, this technique has still not been rejected and it remains a recommended EURL method (EURL for fish diseases 2015). For future investigation, it would be important to keep in mind this negative property of pooling, especially with respect to possible false negative results because of dilution of the virus (Matras et al. 2012; Monagan et al. 2015). Even though we found CyHV-3 DNA in koi tissue only in one sample after cohabitation with topmouth gudgeon (stressed by removal of skin mucus), it seems appropriate to consider topmouth gudgeon as a new potential carrier of this virus. However, in the future it will be necessary to confirm this finding in further experiments utilising diverse experimental set-ups.

Future investigation of this topic should focus not only on the potential asymptomatic susceptibility of the topmouth gudgeon to CyHV-3 (i.e., a carrier), but also on understanding the dissemination of virus through the tissues of this fish. Due to its presence in nearly all fish ponds in Europe, an enhanced understanding of the role of this small and inconspicuous cyprinid fish is vital.

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CHAPTER 4

GENERAL DISCUSSION

CyHV-3 is a highly contagious viral agent causing koi herpesvirus disease with wide possibility of spreading in environment, through fish husbandry, but also among private breeders. For this reason, koi herpesvirus disease belongs to lists of notifiable diseases according to decision of Commission of Europen Union in Animal Health Law, Regulation of The European Parliament and of The Council (EU) No 2016/429 (More et al., 2017) and OIE (OIE, 2019). Such regulation must be strictly implemented at the national level of each country belonging to European Union to minimalize or totally avoid outbreaks of KHVD (Official Journal of the European Union–CS, 2018).

Since CyHV-3 may be transmitted by fish and invertebrate vectors or tools, special regulations are required to minimize the introduction of the viral agent not only to European Union countries, but also to the environment where the viruses might be bioconserved (Cooper, 1995; Matsui et al., 2008; More et al., 2017).

Generally, persistence of virus by "bio conservation" in environment may influence abundance, virulence and invasiveness of the viruses as well as host susceptibility or sensitivity (Cooper, 1995). Such persistence in the environment may be represented by latently infected fish (Penkert et al., 2011; Eide et al., 2011a,b; Xu et al., 2013), fish carriers (More et al., 2017) or by invertebrates (Kielpinski et al., 2010; Minamoto et al., 2011). Next, inanimate factors which can "protect viral agent" against unsuitable conditions may be permissive water temperature (Gilad et al., 2003; Perelberg et al., 2003; Ilouze et al., 2006), muddy sediment (Smith et al., 1978; Honjo et al., 2010) or faeceas (Dishon et al., 2005). It could be assumed that parts of aquatic plants´ bodies may also protect virus to survive in the environment (Cooper, 1995), but studies on CyHV-3, which might be protect by aquatic plants, are missing so far.

All in all, cases of long-term persistence of virus in environment as well as short–term period exist (More et al., 2017). For example, some studies confirmed presence of CyHV-3 DNA in the brain of fish for as long as one year post-infection (Miwa et al., 2007; Yuasa et al., 2012;). Next, in wild populations, there was detection of CyHV-3 DNA in the brain of both seropositive and negative carp for over two years post-outbreak (Uchii et al., 2009). And, furthermore, serological testing of fish from lakes that experienced KHVD, detected seropositive fish even nine years after an outbreak (More et al., 2017). Although the presence of the pathogen has not been proven, this duration of antibody persistence may suggest continued low level exposure to CyHV-3 and therefore persistence (More et al., 2017).

Compared to the above, relatively short-term persistence is represented in experimental studies that detected low levels of CyHV-3 DNA up to 2 months post infection in the gills, kidneys and brain of fish that survived primary infection and no longer showed clinical signs (Gilad et al., 2004). Reactivation of the virus in persistently infected fish was proven after influence of stress raised by manipulation and after the application of temperature stress several months after exposure to the virus (St-Hilaire et al., 2005; Bergmann and Kempter, 2011; Baumer et al., 2013). Despite mentioned results, there is still little knowledge about other factors of reactivation of the virus in latently infected fish (More et al., 2017).

Once the virus is introduced into the aquaculture or environment, removal of CyHV-3 is hard and complicated task connected with high economical losses (Pokorova et al., 2005; More et al., 2017). Methods to control disease can consist of medication or eradication of diseased animals in combination with disinfection of facilities (Van Muiswinkel et al., 1999; Piackova et al., 2015; Bergmann et al., 2017). Mostly, removal and killing of virus is connected with removal of affected fish or fish which experienced KHVD by various methods such as overdose by phenoxyethanol (More et al., 2017), percussive stunning, electrical stunning or spiking and decapitation (Algers et al., 2009). On the other hand, killing by exsanguination or evisceration

will result in further contamination and in this respect is less preferable (More et al., 2017). As it was mentioned above, removal of fish could not be assumed as a final step of virus removal, mainly due to persistence of virus in the environment. Therefore, next steps of eradication should be disinfection, cleaning, decontamination of water and equipment (Brauer et al., 2004; Piackova et al., 2015; Bergmann et al., 2017; More et al., 2017) that may eliminate spread of the virus (More et al., 2017). Moreover, as Bergmann et al. (2017) found out, the water treatment with a disinfectant may prevent transmission of infectious CyHV-3 to naïve carp cohabited with infected carp. If such procedure is not respected, water, sediment, etc. might remain contaminated and thus all these parameters may become a source of infection for long time after the farm has been destocked (More et al., 2017).

For these reasons, it is suitable and necessary to avoid the introduction of CyHV-3 into all aquaculture system. Respect of Directives and Regulation of Europen Union (Official Journal of the European Union–CS, 2016, 2018; More et al., 2017) should lead to insurance of perfect prevention. In addition, implement of results of genetic or vaccine researches (Aonullah et al., 2016; Palaiokostas et al., 2018), and last but not least wider knowledge of susceptibility of other cyprinid and non-cyprinid fish species helps to implement such task of prevention. Eventhough the improving of the immune capacity of fish by vaccination or genetic selection (Van Muiswinkel et al., 1999., Ronen et al., 2003; Perelberg et al., 2005, 2008; Palaiokostas et al., 2018) are very promising and helpful methods, there exists a danger of pathogenesis and reactivation of attenuated vaccines (Yasumoto et al., 2006; Boutier et al., 2015). Moreover, researches in this field might be time-consuming (Lubroth et al., 2007) and thanks to the vaccine´s unrealibiliy, usage of vaccination against KHV for breeds is is not allowed within the EU as the disease is included in Annex IV, Part II of the European Directive 2006/88/EC (Pretto et al., 2013).

From these reasons, the other way to secure fish stock against KHVD may be testing of susceptibility of various fish species to CyHV-3. This method can provide results relatively soon, and it is also cheap. Thus, the information about susceptibility or non-susceptibility of other fish species to CyHV-3 might be more useful for all breeders whose resources are limited and who need to get a solution of danger of infection relatively fast. In addition, the knowledge about susceptibility can also help breeders to insure that they can stock those fish species in one pond together with common carp and koi. Such information about "CyHV-3-free-ability" or "CyHV-3-carrier-disability" of fish stocked with common carp or koi is not only the most useful or the most valuable, but also the cheapest way how to manage their broodstock.

Various species of cyprinid and non-cyprinid fish may share water environment with affected fish naturally or in husbandry polyculture. This situation resulted in the requirement to verify possibility of infection and consequent transmission of CyHV-3. Since KHVD was reported first, it did not take long time when some authors published such investigation. Authors of those studies performed these experiments by cohabitation method (e. g. Perelberg et al., 2003; El-Matbouli et al., 2007). Cohabitation of the same water system is useful because it imitates natural environment where affected (infected) common carp or koi release virus into surrounding water which consequently transmits CyHV-3 to next fish). Perelberg et al. (2003) were probably the authors, who investigated transmission of CyHV-3 to Nile tilapia (*Oreochromis niloticus*), silver perch (*Bidyanus bidyanus*), silver carp (*Hypophthalmichthys molitrix*), goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon idella*). Results showed that all fish of these species survived and, none of them were able to transfer the virus to healthy carps (Perelberg et al., 2003).

Furthermore, no natural outbreaks of KHVD were observed in commonly farmed cyprinid species, such as silver carp (*Hypophthalmichthys molitrix*), and non-cyprinids such as channel

catfish (*Ictalurus punctatus*) (Takashima et al., 2005). These herbivorous species are often breed in polyculture with common carp, but no signs of disease or mortalities were observed in these species either under standard polyculture conditions nor under experimental cohabitation with infected fish (and direct exposure to the virus) (Takashima et al., 2005; OIE, 2019).

There were strong debates about goldfish (*Carassius auratus*), if it was susceptible species or not. Finally, this species was confirmed as asymptomatically susceptible (McColl et al., 2013). Authors, who as first investigated susceptibility of goldfish were as follows, El-Matbouli et al. (2007), Sadler et al. (2008). But from results of these studies it was not clear if CyHV-3 was able to replicate in goldfish tissue or if goldfish represented only a mechanical vector (McColl et al. 2013). Subsequent a study of El-Matbouli and Soliman (2011) focused on goldfish again due to overcome these uncertainties. They demonstrated that CyHV-3 replicated in the goldfish (without causing clinical signs of disease), and they also found out that goldfish could disseminate the virus to susceptible carp (El-Matbouli and Soliman, 2011).

Kempter and Bergmann (2007) examined fish from wildlife water environment from the Northern Poland region (species from lakes, river firths and rivers) for susceptibility to CyHV-3 and they found vimba bream (*Vimba vimba)* and common dace (*Leuciscus leuciscus)* positive for presence of CyHV-3 DNA. Next study of Kempter et al. (2009) reported that Atlantic sturgeon (*Acipenser oxyrinchus*) and Russian sturgeon (*Acipenser gueldenstaedtii*) are susceptible to CyHV-3. Our examination of sterbel, the hybrid between sterlet *(Acipenser ruthenus)* and beluga *(Huso huso)*, revealed this species positive for CyHV-3 DNA (Pospichal et al., 2016). The susceptibility of stone loach (*Barbatula barbatula*, family *Balitoridae*) might suggest that species of the family *Cobitidae* (previous family of stone loach) could be generally susceptible to CyHV 3; for instance, Kempter et al. (2012) reported susceptibility of spined loach. Nevertheless, we did not find that those fish are able to transmit CyHV-3 to naïve fish (Pospichal et al., 2016).

Among next species examined for susceptibility to CyHV-3, grass carp *(Ctenopharyngodon idella)* (Bergmann et al., 2009; Radosavljević et al., 2012), common bream *(Abramis brama)* (Kempter et al., 2012), tench (Fabian et al., 2012; Kempter et al., 2012; Radosavljević et al., 2012), rudd (Fabian et al., 2012), ruffe *(Gymnocephalus cernua)* (Kempter et al., 2012), European perch *(Perca fluviatilis)* (Fabian et al., 2012; Kempter et al., 2012), atlantic sturgeon *(Acipenser oxyrhynchus)* (Kempter et al., 2009), bushymouth catfish *(Ancistrus dolichopterus)* (Bergmann et al., 2009), brown bullhead *(Ameiurus nebulosus)* (Fabian et al., 2012), three spined stickleback *(Gasterosteus aculeatus)* (Fabian et al., 2012) and northern pike *(Esox lucius)* (Fabian et al., 2012) were found positive for viral DNA. Moreover, ongoing experiments focused on dissemination of CyHV-3 to naïve carp revealed grass carp *(Ctenopharyngodon idella)*, silver carp *(Hypophthalmichthys molitrix)* (Kempter et al., 2012; Radosavljevic et al., 2012), common bream *(Abramis brama)* (Kempter et al., 2012), tench *(Tinca tinca)* (Fabian et al., 2012; Kempter et al., 2012; Radosavljevic et al., 2012), ruffe *(Gymnocephalus cernua)* (Kempter et al., 2012) and European perch *(Perca fluviatilis)* (Kempter et al., 2012) as species able to transmit this herpesvirus.

Our previous cohabitation experiments with topmouth gudgeon *(Pseudorasbora parva)* which represents a significant threat to Czech aquaculture thanks to its invasiveness and carrier-ability of various bacterial diseases (Margaritov and Kiritsis, 2011) showed that topmouth gudgeon was not susceptible to CyHV-3 under standard conditions imitating natural aquaculture environment (Pospichal et al., 2018). Regarding the fact, that a physical stress (such as rough handling, scaring by net or other injuries) may affects the skin mucus followed by an increase of fish susceptibility to infection by pathogens (Roberts and Ellis, 2012), we treated topmouth gudgeon by various methods which could mimic the most common

stresses occurring on farms, such as escaping reflex inducing by netting, and removing of skin mucus (Pospichal et al., 2018). These conditions resulted slight enhanced the susceptibility of topmouth gudgeon to CyHV-3 if the harder method was performed (Pospichal et al., 2018). From physiological aspect, the mucus layer is produced by goblet cells (Ringo et al., 2007) and mucus covering the epidermis provides mechanical, chemical and nonspecific immune protection against pathogenic microorganisms (Ellis, 2001; Fontenont and Neiffer, 2004). The main components of mucus are large filamentous, highly glycosylated glycoproteins which make a viscous matrix (McGuckin et al., 2011) composed of a mixture of ions, lipids and proteins (Thornton and Sheehan, 2004; Bansil and Turner, 2006). Antimicrobial peptides (AMPs) (Schauber and Gallo, 2008; van der Marel et al., 2012), beta defensins, mucins and claudins (Adamek et al., 2013) are crucial elements of mucus as well. The expression of all of these molecules is diminished and in the case of claudin marked downregulation of expression was observed during CyHV-3 infection (Adamek et al., 2013; Adamek et al., 2014). Claudin is a major protein of epithelial tight junctions and plays an important role in the regulation of mucosal permeability (Adamek et al. 2013). Its decreased expression leads to a high loss of ions and disintegration of skin (Negenborn, 2009) and also to secondary microbial infections (Adamek et al., 2013). Thus, as Negenborn, (2009), Raj et al. (2011), Adamek et al. (2013) and Adamek et al., (2014) suggested, there is no doubt that the loss of skin mucus enhances the entry of pathogens into the fish body. Furthermore, if consequent cohabitation tests focusing on transmission of CyHV-3 are applied, the usage of stress such as nesting may induce release of virus from carrier fish (Bergmann and Kempter, 2011) and it seems to be a very important part of experiments to examine potential transmission of CyHV-3 to healthy cohabitated fish.

Debates about susceptibility and "transmission-ability" of goldfish which had to be repeated for confirmation (McColl et al., 2013) suggest that the right method for examination of susceptibility and transmission of virus must be considered. Unfortunately, similar questions are raised also in case of topmouth gudgeon. Obviously, this species reacted positively on loss of skin mucus and qPCR revealed presence of CyHV-3 DNA (Pospichal et al., 2018). On the other hand, only one naïve koi was found as CyHV-3 DNA positive after cohabitation with a group of topmouth gudgeons, but none of the topmouth gudgeons from this group was found viral DNA positive (Pospichal et al., 2018). This finding shows similarity to results of Sadler et al. (2008) and El-Matbouli et al. (2007) and may suggested that topmouth gudgeon may play a role as a mechanical vector (McColl et al., 2013). Other possible doubt might be a dilution factor during preparation of samples and negative pooling effect when various tissues of affected fish are pooled. Samples of gill, kidney and spleen are recommended (More et al., 2017; OIE, 2019). Moreover, especially in case of suspicion of latency, pooling of intestine which beared viral particles (Dishon et al., 2005; OIE, 2019) and brain (Eide et al., 2011a,b; OIE, 2019) may be also acceptable (OIE, 2019). We must keep in mind that although sensitivity of PCR based methods is commonly high (it is different according to used type) (Bergmann et al., 2010), dilution of virus thanks to excessive pooling or during preparation of samples might lower the chance of detecting CyHV-3 DNA (Matras et al., 2012; Monaghan et al., 2015). Therefore, in our studies (Pospichal et al., 2016; Pospichal et al., 2018), the sample weight (minimum 25 mg of tissue samples) as well as the dilution factor (acceptable ratio is 1 + 9 w/v) followed recommended limits (EURL, 2015). On the other hand, technique of pooling of samples has still not been rejected and it remains as recommended EURL method, and even pooling of samples from two fish maximal is not excluded (EURL for fish diseases, 2015). Although nested PCR and qPCR are highly sensitive assays for CyHV-3 detection (Monaghan et al. 2015, Bergmann et al., 2010), the rate of positive PCRs findings for CyHV-3 DNA in the tissues of sterbel, stone loach and topmouth gudgeon was relatively low (chapter two and chapter three) (Pospichal et al., 2016; Pospichal et al., 2018). The most probable reason for

such a low rate of positive findings might be just mentioned, like the negative pooling effect. On the other hand, especially in case of stone loach and topmouth gudgeon, this pooling method was used with respect to the small body size of those fish and this procedure reflects our efforts to maximise the chance of positive findings (Pospichal et al., 2016; Pospichal et al., 2018).

For future development of aquaculture, modern biological methods focused on selective cross breeding of natural resistant strains with CyHV-3 susceptible strains, as it is published by Tadmor-Levi et al. (2017), Palaiokostas et al. (2018) and Prchal (2018), will play a decisive role. The analysis of resistance on the molecular level revealed connection to polymorphism of genes in MHC loci (Rakus et al., 2012) which involves immune response and genes of interleukin 10 (IL 10) (Kongchum et al., 2011). Results of resistence of some strains may be consequently exploited for more suitable stock selection (Piackova et al., 2015; More et al., 2017; OIE, 2019). Since strains of carp may differ in susceptibility to CyHV-3, there was a strategy of cross-breeding. Shapira et al. (2005) tested the resistance of Israeli domesticated strains of common carp (Dor 70 and Nasice) and their hybrids with wild Sassan. Hybrids with Sassan showed significantly higher survival in comparison with pure strains in laboratory as well as in field condition. Piackova et al. (2013) evaluated the resistance to CyHV-3 of various local strains of common carp and their hybrids. In accordance with Shapira et al. (2005), they found that strains and hybrids consisted of or derived from *Cyprinus carpio haematopterus* (Amur wild carp; Sassan,which is nowadays separate species *Cyprinus rubrofuscus*) are more resistant then those consisted of or derived from *Cyprinus carpio carpio*. The highest survival rate during experimental infection by CyHV-3 achieved Sassan (97–100%), the lowest rate achieved koi (0–20%). Between these edges fourteen hybrids and strains were splitted and those of them that had got more or less genetic parts of the Sassan exhibited higher survival rate over 70% (Piackova et al., 2015). Ropsha scaly carp (95% of survivors) and newly bred Amur mirror carp (73.3% of survivors) seemed to be very promising for establishment of KHV-resistant stocks (Piacková et al., 2013). Tadmor-Levi et al. (2017) successfully introgressed CyHV-3 resistance from the feral strain "Amur Sassan" into two susceptible cultured strains. Such phenotype was transferable between generations with contributions to resistance from both the resistant feral and the susceptible cultured strains without scales (mirror type). Their back-crossed hybrids of BC_1 families [Yugoslavian Našice x (Sassan x Yugoslavian Našice) and Dor-70 x (Sassan x Dor-70)] indicated successful combination of CyHV-3 resistance with the desirable mirror phenotype (Tadmor-Levi et al., 2017). In addition, the CyHV-3 viral load in tissues throughout the infection of susceptible and resistant fish was applied. Eventhough the resistant fish got infected, viral loads in their tissues were significantly lesser than in case of susceptible fish leading them to survival of the disese (Tadmor-Levi et al., 2017).

Although (according to valid legislative) no vaccine against CyHV-3 is registred and used in EU (Piackova et al., 2015), some researches in this field still continue (Bergmann et al., 2018; Schröder et al., 2018). Inactivated vaccine (by heat treatment or repeated passages on cell cultures) from CyHV-3 (Ronen et al., 2003; Perelberg et al., 2005; Perelberg et al., 2008) seemed to be high virulent for spread usage (Gotesman et al., 2013; Pretto et al., 2013; Bergmann et al., 2018). Moreover, also the questions about function of vaccine and relationship between vaccine against CyHV-3 and its novel strains with their unclear low- or non-pathogenicity, (Engelsma et al., 2013) are rising. It all turns attention of immunoprofylaxis on methods such as genetically engeneered vaccines or usage of immunostimulation with virus eradication (Bergmann et al., 2018) but no more information is available yet. On the other hand, the future in vaccination against CyHV-3 is wide open and can be undoubtly very useful (Bergmann et al., 2018). Evidence of it is research of Schröder et al. (2018), who generated so called deletion mutants of CyHV-3 lacking the nonessential genes encoding

two enzymes of nucleotide metabolism, thymidine kinase (TK, ORF55) and deoxyuridine-triphosphatase (DUT, ORF123). Schröder et al. (2018) performed this genetic intervention in order to obtain safe and efficacious live vaccines. After testing this "mutant CyHV-3" *in vivo*, examination showed that the viral mutant was almost avirulent but still protective. Such kind of vaccine seems to be a prerequisite for economic vaccine production (Schröder et al., 2018) maybe for future global eradication of KHVD.

Conclusions

This dissertation thesis submits results of cohabitation assays of cyprinid (topmouth gudgeon) and non-cyprinid fish species (sterbel – hybrid between sterlet and beluga, stone loach). During these assays, susceptibility of these species to CyHV-3 was examined and consequent transferability of the virus to healthy naïve common carp and koi were also tested. Topmouth gudgeon, were tested under normal (natural) conditions by cohabitation with susceptible species and also under stress conditions that imitated non-natural, but possible situations leading to removing of skin mucus followed by decrease of fish immunity and increase of its susceptibility.

Results of our investigation suggested that all of these species might be asymptomatically susceptible to CyHV-3 (according to positive findings of molecular-biological methods), even though the amount of positive signals were relatively low. Usage of more sensitive PCR (in first study we performed nested PCR, in second study we used also qPCR) also showed low amount of positive fish but, on the other hand, qPCR helped reveal even negative samples tested previously by nested PCR.

Through all scientific articles we can find many results about next possible fish vectors of CyHV-3 which represent concern for common carp aquaculture, but some of them mention doubts on if carrier fish may behave as a mechanical vector or fomite. On the other hand, all of this information about "possible" transmission of CyHV-3 by other fish species may warn future readers at least and recommend not forgetting to include this species into quarantine. Therefore, according to criteria leading to assessment to put suspicious species into the list of susceptible or vector animal species related to CyHV-3 as it is mentioned in EFSA journals (More et al., 2017), I would suggest to enlarge this list with fish species that were examined by us – nominally: stone loach (*Barbatula barbatula*), sterbel – a hybrid between sterlet (*Acipenser ruthenus*) and beluga (*Huso huso*), and topmouth gudgeon (*Pseudorasbora parva*).

Finally, future investigations on this topic should focus not only on the potential asymptomatic susceptibility of mentioned topmouth gudgeon or other species to CyHV-3 (i.e., if they might be carriers), but also on understanding the dissemination of virus through the tissues of examined fish.

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ENGLISH SUMMARY

Susceptibility of cyprinid and non-cyprinid fish species to CyHV-3

Aleš Pospíchal

Cyprinid herpesvirus 3 (CyHV-3) also known as koi herpesvirus (KHV) is a causative agent of highly contagious disease (koi herpesvirus disease) and can cause significant losses in fish stocks. The disease is restricted to koi and common carp, but recent investigations have shown that other cyprinids as well as non-cyprinid species may be asymptomatically susceptible to this virus and might play roles as potential carriers or can contribute to biological conservation leading to persistence of this virus in environment. Therefore, it seems to be important to verify not only the susceptibility of other cyprinid and non-cyprinid species, but also their ability to transmit CyHV-3 infection to susceptible species.

At the beginning of our investigation, the susceptibility of stone loach (*Barbatula barbatula*) and sterbel – a hybrid between sterlet *(Acipenser ruthenus)* and beluga *(Huso huso)* to cyprinid herpesvirus 3 was tested by means of their cohabitation together with naïve koi and intraperitoneally KHV-infected koi (this part of cohabitation is called primary challenge). When cumulative mortality of koi reached 100% in at least one experimental group, a secondary challenge of cohabitation was started (a portion of the surviving stone loach and sterbel were transferred to tanks with other naïve koi). All dead as well as surviving fish were investigated for the presence of KHV DNA in pooled samples of tissue from individual fish by nested PCR. Sampling for PCR from surviving fish was performed on the 15th dpi and on the 30th dpi of the primary challenge, and on the 30th dpi of the secondary challenge. During the primary challenge (up to the 30th dpi), average cumulative mortality in duplicated experimental groups was as follows: koi 100%, sterbel and stone loach both 5%. In the primary challenge, no surviving stone loach or sterbel sampled on the 15th dpi or those that died previously were found to be positive for viral DNA. Results of PCR revealed the presence of KHV DNA in 95% of cohabited naïve koi samples. PCR analysis of tissues taken from surviving fish on the 30th dpi revealed the presence of viral DNA in 77.8% (7/9) of stone loach and in 22.2% (2/9) of sterbel. Cumulative mortality of fish in the secondary challenge was 100% for stone loach and for koi co-habitating with them, and 50% for koi cohabitating with sterbel, which all survived. The reason of such mortality was malfunction of biofilters or bacterial infection which occurred during secondary challenge in these experimental groups.

Our next investigation focused on assessment of the susceptibility of highly invasive cyprind fish species – topmouth gudgeon (*Pseudorasbora parva*). In this case, we performed two different challenges, but both of them were based also on cohabitation. First assay, when tested topmouth gudgeon were kept under standard conditions, did not reveal the presence of CyHV-3 DNA in the tissues of this species after cohabitation with infected koi. Consequently, we changed the experimental conditions and we applied two stress factors (scaring by net and removal of skin mucus) which would presumably mimic the stress most commonly encountered in the wild. Both experiments (without and with stress factors) consisted of primary and secondary challenges as well as in all previous cases. In both the no-stress and stress experiments, the first challenge was focused only on testing the susceptibility of the topmouth gudgeon to the virus. With the secondary challenge, we investigated potential viral transmission from the topmouth gudgeon to healthy naive koi after exposure to stress factors. All fish (dead, surviving and sacrificed) were tested for the presence of CyHV-3 DNA using nested PCR (no-stress experiment) and real-time PCR (stress experiment). After the primary challenge of the no-stress experiment, PCR did not reveal the presence of CyHV-3

DNA in any specimen of cohabited topmouth gudgeon, but all specimens of dead koi were CyHV-3 DNA-positive. PCR of fish tissues subjected to the secondary challenge did not show the transfer of virus to naive fish. After exposure to stress (removal of skin mucus), qPCR revealed four out of five samples (80%) of topmouth gudgeon to be positive for CyHV-3 DNA. Two out of five samples (40%) of topmouth gudgeon treated by scaring were found to be positive for the presence of viral DNA. Real-time PCR after the secondary challenge did not reveal any viral DNA positivity in specimens of topmouth gudgeon from groups previously exposed to stress. The stress experiments show that removal of skin mucus might potentially lead to susceptibility of topmouth gudgeon to CyHV-3 infection, but the transmission of the virus to koi carp was not observed.

The presented results of cohabitation assays of cyprinid and non-cyprinid fish species (nominally above) indicate other fish species showing their possible asymptomatic susceptibility to CyHV-3. Even though PCRs positive findings of KHV DNA in tissues of fish were relatively low, the hybrid between sterlet and beluga, the stone loach and topmouth gudgeon (exposed to loss of skin mucus) seem to might be slightly susceptible to cyprinid herpesvirus 3. On the other hand, despite the high mortality of koi and stone loach which occurred in the secondary challenge, our results do not suggest that any fish of koi and stone loach, nor any of the sturgeon hybrids might be considered to be positive for KHV DNA. Thus, we could not prove that they can transfer this virus to naïve koi. In a similar way, also in case of topmouth gudgeon, we found possible enhancement of susceptibility to CyHV-3 when skin mucus was removed, but viral transmission in such way treated topmouth gudgeon was not proven too.

CZECH SUMMARY

Vnímavost kaprovitých a nekaprovitých druhů ryb k CyHV-3

Aleš Pospíchal

„Kapří herpesvirus 3" – Cyprinid herpesvirus 3 (CyHV-3) známý také jako koi herpesvirus (KHV) je původcem vysoce nakažlivé choroby (koi herpesviróza) a může v populacích ryb způsobit značné ztráty. Tato choroba je v přirozených podmínkách hostitelsky omezena na barevnou varietu kapra – koi a na kapra obecného. Nedávné výzkumy však odhalily, že i jiné kaprovité a nekaprovité druhy ryb mohou být vůči tomuto viru asymptomaticky vnímavé a mohou hrát roli potenciálních nosičů viru nebo mohou přispět k biologickému „zakonzervování" viru v životním prostředí. Proto se ukazuje za důležité ověřit nejen vnímavost jiných kaprovitých či nekaprovitých druhů ryb, ale také ověření jejich schopnosti přenášet infekci způsobenou KHV na vnímavé druhy.

Na počátku našeho výzkumu byla testována vnímavost dvou, zatím nezkoumaných druhů, a to mřenky mramorované (*Barbatula barbatula*) a takzvaných sterbelů – hybridů mezi jesetery malými (*Acipenser ruthenus*) a vyzou velkou (*Huso huso*) vůči KHV pomocí metody kohabitace (sdílení stejného vodního prostředí spolu s naivními koi kapry a koi kapry intraperitoneálně infikovanými KHV – primární část pokusu). Když kumulativní mortalita koi kaprů dosáhla 100 % alespoň v jedné experimentální skupině, byla zahájena sekundární část pokusu (část přeživších mřenek a sterbelů byla přenesena do tanků s jinými naivními koi kapry). Všechny uhynulé i přeživší ryby byly vyšetřovány na přítomnost KHV DNA ve směsných vzorcích tkáně z jednotlivých ryb pomocí nested PCR. Odběr vzorků pro PCR z přeživších ryb byl prováděn v 15. den po infekci (dpi) a v 30. dpi prvotní části testu a v 30. dpi sekundární části testu. Během prvotní části testu (až do 30. dpi) byla průměrná kumulativní mortalita v duplikovaných experimentálních skupinách následující: koi 100%, sterbel a mřenka mramorovaná – oba 5%. V primární části pokusu, žádná přeživší mřenka nebo sterbel, který byl odebrán v 15. dpi nebyl pomocí PCR analýzy shledán KHV DNA pozitivní. Stejně tak i ti jedinci těchto druhů, které dříve uhynuly, nebyly pozitivní na virovou DNA. Výsledky PCR ukázaly přítomnost KHV DNA v 95 % vzorků koi kaprů. PCR analýza tkání odebraných z přeživších ryb k 30. dpi odhalila přítomnost virové DNA v 77,8 % (7/9) mřenky a 22,2 % (2/9) sterbela. Kumulativní mortalita ryb v sekundární části pokusu byla 100% pro mřenky mramorované a pro koi kapry s mřenkami kohabitovaných a 50% pro koi kapry kohabitovaných se sterbelem, kteří přežili všichni. Nejpravděpodobnějším důvodem těchto úhynů byla porucha biofiltrů nebo přítomnost bakteriální infekce, která se vyskytla během sekundární části pokusu v těchto experimentálních skupinách.

Náš další výzkum byl zaměřen na zhodnocení vnímavosti vysoce invazivního druhu – střevličky východní (*Pseudorasbora parva*). V tomto případě jsme provedli různé způsoby zacházení s rybou. Ve všech případech však byly testy založeny na kohabitaci. V prvním pokusu byla střevlička východní ve standardních (přirozených) podmínkách, což však neodhalilo možnost nákazy KHV a přítomnost virové DNA v tkáních po kohabitaci s infikovanými koi kapry. Následně jsme změnili experimentální podmínky a aplikovali dva stresové faktory (plašení sítí a odstranění kožního hlenu), což byly stresy zvolené k napodobování nejčastěji se vyskytujících stresů ve volné přírodě. Oba experimenty (bez a se stresovými faktory) se skládaly z prvotních a sekundárních částí testů, stejně jako v případě prvního pokusu, kde byly zkoumány mřenky a hybridi. V experimentech bez stresu i ve „stresových experimentech" byla prvotní část pokusu zaměřena pouze na testování vnímavosti střevličky ke KHV. V sekundární části pokusu jsme opět zkoumali potenciální přenos viru ze stresovaných střevliček na zdravé naivní koi kapry. Všechny ryby (mrtvé, přeživší a usmrcené) byly testovány na přítomnost DNA KHV pomocí

nested PCR (v pokusu bez použití stresu) a kvantitativní – real-time PCR, zkráceně: qPCR (stresový experiment). Po primární infekci v pokusu bez stresu, PCR neodhalila přítomnost virové DNA v jakémkoli vzorku kohabitovaných střevliček. Navzdory tomu však všechny vzorky mrtvých koi byly pozitivní na přítomnost virové DNA. PCR rybích tkání podrobených sekundární části pokus (testu) neprokázala přenos viru na naivní koi kapry. Po vystavení střevliček východních stresu (kdy byl odstraněn kožní hlen), odhalila qPCR čtyři z pěti vzorků (80 %) střevliček východních pozitivních na KHV DNA. Dále jsme zjistili, že dva z pěti vzorků (40 %) střevliček východních, které byly plašeny pomocí síťky, byly pozitivní na přítomnost virové DNA. Real-time PCR prováděná po sekundární části testu neodhalila žádnou virovou DNA ve vzorcích střevličky východní pocházejících ze skupin dříve vystavených stresu. Stresové experimenty naznačují, že odstranění kožního hlenu by mohlo vést k potenciální vnímavosti střevličky východní vůči KHV. Případný přenos viru na koi kapra střevličkou východní se nám však nepodařilo prokázat.

Prezentované výsledky kohabitačních testů kaprovitých a nekaprovitých druhů ryb (jmenovitě výše) tedy poukazují na další druhy vykazující jejich možnou asymptomatickou vnímavost vůči KHV. I když PCR pozitivní nálezy virové DNA v tkáních ryb byly relativně nízké, zdá se, že hybridi mezi sterletem a belugou, mřenka mramorovaná a střevlička východní (které byl otírán kožní hlen) by mohli být mírně vnímavé vůči koi herpesviru. Na druhou stranu, i když byla mortalita naivních koi kaprů v sekundárním testování prvního pokusu vysoká, žádná z ryb s nimi kohabitovaných druhů (mřenky mramorované a hybridů jesetera), nebyla KHV DNA pozitivní. Nemohli jsme tudíž dokázat, že tyto druhy mohou KHV přenést na naivní koi kapry. Podobně tomu bylo i v případě střevličky východní, kdy jsme sice zjistili možné mírné zvýšení vnímavosti tohoto druhu vůči KHV po odstranění kožního hlenu, avšak přenos viru na vnímavé druhy (koi kapr) těmito střevličkami nebyl také prokázán.

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LIST OF PUBLICATIONS

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Pospichal, A., Pokorova, D., Vesely, T., Piackova, V., 2018. Susceptibility of the topmouth gudgeon (*Pseudorasbora parva*), and hybrids to CyHV-3 under no-stress and stress conditions. Veterinarni Medicina 63: 229–239. (IF 2018 = 0.489)

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