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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.

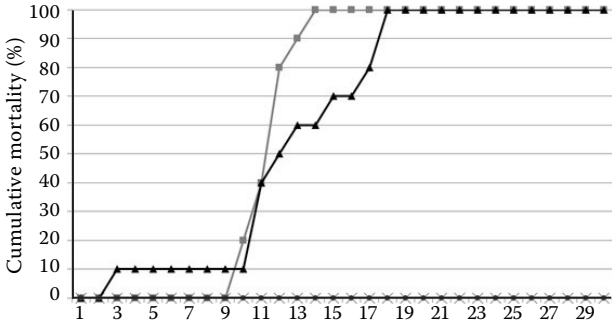

Figure 1. Graph of cumulative mortality of koi and topmouth gudgeon under no-stress conditions

= 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

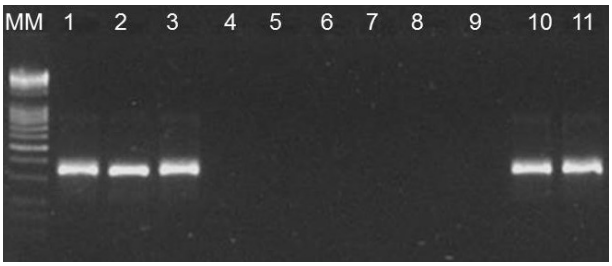

Figure 2. Electrophoresis of PCR products from the non-stress experiment (positive band is 348 bp)

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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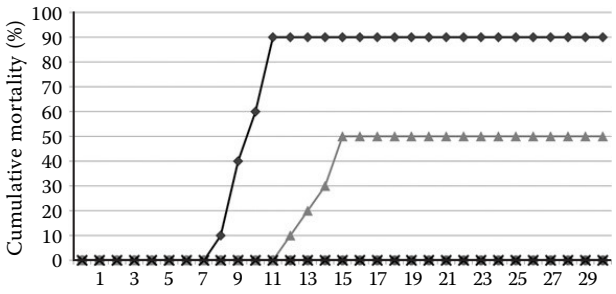

Figure 3. Graph of cumulative mortality of koi and topmouth gudgeon under stress conditions

—◆— = "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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