Serological responses to koi herpesvirus (KHV) in a non-cyprinid reservoir host

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Abstract

Koi herpesvirus (KHV) is a highly contagious virus that causes KHV disease (KHVD) inducing high mortality in carp and koi (Cyprinus carpio L.). In the late stage, latency occurs with very low, often non-detectable virus concentrations, which represents a challenge for virus detection. After validation according to OIE recommendations, an antibody ELISA was established to recognize antibodies of C. carpio against KHV infection. In this study, the ELISA was modified to detect anti-KHV antibodies from a non-cyprinid fish. Experimentally infected rainbow trout (Oncorhynchus mykiss) were able to transmit KHV to naïve carp at two different temperatures, demonstrating their potential as a reservoir host. At 20°C, KHVD was induced in carp but not at 15°C. Unexpectedly, rainbow trout developed humoral response against KHV at both temperatures. In contrast to carp, at 15°C trout produced neutralizing antibodies but not at 20°C. While antibodies obtained from infected carp sera reacted in a similar way against all KHV, antibodies from rainbow trout sera reacted differently to the same isolates by ELISA. The data show that even when non-cyprinid fish species are infected with KHV, they can produce antibodies that differ from those observed in carp.

Keywords: carp/rainbow trout sera, enzyme-linked immunosorbent assay (ELISA), koi herpesvirus (KHV) isolates, antibodies, neutralising antibodies

Introduction

Koi herpesvirus (KHV), taxonomically cyprinid herpesvirus 3 (CyHV-3), is a highly contagious viral agent lethal to carp or koi (Cyprinus carpio L.) of all ages (Dishon et al., 2005) and induces KHV disease (KHVD) (Bergmann et al., 2009; Gilad et al., 2002). Since clinical outbreaks were reported from Israel in 1998 (Hedrick et al., 2000), it spread globally to countries with carp or koi industry (Haenen et al., 2004). Generally, disease outbreaks are observed at water temperatures above 17°C (Gilad et al., 2004), but there is also evidence of disease or virus reactivation at 10°C (Baumer et al., 2013) or even lower at 4°C (pers. comm., A. Nilz). One characteristic of all known herpesviruses is that they induce latency or persistence (Roizman & Pellett, 2001; St-Hilaire et al., 2009). There is also strong evidence that survivors or infected but apparently healthy fish can become asymptomatic carriers (Uchii et al., 2009), which are able to release infectious virus to susceptible hosts. Transmission of infectious KHV has also been demonstrated in fish species resistant to KHV, as detected by molecular methods (Bergmann et al., 2010; Matras et al., 2019). While detection of the virus or its DNA in the latent/persistent stage of infection is difficult, a possible screening of fish populations can be achieved using serological methods such as serum neutralization assay (SNT), enzyme-linked immunosorbent assay (ELISA) or Western blot (WB). SNT and WB are specific and sensitive but very labour-intensive. The antibody ELISA is a very fast and sensitive assay, but specificity can be a challenge for low antibody titres. The ELISA method is, however, very effective for large numbers of samples, that is to obtain an overview of the reaction status of the tested population. The World Organisation for Animal Health (OIE) recommends ELISA testing not only for targeted surveillance of adults and presumptive diagnosis in terms of virus detection, but also for disease freedom declaration (OIE, 2019a) if validated assays are available. According to the OIE guidelines OIE. (2019b), an ELISA for the detection of antibodies against KHV in carp/koi was

established (Bergmann et al., 2017). By applying this ELISA with modifications, and using SNT, the antibody response of a non-cyprinid reservoir host (rainbow trout) was investigated at temperatures natural to both species.

Materials and methods

Viruses, cells and antigen preparations

Different KHV isolates (n = 5, Table 1) were replicated onto common carp brain cells (CCB cells; Neukirch et al., 1999). After 90% cytopathic effect (CPE) had been observed, the viruses were harvested by freezing at −80°C, thawed once and prepared for ELISA according to Bergmann et al. (2017).

Table 1 KHV isolates used and prepared for the ELISA as antigen

Isolate	**Origin**	**KHV lineage***	**Remarks**
KHV-T	Taiwan	Asian	cloned isolate from koi
KHV-E	England	European	isolate D 132 from koi
KHV-Israel	Israel	Asian/European	new isolate** from koi
KHV-G 1	Germany	European/Asian	isolate from koi
KV3***	Israel	European	attenuated vaccine virus

* according to Klafack et al. 2017

** not the reference isolate KHV-I (Aoki et al. 2007) which was used for SNT

*** commercial vaccine against KHVD (Phibro Animal Health)

After preparation of the antigen obtained following ultracentrifugation virus purification from the sucrose gradient layers at 40 and 50% (Table 2), the protein content was determined (Bergmann et al., 2017). Not only the virus obtained from the 50% layer but also from the

40% layer had proved to be useful as antigen for the assay. The viruses were confirmed by electron microscopy after concentration by ultracentrifugation (data not shown).

Table 2 Protein concentration from the purified KHV isolates used as ELISA antigen

Isolates	40% sucrose layer (mg/ml)	50% sucrose layer (mg/ml)	Remarks
KHV-T	0.2	6.1	highest
KHV-E	0.2	1.4	-
KHV-Israel	0.6	1.4	-
KHV-G 1	0.2	1.1	lowest
KV 3*	0.8	1.8	-

* KV3 is the commercial KHV vaccine (live attenuated) produced by Phibro LtD, former KoVax LtD in Israel.

Animal experiment

Fish

Steelhead trout (Oncorhynchus mykiss, n = 30, 80–100 g) from a commercial farm in Thuringia (Germany) were kept in a recirculating system (450 L) at 15°C and fed twice a day with commercial trout food. Up to 100 L water was exchanged daily. It was confirmed that all rainbow trout were free of KHV and all notifiable diseases according to EU legislation, for example viral haemorrhagic septicaemia (VHS), infectious haematopoietic necrosis (IHN) and infectious salmon anaemia (ISA). From the latter disease, Germany is proven free of ISA. From the stock, 10 rainbow trout remained at 15°C (group 1). Another 10 rainbow trout (group 2) were selected to adapt to 20°C for 14 days. Group 3 (n = 10) was kept as a negative control at 15°C.

A similar temperature regime was applied with common carp (n = 16). First, they were all adapted to 15°C for at least four weeks. Then, eight carp were selected for adaptation to 20°C

for 14 days. At each water temperature treatment, three carp were tested for freedom from spring viraemia of carp virus (SVCV), carp poxvirus, goldfish herpesvirus and KHV by RT-PCR (Koutná et al., 2003) and PCR using sequence analysis (Bergmann, Riechardt, et al., 2010; Bergmann et al., 2006; Engelsma et al., 2013; Gilad et al., 2002).

Experimental scheme

After adaptation, rainbow trout were kept and adjusted to 15 and 20°C. Non-lethal samples were collected for virology (gill swabs for PCR) and serology (blood for serum or plasma after leucocyte separation). At each temperature, ten rainbow trout were infected by immersion with KHV-E at a concentration of 103 TCID50/mL in 20 L at 15 or 20°C, respectively, for one hr. After that, rainbow trout were returned to their aquarium. Every day, approximately 100 to 120 L of water was exchanged up to day seven post-infection (dpi). On 7 dpi, five carp adapted to 15 and 20°C, respectively, were transferred to the aquarium with the rainbow trout but separated from these fish by a perforated slice beyond the rainbow trout in a flow direction of the water. This approach facilitated the induction of a waterborne infection. Then, the rainbow trout were caught by dip net, retained out of the water for approximately 30 s and returned to the same aquarium at the same temperature for KHV reactivation. Samples (gill swabs and blood) were collected on 0, 2, 3, 7, 14 and 31 dpi from rainbow trout and 0, 14 and 21 dpi (post-cohabitation) from carp, corresponding to rainbow trout from the experiment at 14 and 31 dpi.

Sample testing

Gill swabs and separated leucocytes, adjusted to 107 cells/mL, were tested by KHV qPCR (Gilad et al., 2002, modified according to Bergmann, Riechardt, et al. (2010)) and a one tube semi-nested KHV PCR (Bergmann, Riechardt, et al., 2010). Sera or plasma obtained from

carp were tested by KHV antibody ELISA (Bergmann et al., 2017). The ELISA for carp was only slightly modified as sera or plasma from rainbow trout was examined by the same procedure, but instead of anti-carp IgM, anti-trout IgM monoclonal antibody (mab) 4C10 was used in a dilution of 1:1.000 in PBS-T. The basic dilution of carp sera was 1:300, and that of rainbow trout sera was 1:100. The serum neutralization assay (SNT) was carried out according to Matras et al. (2012) using the antigen KHV-I Aoki et al. (2007), European lineage).

Antibody ELISA procedure

The ELISA was carried out according to Bergmann et al. (2017) with slight modifications made with regard to the antigen and the mabs used. The antigens were prepared and adjusted to 10 µg/ml and plated in a final concentration of 1.0 µg/100 µl per well. For the antibody ELISA with carp sera, the conjugated monoclonal antibody against carp IgM C16-HRP (Aquatic Diagnostics, UK) was used. With sera from rainbow trout, a double indirect ELISA was carried out with mab 4C10 and the goat anti-trout IgM HRP conjugate (Sigma, Germany) due to a missing direct conjugated secondary mab.

Statistical analysis

To evaluate the modified assays, results from the carp and rainbow trout sera values, variances, standard deviation and assay values were again calculated, normalized against the background of the plates and OD values of proven negative sera, and compared with each other to estimate the cut-off value of the ELISAs. The mean values and significant differences were calculated using the t test for the OD values and reactions by SNT.

Results

All sera were tested with both secondary antibodies, anti-carp-IgM and anti-trout-IgM. No cross-reaction occurred by ELISA between the two species using the heterologous mab.

Statistical evaluation and cut-off determination

To determine an appropriate OD cut-off for the ELISA with rainbow trout sera, 10 serum samples from specific pathogen-free (SPF) rainbow trout (Japanese female clones at FLI) and 10 serum samples from a commercial farm in Mecklenburg-Western Pomerania were used. All serum samples were tested by SNT with negative results. Gill swabs were taken and tested by qPCR (Bergmann, Riechardt, et al., 2010) to detect KHV infection. Again, only negative results were obtained. All rainbow trout sera were diluted 1:50 to 1:400 and were tested at least three times, always including replicates. For the assessment, 1:100 dilutions of the sera were used. The OD never reached the absolute values >OD 0.1. Results for rainbow trout sera are summarized in Table 3. The "grey zone" OD value for the rainbow trout sera was determined to be between 0.1 and 0.2. From OD 0.201 at a dilution 1:100, the sera were considered to be KHV antibody-positive, which included a safety value for negative sera of OD 0.072.

Table 3 Analysis of detection cut-off using KHV negative sera from rainbow trout

Sera	mean value	variance	standard deviation (SD)	SD x 3	absolute OD value
1-10*	0.079	0.00027721	0.01664966	0.049948974	0.128
11-20**	0.085	0.00040711	0.00638053	0.019141578	0.104
all plates	0.082	0.00037416	0.01151509	0.034545276	0.116

* steelhead (rainbow) trout form a commercial farm

** Japanese rainbow trout clones

Animal experiment at 15°C

Carp and rainbow trout stayed healthy, unaffected and without any pathological findings over the entire period of the experiment, that is 31 dpi and 21 dpi, respectively. In rainbow trout, all samples from infected fish collected for detection of KHV were considered to be negative until 6 dpi. From 6 dpi, by the end of the experiment, KHV-positive signals were detected in gill swabs and in separated leucocyte samples by semi-nested PCR (Bergmann, Riechardt, et al., 2010) and qPCR (Gilad et al., 2004, modified according to Bergmann, Riechardt, et al. (2010)), respectively. At the end of the experiment at 15°C, 100% of the rainbow trout and carp samples were considered to be KHV qPCR-positive, but only four of five samples from carp were tested positive by semi-nested PCR. The averages of the cq values were very similar from gill swab samples (Table 4). KHV was only found in gill swab samples, but not from separated leucocyte samples, of both species at 31 (rainbow trout) or 21 (carp) dpi.

Table 4 Detection of KHV using non-lethal sample collection (gill swabs)

Samples	Temperature	Positive ratio	Mean cq value*	Semi-nested PCR pos.
rainbow trout (31 dpi)	15°C	10/10	30.20	10/10
carp (21 dpi)	15°C	5/5	29.33	4/5

* KHV qPCR (Gilad et al. 2004, modified according to Bergmann et al. 2010)

From 7 dpi, positive reactions were found by antibody ELISA using rainbow trout sera (Figure 1) but not with carp sera. Positive reactions with carp sera first started on day 15 after cohabitation. The rainbow trout antibody reaction against the Asian lineage was considerably stronger compared with that against the European lineage.

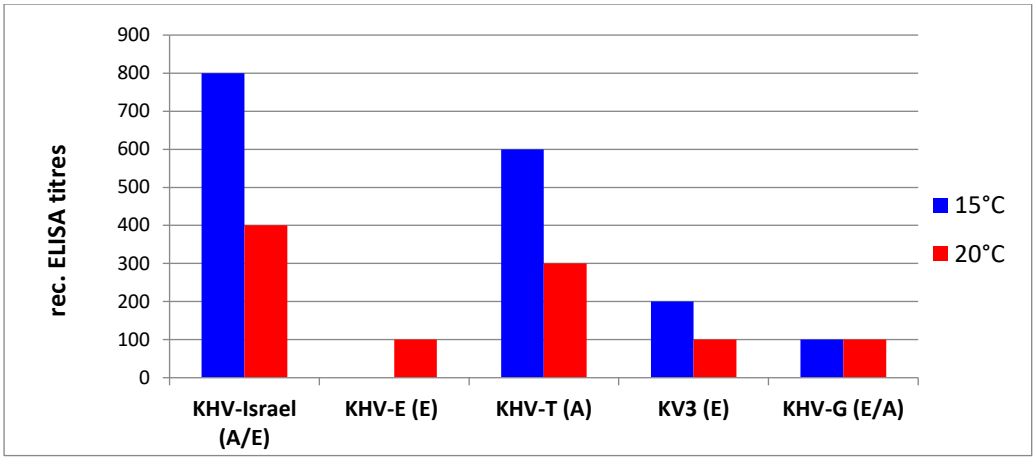

Figure 1 Reaction of sera from rainbow trout (n = 10) infected by KHV-E on 7 dpi at two different water temperatures.

Generally, 26% of the rainbow trout sera were considered to be KHV antibody-positive. In contrast, 100% of the carp that were kept at 15°C developed antibodies against KHV.
Carp sera reacted positively with much higher titres compared with rainbow trout sera. Seven of the ten rainbow trout sera samples reacted positive by SNT, but only one carp serum sample out of five developed neutralizing antibodies at 15°C. However, where fish were positive the average SNT titres were similar for both species (Table 5).

Table 5 General serological reactions of rainbow trout and carp sera by ELISA and SNT

Sera	Temperature	Positive ratio ELISA	Rec. average ELISA titre*	Positive ratio SNT	Rec. average SNT titre**
rainbow trout (31 dpi)	15°C	13/50	98	7/10	11,2
carp (21 dpi)	15°C	25/25	5.100	1/5	12,8

* value against five antigens (KHV-Israel, KHV-E, KHV-T, KV3 and KHV-G)

** SNT with KHV-I (Aoki et al. 2007, European lineage)

The reactions of rainbow trout and carp sera against the five ELISA antigens differed considerably. While antibodies with very high titres against all KHV antigens were detectable by ELISA from carp sera, rainbow trout sera were considered to be positive with three antigens only (KHV-Israel, KHV-E and KHV-T). Using the antigens from KV3 and KHV-G, only one serum sample contained antibodies against KHV. Rainbow trout sera showed the strongest reaction with KHV-Israel (Asian/European (A/E) linages), followed by KHV-T (Asian lineage (A)) and KHV-E (European lineage (E), used for infection) and hardly reacted with antigens from KV3 (E) and KHV-G (European/Asian lineages (E/A)). In contrast, carp antibodies against KHV most strongly reacted with the antigen from the European lineage (KHV-E and KV3), less but still strong with the Asian (KHV-T) and European/Asian lineages (KHV-G) and least with the antigen from KHV-Israel (Asian/European lineages) (Table 6).

Table 6 ELISA reaction of rainbow trout and carp sera with antigens of different KHV lineages from the 15°C experiment

	KHV-Israel (A/E)		KHV-E (E)		KHV-T (A)		KV3 (E)		KHV-G (E/A)	
	Pos.	titre*	Pos.	Titre	Pos.	titre	Pos.	titre	Pos.	titre
rainbow trout	4/10	1:210	4/10	1:110	3/10	1:130	1/10	1:20	1/10	1:20
carp	5/5	1:140	5/5	1:7,040	5/5	1:5,520	5/5	1:7,040	5/5	1:5,760

* average titres of 10 rainbow trout and five carp sera

With KHV-Israel and KHV-T, rainbow trout sera reacted positively with titres between 1:100 and 1:800, with KHV-E 1:100 to 1:400 and with KV3 and KHV-G 1:200 only. In contrast, carp sera reacted positively with titres between 1:3,200 and 12,800 against the antigens from the European lineage (KHV-E and KV3), with 1:3,200 to 6,400 against KHV-G, followed but

not different from KHV-G with titres between 1:400 and 1:12,800 against KHV-T. Carp sera reacted very weakly with the antigen from KHV-Israel with titres between 1:100 and 1:200. For rainbow trout, the antibody reaction to the Asian lineage was the strongest, whereas for carp sera, antibody reactions to the European lineage antigens (KHV-E and KV3) were the strongest, corresponding to an enhanced response to the challenge isolate, as KHV-E was used for infection of rainbow trout.

Animal experiment at 20°C

At the end of the experiment at 20°C, which is a non-physiological temperature for salmonids, eight out of 10 gill swabs from rainbow trout and two out of four carp samples were considered to be KHV qPCR-positive. Using the semi-nested PCR as a detection method, nine out of 10 rainbow trout samples were positive but only two out of four carp gill samples (Table 7). In contrast to the results from the 15°C experiment, KHV DNA was also found in separated leucocytes of both species. In rainbow trout and carp leucocyte samples, a mean cq value of 28 occurred ranging up to cq 32 (rainbow trout) and up to cq 29 (carp).

Table 7 Detection of KHV using non-lethal sample collection (gill swabs)

Samples	Temperature	Positive ratio	Mean cq value*	Semi-nested PCR pos.
rainbow trout (31 dpi)	20°C	8/10	28.75	9/10
carp (21 dpi)	20°C	4/4*	29.33	2/4

* KHV qPCR (Gilad et al. 2004, modified according to Bergmann et al. 2010)

As shown in Figure 1, rainbow trout developed antibodies against KHV from 7 dpi at 20°C, but on average with a lower titre compared with the serum samples from 15°C. At 20°C, rainbow trout also developed a low antibody concentration against KHV-E. As already

presented at 15°C, the reaction of rainbow trout sera against the Asian lineage antigens (KHV-Israel, KHV-T) was stronger than against the antigens prepared from European lineage KHV.

Generally, 54% of the rainbow trout sera reacted positively by KHV antibody ELISA with relatively low average titres. Much higher ELISA titres were found from 100% of the carp sera. While in carp sera, neutralizing antibodies were present, no positive SNT reaction was found using rainbow trout sera from the 20°C experiment (Table 8). Again, carp on average developed a stronger humoral immune response compared with rainbow trout.

Table 8 General serological reactions of rainbow trout and carp sera by ELISA

sera	temperature	positive ratio ELISA	rec. average ELISA titre*	positive ratio SNT	rec. average SNT titre***
rainbow trout	20°C	27/50	284	0/10	0
carp	20°C	20/20	1,000	3/4 **	64

* value from five antigens (KHV-Israel, KHV-E, KHV-T, KV3 and KHV-G)

** a carp died due to KHVD on 16 dpi after cohabitation with KHV-E infected rainbow trout

*** SNT against KHV-I (Aoki et al. 2007, European lineage)

On 31 dpi, in eight out of 10 sera samples from rainbow trout, antibodies against KHV-E and KHV-T were detected. By ELISA using KV3 as antigen, only five rainbow trout sera were considered to be KHV antibody-positive. Using KHV-Israel and KHV-G, three rainbow trout sera were positive by ELISA only. With the average titre of three rainbow trout sera against KHV-G, one antibody titre was determined to be positive at 1:6,400, and the other two with 1:400 and 1:100, respectively. Therefore, the average titre is different from the other ELISAs

with an obvious high outlier, but nonetheless different. Nevertheless, the strongest reaction of rainbow trout sera was against KHV-E, followed by KHV-T, KHV-G, KHV-Israel and KV3.

All sera from the surviving four carp developed antibodies, which reacted very similarly with all five antigens. Compared with the reaction of carp sera, antibody titres of rainbow trout sera were much weaker. On 21 dpi (post-cohabitation), carp developed similar antibody titres against all antigens which were higher than those in rainbow trout (Table 9). The reaction of carp sera was slightly higher against KHV-T by ELISA.

Table 9 ELISA reactions of rainbow trout and carp sera with antigens of different KHV lineages from the 20°C experiment

	KHV-Israel (A/E)		KHV-E (E)		KHV-T (A)		KV3 (E)		KHV-G (E/A)	
	pos.	titre*	pos.	titre	pos.	titre	pos.	titre	pos.	titre
rainbow trout	3/10	1:40	8/10	1:210	8/10	1:190	5/10	1:110	3/10	1:690
Carp	4/4	1:700	4/4	1:800	4/4	1:1,100	4/4	1:650	4/4	1:750

* average titres from 10 rainbow trout and four carp sera

Discussion

Serological investigations within a population can be a very powerful tool in combating disease or epidemics. The major advantage of a validated serological ELISA in virology is the indirect detection of exposure to a present or already eliminated viral agent. The assay is fast, relatively simple, and cost- and time-effective. It allows non-lethal sampling and can be used in surveillance and monitoring. It is particularly valuable for fish broodstock (Jaramillo et al., 2017). On the farm or pond level, it has been shown that control measures can be

implemented based on recognizing KHV prevalence indirectly by antibody assays (Taylor et al., 2011).

Due to the required validation process including controls, standard materials, reliability, repeatability, and intra- and interassay controls (OIE, 2019b), disadvantages related to possibly low analytical sensitivity are minimized. In this study, a validated antibody ELISA recognizing antibodies against KHV in carp or koi sera (Bergmann et al., 2017) was successfully adapted to salmonid sera, for example from rainbow trout infected with KHV. Rainbow trout originated from the west coast of the USA mostly with a high thermal tolerance. The optimal temperature for steelhead trout is 13°C in the summer when the air temperature is approximately 22°C (Dunham, 1999). There are rainbow trout strains tolerating a water temperature of up to 26°C in different areas of the USA (Chen et al., 2015). In European rainbow trout aquaculture, 15°C is considered to be the optimal water temperature. According to FAO (2021), temperatures can range from 12 to 21°C, while lower temperatures are optimal for spawners and fingerlings. It was unclear whether rainbow trout can react immunologically at higher temperatures against KHV compared with their biologically normal 15°C, which is desired in Europe. The optimal temperature for keeping common carp is between 18 and 22°C in Europe, but can be as high as 30°C (FAO, 2021). In the winter time, carp stay at the bottom of a pond where a temperature of 4°C is normal. These capabilities were to be explored in the experiment reported here.

While carp in this study behaved physiologically stable at both temperatures, steelhead trout had to be adapted and adjusted to 20°C for at least four weeks. Only after this period did the rainbow trout kept at 20°C start to feed normally.

It was unknown whether rainbow trout can be infected with KHV and support replication of the virus or at which temperature rainbow trout show a humoral reaction against the virus. An additional question was whether rainbow trout can respond immunologically and whether antibodies can discriminate between viruses of different origin or lineages.

Rainbow trout are known to show humoral immune responses against disease agents, optimally with, for example, neutralizing antibodies at around 12 to 15°C but carp between 19 and 26°C, then losing this ability below 18°C water temperature (Abram et al., 2017).

Unexpectedly, the trout responded to the virus with antibody production from 7 dpi not only at 15°C but also at 20°C. This phenomenon was not observed in carp. The latter started to produce antibodies from approximately 14 to 15 dpi/post-cohabitation. These early antibodies in rainbow trout did not react against KHV-E belonging to the European lineage of KHV, but mainly against the two Asian lineage isolates, KHV-Israel and KHV-T. Later on, at the end of the experiment, humoral reactions against antigens of the Asian and the European lineages were seen, but this was limited to only one serum sample, respectively, against KHV-G (E/A) and KV3 (E) antigens. Generally, only 26% (four sera each) of the rainbow trout sera were considered to be positive by ELISA. In carp, five sera from five fish became antibody-positive by ELISA. While rainbow trout also developed neutralizing antibodies against KHV-I to a much higher extent (7 out of 10 sera), in carp sera obtained from the 15°C experiment only one sample contained neutralizing antibodies against KHV. For unknown reasons, more rainbow trout sera were found to contain neutralizing antibodies than were detected for antibodies by ELISA. It was also unusual to recognize that carp reacted with antibody production at 15°C, which confirmed the investigation by Lorenzen et al., (2010) and Perelberg et al., (2008). It was expected that there would be no neutralizing activity in carp

serum at 15°C; however, a clearly positive titre of 1:64 was observed. It seems that carp also can develop antibodies and neutralizing antibodies at much lower temperatures than 18°C water temperature. Additionally, it was proven by virus transmission to naïve carp that rainbow trout are able to permit replication of KHV, which also can induce a humoral immune response in the host without any clinical signs. Compared with the 15°C experiment, the antibody titres against KHV in carp sera were obviously weaker in the 20°C experiment, which can be explained by the KHVD outbreak with mortality (16 dpi, one carp). Morbidity was observed from 7 dpi in carp only. Disease signs in carp were darkening, round white patches on the skin, bleedings of the skin, petechial bleeding in gill tissue and increased mucus production until 9–10 dpi (post-cohabitation). With antibody titres of 1:700 to 1:1,100, carp developed a relatively uniform average antibody titre against all antigens from different KHV lineages, whereas reactivity to the antigens varied for rainbow trout sera at 20°C. Five to eight serum samples from rainbow trout reacted against KHV-E (E), KHV-T (A) and KV3 (E) but only three against KHV-Israel (A/E) and KHV-G (E/A). The lowest average titres were found, in contrast to the antibody titres from 7 dpi, against KHV-Israel, and the highest, against KHV-G. The latter average titre might be misleading because of the unusually extreme strong reaction of one serum sample (1:6.400), which strongly increased this average titre compared with the other titres against the other antigens.

Generally, both species developed antibodies at both temperatures. Rainbow trout were able to release infectious KHV after 7 dpi by the natural route, immersion, proven by virus detection from internal organs, for example leucocytes. Rainbow trout were therefore also able to replicate the virus at least in the leucocytes and the other collected organ tissues (Bergmann et al., 2017). Even if rainbow trout are not susceptible to the disease, they can be a threat for carp and koi in terms of a natural virus reservoir. Although molecular methods are

useful for the detection of KHV in such reservoir hosts, the sensitivity of such testing may be limited where there is no clinical disease present (Bergmann, Riechardt, et al., 2010; Matras et al., 2012). Antibody testing, as shown here, may provide an alternative approach to detecting possible non-cyprinid carrier species.

Also at 15°C, it might be possible, especially for carp or koi, to induce a protective immunity based on humoral immune response that can be used for vaccination in autumn at least in Europe. It is likely that the immunity will last over winter time and may appear again in spring. This may be helpful if live attenuated vaccines are used, but questionable if inactivated vaccines are applied.

However, the animal experiment does not show the reaction of both species against the other KHV isolates because they were infected with KHV-E only. It is expected that once the virus is in the fish, it might develop a latent or persistent phase of the infection. This is known for carp or koi (Bergmann et al., 2009) but has never been proven for salmonids. All these aspects require clarification.

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