RESEARCH **6**

2020-104

WILL CARP VIRUS BIOCONTROL BE EFFECTIVE?

NATIONAL CARP CONTROL PLAN

Evaluating the role of direct fish-to-fish contact on horizontal transmission of koi herpesvirus

This suite of documents contains those listed below.

NCCP TECHNICAL PAPERS

1. Carp biocontrol background
2. Epidemiology and release strategies
3. Carp biocontrol and water quality
4. Carp virus species specificity
5. Potential socio-economic impacts of carp biocontrol
6. NCCP implementation
7. NCCP engagement report
8. NCCP Murray and Murrumbidgee case study
9. NCCP Lachlan case study

NCCP RESEARCH (peer reviewed)

Will carp virus biocontrol be effective?

1. 2016-153: Preparing for Cyprinid herpesvirus 3: A carp biomass estimate for eastern Australia
2. 2018-120: Population dynamics and carp biomass estimates for Australia
3. 2017-148: Exploring genetic biocontrol options that could work synergistically with the carp virus
4. 2016-170: Development of hydrological, ecological and epidemiological modelling
5. 2017-135: Essential studies on Cyprinid herpesvirus 3 (CyHV-3) prior to release of the virus in Australian waters
6. 2020-104: Evaluating the role of direct fish-to-fish contact on horizontal transmission of koi herpesvirus
7. 2019-163 Understanding the genetics and genomics of carp strains and susceptibility to CyHV-3
8. 2017-094: Review of carp control via commercial exploitation

What are the carp virus biocontrol risks and how can they be managed?

9. 2017-055 and 2017-056: Water-quality risk assessment of carp biocontrol for Australian waterways
10. 2016-183: Cyprinid herpesvirus 3 and its relevance to humans
11. 2017-127: Defining best practice for viral susceptibility testing of non-target species to Cyprinid herpesvirus 3
12. 2019-176: Determination of the susceptibility of Silver Perch, Murray Cod and Rainbow Trout to infection with CyHV-3
13. 2016-152 and 2018-189: The socio-economic impact assessment and stakeholder engagement
    Appendix 1: Getting the National Carp Control Plan right: Ensuring the plan addresses community and stakeholder needs, interests and concerns
    Appendix 2: Findings of community attitude surveys
    Appendix 3: Socio-economic impact assessment — commercial carp fishers
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15. 2017-054: Social, economic and ecological risk assessment for use of Cyprinid herpesvirus 3 (CyHV-3) for carp biocontrol in Australia
    Volume 1: Review of the literature, outbreak scenarios, exposure pathways and case studies
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3. 2017-222: Engineering options for the NCCP
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5. 2018-209: Various NCCP operations case studies for the Murray and Murrumbidgee river systems (refer to Technical Paper 8)

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DRAFT

Evaluating the role of fish-to-fish contact on horizontal transmission of Koi herpesvirus

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Evaluating the role of fish-to-fish contact on horizontal transmission of Koi herpesvirus
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Abbreviations

ACDP, Australian Centre for Disease Preparedness

BC2, Biosecurity containment level 2

CPE, Cytopathic effect

Ct, Cycle threshold

EMAI, Elizabeth Macarthur Agriculture Institute

IP, Intraperitoneal

KHV, Koi herpesvirus

NCCP, National Carp Control Program

qPCR, quantitative polymerase chain reaction

$TCID_{50}$, 50% tissue culture infective dose

Executive Summary

Koi herpesvirus (KHV) is being considered as a biocontrol agent to assist in management of the feral carp (*Cyprinus carpio*) which are damaging freshwater ecosystems in south-eastern Australia. This virus emerged as the cause of high mortality disease of wild and farmed carp in many parts of the world over the previous 25 years. Given the high value of this fish species in aquaculture and native ecosystems, KHV was listed by the World Organisation for Animal Health (OIE) as a notifiable pathogen with guidelines to reduce its spread. In contrast, a virulent and host specific virus may be suitable as a biocontrol agent in Australia where invasive carp are responsible for extensive environmental damage and degradation of vulnerable freshwater ecosystems. Epidemiological modelling has been undertaken to evaluate the impact of KHV in a biocontrol program. The present project provides information to support key components of the mechanisms of virus transmission which underpin the epidemiological model. Specifically, it was shown that direct contact between carp was necessary for efficient transmission of KHV. Studies using feral carp held in aquaria demonstrated highly efficient transmission of KHV when fish were cohabited to allow close/direct contact for short periods of time and with a small proportion of infected individuals. Conversely, water-borne transmission of KHV was inefficient. Infection by immersion was achieved by using high concentrations of cell culture amplified KHV. However, a concentration of KHV that was sufficiently high to establish infection by immersion was rarely generated by infected carp, even when aquarium water was contaminated by carp with clinical signs of disease and very high viral loads. The quantities of KHV in tissues, skin swabs and water were monitored throughout the course of infection from pre-clinical stages, through the development of lesions and after death or recovery. Despite very high loads of KHV in tissues and skin mucus, the concentration of virus released into water rarely reached a concentration that was needed to establish infection. These results confirm that epidemiological models for KHV transmission should focus on transmission by fish-to-fish contact. The role of water-borne transmission under field conditions is unlikely to contribute significantly to the spread of KHV where the large volume of natural waters result in a high dilution of virus and there are adverse conditions for the maintenance of infective KHV.

Keywords

Koi herpesvirus (KHV, *Cyprinid herpesvirus 3*); carp (*Cyprinus carpio*); biological control

Introduction

Carp (*Cyprinus carpio*) are an invasive environmental pest in Australia (Koehn, 2004) that negatively impact freshwater ecosystems (Vilizzi et al., 2015). Control of feral carp is an important national priority being addressed through the Australian Government's National Carp Control Program (NCCP; https://carp.gov.au/). Koi herpesvirus (KHV, *Cyprinid herpesvirus 3*) emerged in 1997 and has spread to many parts of the world where it causes a fatal disease of wild and farmed carp (Hanson L et al., 2016). KHV is a notifiable pathogen to the World Organisation for Animal Health and is exotic to Australia (OIE, 2021). Disease resulting in a high mortality (up to 90% in naïve carp) together with the host specificity of KHV have led to consideration of this virus as a biocontrol agent for management of freshwater ecosystems in south-east Australia, where the carp population density is very high. Epidemiological modelling of the impact of KHV on carp in SE Australia considers the multifactorial nature of the disease with a strong influence of water temperature and host factors such as age (Durr et al., 2019). A particularly important aspect of the model are the underlying assumptions for modes of transmission of KHV, especially the relative importance of direct fish-to-fish contact compared to water-borne transmission of KHV. Aspects of carp biology such as aggregation at the time of breeding will be important determinants of disease outcomes as they will influence transmission mechanisms. Experimental studies evaluating the susceptibility of carp to infection by immersion have found a dose response with a mortality ranging from 10% to 95% with < 1 - 630 $TCID_{50}$ (50% tissue culture infective doses) of KHV per mL of water (McColl & Crane, 2013). Immersion of various species including grass carp (*Ctenopharyngodon idella*) for 45 min in water containing KHV from cell culture with at 0.4 $TCID_{50}$ per mL reliably transferred infection resulting in fatal disease in common carp when these fish were cohabited with the common carp 7 days after the immersion challenge (Matras et al., 2019). Detection of KHV in water during outbreaks of disease and in endemic waterways has been described (Haramoto et al., 2007). However, it remains unclear if this represents enough infectious KHV to establish infection by the indirect, water-borne route.

The transmission coefficient (β) is the rate at which a pathogen is spread from infected to susceptible individuals in a population (Kirkeby et al., 2017). This single parameter used in disease models encompasses many complexities of the biology of pathogen transmission relating to the contact rate between susceptible (S) and infectious (I) individuals and the likelihood that transmission will occur during a contact. Pathogen specific (e.g. infectious dose, duration of infectivity) and scenario specific factors (e.g. population density) influence β (Reno, 1998). There are limited data from field outbreaks to determine β for KHV as this requires longitudinal disease monitoring (Kirkeby et al., 2017). The transmission coefficient is an important parameter in epidemiologic models of KHV in Australia.

The present study was designed to inform epidemiological modelling to support the suitability of KHV as a biocontrol agent for feral carp in Australia (Durr et al., 2019). Specifically, the objective was to assess the role of direct contact between carp compared to water-borne virus for the transmission of KHV. The present study used individual housing of carp to limit the potential for transmission to short time intervals with carefully defined exposure parameters. The outcome was transmission of KHV infection as monitored by qPCR to detect amplification of the viral genome. The study was not designed to predict mortality from KHV in a biocontrol scenario.

Objectives

Define dose-response parameters for KHV infection after challenge of wild Australian carp by immersion or injection challenge, including mortality, median survival time and 50% lethal dose estimates in a defined experimental setting.

Measure the infectivity of KHV for carp when the virus is derived from cell culture compared to virus excreted from an infected carp by using qPCR quantification of KHV DNA. This would allow comparison of the virus load in samples with potentially different infectivity and that cannot be readily determined by conventional titration in cell culture systems.

Quantify the excretion of KHV from carp infected by IP injection when the infection is subclinical and during clinical disease up-to near death and for 24 hours after death.

Measure the transmission parameter β for horizontal transmission of KHV through direct and indirect contact. This parameter will be assessed under two levels of force of infection depending on the time before indirect exposure to KHV and the proportion of infected individuals for direct transmission.

Methods

2.1 Fish

Acquisition and transport.

Feral carp (*Cyprinus carpio*) were obtained by Keith Bell (K&C Fisheries Global P/L) in June 2021. Young of the year carp were reported to be more difficult to find than expected and were accumulated from several small lakes in Victoria (Horsham area: Taylors Lake; Greens Lake) and New South Wales (Griffith area: Lake Wyangan). The fish were visually assessed as being carp with potential goldfish hybrids excluded by the experienced carp fisher. The fish were transported in dechlorinated municipal water in an intermediate bulk container (IBC) with battery powered aerators.

On arrival at EMAI the carp were purged in aquarium water before being transferred to recirculating aquarium tanks for holding prior to KHV challenge. There were 2 distinct groups based on size: smaller (5 – 10 cm; n=147) and larger (>12 and up to 22 cm fork length; n=80).

All fish were treated twice with a formalin bath (75 mg/L for 1 hour) at 1-week intervals within the first 3 weeks of acquisition to treat a *Dactylogyrus* sp. infestation of the gills (informed by Tancredo et al., 2019). This parasite infestation was associated with disease and mortality of ~5% of predominantly smaller individuals. It was also managed by maintaining aquarium salt (Ocean Nature, Aquasonic) in the water at 2 parts per thousand (ppt).

Fish care prior to challenge studies.

The fish were grouped by size and kept at approximately equal stocking density by body weight in 3 plastic tanks, each containing 400 L water at 2 ppt salinity with temperature maintained at 22°C with thermostatically controlled aquarium heaters. Each tank had 3 external cannister biofilters (Fluval) and aeration via 3 large aquarium air stones. The temperature and pressure-controlled room met BC2 containment conditions and was provided with 11h artificial light per day.

Daily care included feeding with commercial koi pellets (Kirameki) at approximately 0.5-1% body weight per day and water quality monitoring with an API Freshwater Master Test Kit (Aquarium Pharmaceuticals Incorporated). Water exchange, cleaning of filters and addition of bicarbonate was undertaken as needed to maintain water quality. Aquarium water was prepared prior to exchange by drawing municipal water into a reservoir where it was treated with aquarium water conditioner (Prime, Seachem) and circulating through a cannister filter (Eheim) containing activated charcoal (API) and equilibrating to 22°C for at least 12h.

The carp were held and used for *in vivo* studies according to the EMAI Animal Ethics Committee Research Authority M21/10.

2.2 Koi herpesvirus

This study used the Indonesian KHV isolate CyHV-3 C07 that was supplied by the Australian Fish Diagnostic Laboratory (AFDL, ACDP, Geelong under import permit IP06014396). This isolate originated from Indonesia in 2007 and was used in other research for the NCCP (Moody, 2021). The original imported isolate underwent 3 additional passages on KF-1 cells at ACDP prior to supply to EMAI as tissue culture fluid with a titre of $10^{4.3}$ $TCID_{50}$/mL. At EMAI this material (O333) was inoculated directly onto monolayer cultures of KF-1 cells to produce a master stock of virus that was stored in aliquots at -80°C. The working stocks of virus used for challenge of carp underwent no more

than 4 additional passages. The KHV isolate and KF-1 cells supplied by ACDP were certified to be free from adventitious agents.

Approval to use restricted imported biological material for *in vivo* use was provided by the Department of Agriculture Water and Environment, Animal Biosecurity (DAWE, Approval number 2021/008). The EMAI Biosecurity Committee approved the work (IBC Approval M21/02). These approvals provided guidance on biosecurity requirements for *in vitro* and *in vivo* work with KHV at BC2 containment.

2.3 *In vitro* amplification and titration of KHV

KF-1 cells were imported by CSIRO under Import Permit IP7003748 and were supplied at passage 108. These were grown in minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS) at 25°C and sub-cultured with a 1/4 split ratio after trypsinisation every 5 to 10 days. A seed-lot system consisting of Master, Daughter and Working stocks of the KF-1 cells was cryopreserved in liquid nitrogen. The cells were seeded into a variety of vessels including 75cm^2 cell culture flasks, 10mL culture tubes, 10-deck (6320cm^2) cell culture factories and 96-well culture plates depending on requirements for KHV isolation, propagation, and titration.

Pre-master stocks of KHV (O456, O457; O734) were produced by inoculating near confluent monolayers of KF-1 cells in 4 x 75 cm^2 flasks with a 1/5 dilution of O333 in MEM (no serum) by adsorption for 1 hour at 25°C. The cells were incubated at 25°C with 30 mL maintenance medium (MEM, 2% FBS) and observed daily with an inverted microscope for cytopathic effect (CPE) which was consistent with previous reports (Mahardika & Yasuda, 2011). Tissue culture fluid (TCF) was collected when CPE affected >80% of cells but with the monolayer remaining largely intact. This stock of virus was used for challenge of fish by intraperitoneal injection and to produce working stocks of KHV in cell culture factories.

A concentrated preparation of KHV (O680) was used for challenge by immersion and also for intraperitoneal injection of carp. After removal of the growth medium, cell culture factories were inoculated by adding 10 mL KHV TCF (O147) to 1.5 L maintenance medium when monolayers were near confluence. The TCF was harvested after 7-days incubation at 25°C when CPE affected >80% of cells and the monolayer remained intact. Factory derived TCF was concentrated approximately 100-fold by centrifugation at 10,000 rpm for 22 hours (no brake) at 4^{o}C. For every 100mL of TCF, the cell/virus pellet was resuspended in 1 mL of MEM and frozen at approximately -80^{o}C until thawed for use.

Quantification of KHV by titration

Working stocks of KF-1 cells seeded into 96-well tissue culture plates were grown to near confluent monolayers over 3-5 days before inoculation with 10-fold serial dilution of the test sample in growth medium with 8 replicate wells per dilution. The cells were assessed for CPE after 10 days and the titre was calculated to determine the 50% end point (Reed & Muench, 1938).

Virus isolation to test for infectivity of KHV in tissue, swab and water samples

Selected water samples were collected from aquaria containing infected fish and were held frozen at approximately -80^{o}C. Subsequently, these samples were tested for the presence of infectious KHV. After thawing they were diluted 1/1 in double strength MEM (no serum) and filtered at 0.45 µm. Two hundred µL of sample was then adsorbed for 1h at 25°C onto monolayers of KF-1 cells in duplicate tubes which were examined at least on days 3, 5 and 7 for CPE. In the absence of cytopathology, the TCF was passaged twice more using fresh KF-1 cells in tubes. Tissue culture fluid at each passage was

tested by qPCR to assess replication of KHV. Positive and negative control samples were included at each passage during the virus isolation attempts.

2.4 KHV Challenge studies with feral carp

Figure 1 provides an overview of the *in vivo* experiments.

Intraperitoneal injection

A series of virus dilutions (expressed as $TCID_{50}$/fish) were prepared in MEM (no serum); MEM was also used for the negative control injection. Carp were anesthetised in a solution of benzocaine (60 mg/L) in water prior to injection of 200µL by the intraperitoneal route using a 1mL syringe and 25-gauge needle.

Individual housing of carp to assess infection outcome

Plastic aquaria (Keji) containing 40L of conditioned aquarium water were supplied with aeration by an air stone and a small thermostatically controlled aquarium heater (Aquasonic) was used to maintain the temperature at 22°C. Water was exchanged at the rate of 1/3 total volume per day. Challenged carp were examined twice a day for clinical signs (Appendix 1) and were offered food once a day.

In summary, mild disease was characterised by increased skin mucous and focal reddening of the skin. These mild signs were always accompanied by complete loss of appetite. Moderate disease was characterised by exacerbation of the skin lesions with ulceration and rough, sandpaper lesions observed. Activity was reduced at this stage but the carp would still swim to avoid a stimulus. Carp that were in this category for 1 or more days sometimes recovered to have no clinical signs at the completion of the trials. Severe disease that was also a trigger for euthanasia included similar skin lesions to the moderate classification but in carp that had rapid shallow opercula movements and no attempts to swim. Tail fin degradation was seen frequently in fish that reached this stage.

The carp were challenged with KHV across 3 temporally distinct experiments:

(i) Experiment 1. KHV Excretion dynamics and titration by intraperitoneal injection

Carp (n=4 per treatment) were injected with 1 x 10^4, 1 x 10^3, 1 x 10^2 or 10 $TCID_{50}$/fish (O456) or the negative control by the intraperitoneal route (IP) and placed in individual aquaria where they were observed for 21 days. The fish were selected to provide 1 large and 3 small individuals for each group. Water samples were collected twice a day and a skin swab was collected once a day. Water samples and skin swabs were collected for 24h after death from carp that died or were euthanised but were left in the aquaria to assess post-mortem KHV release. Cotton swabs were used to sample skin mucus along the length of the lateral line on one side of the carp while briefly out of the water. Water samples (1.5 – 2 mL) were collected with a transfer pipette from the mid water column.

(ii) Experiment 2. Challenge by cohabitation and immersion

A source of naturally amplified KHV (i.e., from infected carp) for challenge by immersion in water or by cohabitation was generated by IP injection of 11 carp with 200 µl containing 10^4 $TCID_{50}$ of KHV (O456). These carp were housed individually and excretion of KHV was assessed by qPCR on tank water and skin swabs collected on Day 4 (pm), Day 5 (am and pm) and Day 6 (am). The results were used to select aquarium water that would be suitable for challenge by immersion and to identify individual fish to use in the challenge of additional naïve carp by cohabitation.

Immersion challenge. Water collected from the aquaria holding the selected donor fish was mixed well, including debris, pooled, and then transferred in 40L volumes to 2 clean aquaria. A third aquarium was set up with clean, KHV-free aquarium water that had contained a negative control carp. Exposure by immersion involved addition of 6 carp per tank for 4h with light aeration and maintenance of 22°C water temperature.

Cohabitation challenge. Challenge by cohabitation involved adding 6 naïve fish to an aquarium with 40L of water and 1 selected infected donor (2 replicates) or 2 infected donors (2 replicates) for 4h. The negative control involved cohabitation of 6 carp and a single negative control donor fish in a 40L tank. There was potential for direct contact between carp for the duration of the cohabitation.

The donors were euthanised at the end of the cohabitation period and all challenged, and control fish were moved into individual aquaria to monitor the outcome for a further 21 days.

(iii) Experiment 3. Additional intraperitoneal injection, cohabitation, and immersion challenges

On completion of the preceding experiments, trials to explore the initial outcomes further were undertaken as follows:

- High dose immersion challenge. A concentrated cell culture derived KHV stock (O680; 5.97 x 10^5 $TCID_{50}$/mL) was used to challenge fish by immersion at a higher dose than was achieved by contaminating aquarium water with infected fish. The highest dose was prepared by mixing 13 mL of the concentrated stock (O680; 5.97 x 10^5 $TCID_{50}$/mL) in 11 L aquarium water to give an estimated total virus concentration of approximately 6 x 10^2 $TCID_{50}$/mL and another 10L volume with ten-fold less virus (estimated total virus load of 6 x 10^1 $TCID_{50}$/mL) was also prepared. Challenge involved holding 6 carp in each 10L volume for 1h with light aeration before moving to individual housing. The negative control was prepared using MEM in aquarium water.

Cohabitation and immersion in aquarium water, Part 2.

Potential donor carp were prepared by IP injection of high doses of virus (1 x 10^5 or 1 x 10^4 $TCID_{50}$/fish O680, n=6 per dose). Excretion of KHV was monitored by qPCR on water and skin swabs with the objective of identifying donors that were excreting KHV, prior to the detection of clinical signs, excreting KHV with active skin lesions and generating the highest concentration of KHV in aquarium water. Exposure of 6 naive fish for 1h was achieved in the tank of a single donor fish, either by cohabitation or by immersion after removal of the donor fish. There was potential for direct contact between carp for the duration of the cohabitation.

Additional IP injection challenge. This experiment included larger carp that were group housed in two recirculating aquaria, maintained at 0 or 2 ppt salinity for 7 days (n=13). A dose of 1 x 10^4 $TCID_{50}$/fish, (dilution of O680) was administered by IP injection.

Calculation of the transmission coefficient

The transmission coefficient (β) was calculated for cohabitation challenges according to the formula:

$$\beta = 1 - \exp((\ln(S_i/S_0)/I_0))$$

where S_0 is the number of susceptible carp at the start of the trial, I_0 is the number of KHV infected carp at the start of the trial and S_i is the number of susceptible carp that remained uninfected at the completion of the experiment. A nominal value of 0.001 was added when any value was 0 to enable a calculation from the formula. This calculation has been used in previous experimental aquatic animal studies (Butler et al., 2008).

2.5 Molecular detection of KHV

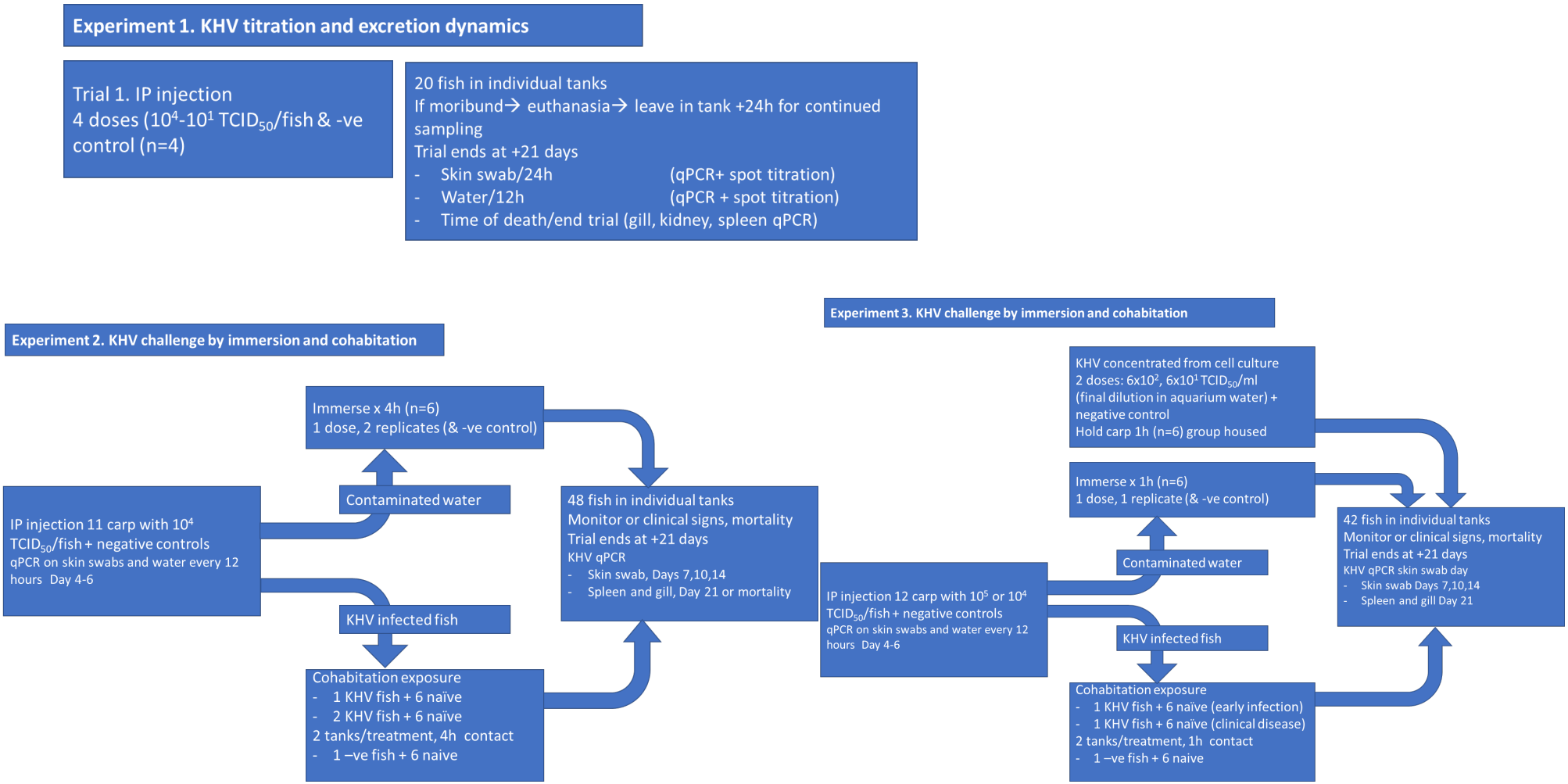
Sample preparation

Subsamples of tissues (~0.1 g) were homogenised by bead beating with 1 mL phosphate buffered saline. Swabs were placed in 2 mL phosphate buffered gelatin saline (PBGS) at the time of collection and swab fluid was tested after being held for at least 2 h at room temperature. Water samples were stored in 5 mL screw cap vials on ice or at 4°C and mixed thoroughly before a subsample was removed for testing. Nucleic acids were purified from 50 µl of swab fluid, TCF or tissue homogenate or 100 µl of aquarium water using the MagMAX™ Viral RNA Isolation Kit (Applied Biosystems, ThermoFisher) using a semi-automated magnetic bead-based nucleic acid extraction system. Water and swab samples were tested on the day of collection without a freeze/thaw cycle, tissue samples were prepared for testing after storage at -80°C.

Real-time PCR (qPCR) for KHV

Samples were tested for KHV DNA using a real-time PCR assay based on Gilad et al., (2004) using AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems). This assay amplifies a target in the KHV genome, open reading frame 89 (ORF89, encoding a hypothetical protein) with methods that detect combined DNA and RNA copies of the KHV genome in the samples. The reactions were conducted according to the standard conditions specified by the manufacturer using an ABI 7500 qPCR thermocycler (Applied Biosystems). Prior to testing of samples, the assay was evaluated using a synthetic nucleic acid construct as a quantitative positive control to determine the limit of detection. Samples were tested in 96-well plates and each batch of assays included two KHV genomic DNA samples as positive controls (including a low concentration control near the limit of detection of the assay) and a tRNA negative control sample. Assays were duplexed with an exogenous internal control (XIPC) to monitor assay efficiency and the presence of reaction inhibitors. Assays were run for a total of 45 cycles. Results were analysed using the 7500 Software v2.3 (Thermofisher Scientific) with a fixed manual threshold (0.05). Results were reported as positive or negative based on examination of the normalised FAM amplification curve and a cycle threshold value (Ct) was assigned to positive samples to indicate the relative quantity of KHV DNA present in experimental samples.

Figure 1. Overview of experiment design and in vivo experiments with KHV infection of carp.



Results

3.1 *In vitro* propagation of KHV

The KHV premaster stock produced characteristic cytopathology (CPE) and had a titre of 4.5 x 10^4 $TCID_{50}$/mL. The titre of virus used for IP injection prior to dilution was 4.6 x 10^4 $TCID_{50}$/mL (O456; 1st passage O333). The titre of cell culture supernatant concentrated by centrifugation from cell factories was 5.97 x 10^5 $TCID_{50}$/mL (O680; 4th passage in cell culture factory).

3.2 KHV challenge by intraperitoneal injection

A total of 65 carp were challenged by IP injection across the 3 experiments, including fish used as donors for cohabitation experiments. There was a high rate of infection by this route (88%), which was dose responsive (Table 1). The 16 negative controls for IP injection remained negative for KHV and healthy for the duration of the experiments. In a titration of KHV by IP injection, infection was established in 8/8 fish with a dose of 1 x 10^3 or 1 x 10^4 $TCID_{50}$ and 6/8 fish with 1 x 10^2 or 1 x 10^1 $TCID_{50}$. Morbidity and mortality in this experiment was also dose responsive (Table 1). The experiment included individuals that had moderate to severe clinical signs but had recovered by the end of the trial.

Table 1: Results summary for Experiment 1, KHV infection and excretion dynamics after IP injection with carp held in individual aquaria. Water was tested twice a day and skin swabs once a day for 21 days (Appendix 2).

Dose ($TCID_{50}$/fish)	Morbidity*	Mortality	KHV present in water		KHV present in skin mucus		KHV qPCR positive tissue[1]
			Proportion	Days (range)	Proportion	Days (range)	
0	0	0	n/a	n/a	n/a	n/a	0
10	1/3	1/3	1/3	14	2/3	8 - 14	3/3
10^2	1/4	0/4	0/4	n/a	2/4	5 - 20	2/4
10^3	3/4	1/4	4/4	2 - 18	4/4	3 - 16	3/4
10^4	2/4	2/4	3/4	2 - 12	4/4	2 - 13	4/4

[1] Tissues were collected at the time of death (gill biopsy) or at the completion of the trial, 21 days after injection. * Number of carp which had at least mild clinical signs at some time during the trial.

In Experiment 3, large carp cohoused in recirculating systems with salinity 0 or 2 ppt (n=13) were examined and sampled 8 days after injection with 1 x 10^4 $TCID_{50}$/fish. There was an equivalent outcome in each tank: In Tank 1 (salinity=0) 11/13 were qPCR positive; Ct 31.3+/-4.35 (average, st.dev). In tank 2 (salinity=2ppt) the Ct was 31.9 +/- 3.39 for 11 positive carp. KHV DNA was detected in both gill and spleen for all but one of the infected fish and the viral load was equivalent in these tissues: gill Ct 28.1 +/- 5.27 and spleen 29.47 +/- 4.53 (p=0.19). Mild to moderate skin lesions were present in 69% (18/26) of the carp at this time and was equivalent in each tank.

3.2 KHV excretion dynamics

The titration experiment with daily sampling was undertaken to evaluate the excretion of KHV to inform cohabitation experiments and to better understand how donor fish might be used to infect naïve carp. Excretion of KHV was dose dependent with the number of days with positive skin swabs

and the quantity of KHV DNA higher for fish challenged with a higher dose (Table 1, Appendix 2). There was a higher concentration of KHV DNA in skin swabs (when positive) compared to water samples: Ct 30.5 +/- 4.86 compared to 32.6 +/- 2.13 ($p<0.01$). The variability in KHV viral load was also greater in skin swabs compared to water with Ct values sometimes less than 22 (Figure 2). There was a high level of individual and temporal variation in the excretion of KHV (Appendix 2). Excretion was prolonged, ranging from 3 to 18 days after injection from prior to clinical signs of disease until after recovery in surviving carp. Peak KHV excretion preceded death; detection of KHV DNA in skin swabs and water quickly diminished after death suggesting that live carp are the primary source of infectious virus.

The variable timing of KHV excretion was further evident when skin swabs and water samples taken from IP challenged carp assessed for use as cohabitation donors in the second and third experiments (Table 2).

Figure 2: Ct values for water and skin swab samples that tested positive by qPCR during daily monitoring of KHV excretion after IP injection of carp. Samples were collected daily (swabs) and twice daily (water) during Experiment 1. Highlighted data are the median, quartiles and range.

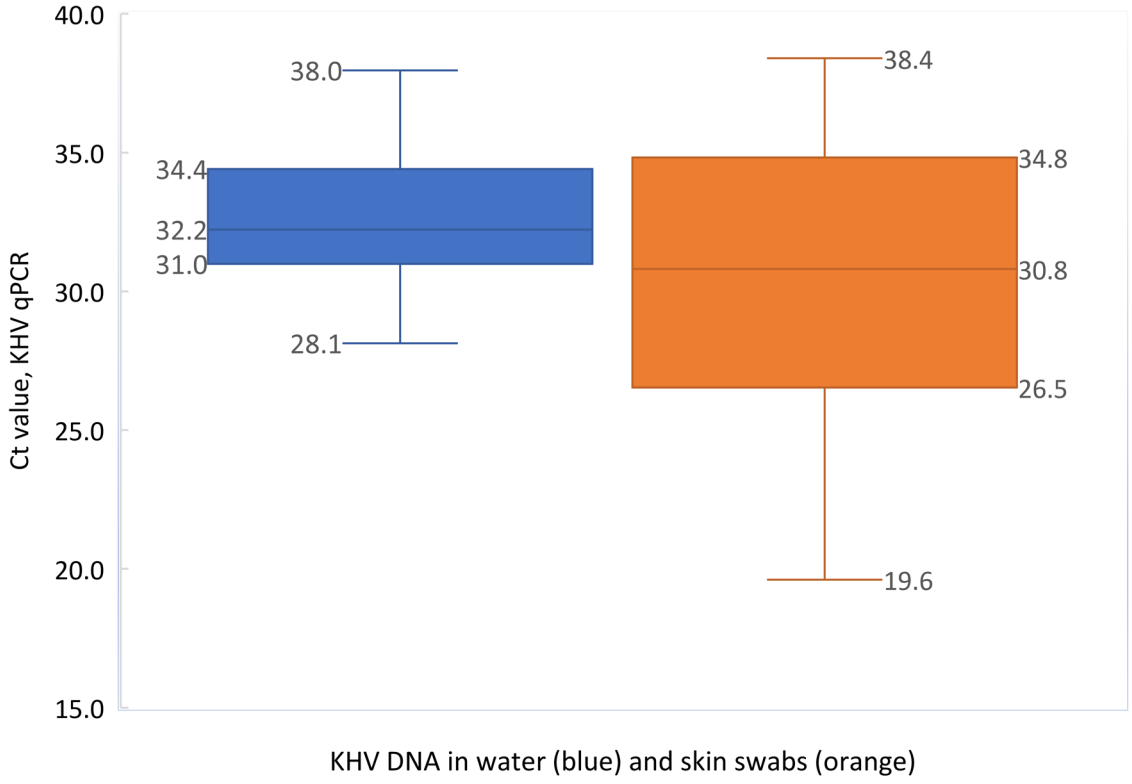


Table 2: KHV excretion for IP injected carp that were assessed for use as donors in cohabitation experiments. Data are Ct values for KHV qPCR.

Experiment	Fish ID	Day 4 am skin swab	Day 5 skin am swab	Day 5 skin pm swab	Day 6 skin am swab	Day 7 skin swab	Day 4 am water	Day 5 am water	Day 5 pm water	Day 6 am water	Day 7 water
2	1	Negative	32.5	29	30.6	n/d	Negative	35.7	Negative	Negative	n/d
	2	Negative	Negative	Negative	Negative	n/d	Negative	Negative	Negative	Negative	n/d
	3	35.8	26.5	25.3	27.8	n/d	35.8	33.6	33.3	32.8	n/d
	4	Negative	38.9	32.2	26.9	n/d	35.9	Negative	Negative	33.8	n/d
	5	Negative	32.8	29.3	23.7	n/d	32.8	33.7	32	31.4	n/d
	6	Negative	25.7	22.8	25	n/d	33.8	33.7	34.9	34	n/d
	7	Negative	Negative	Negative	Negative	n/d	Negative	Negative	Negative	Negative	n/d
	8	35.8	Negative	34.9	32.8	n/d	Negative	Negative	Negative	Negative	n/d
	9	30.4	27.6	26.6	24.8	n/d	33.7	34	33.3	33.5	n/d
	10	36.5	26.1	26.6	Negative	n/d	Negative	Negative	35	Negative	n/d
	11	Negative	29.2	29.5	Negative	n/d	Negative	Negative	36.2	Negative	n/d
3	9	Negative	n/d	n/d	n/d	34.4	n/d	n/d	n/d	n/d	Negative
	10	Negative	n/d	n/d	n/d	Negative	n/d	n/d	n/d	n/d	33.4
	11	28	n/d	n/d	n/d	26.1	n/d	n/d	n/d	n/d	31.3
	12	26.2	n/d	n/d	n/d	31.6	n/d	n/d	n/d	n/d	34.6
	13	Negative	n/d	n/d	n/d	32.6	n/d	n/d	n/d	n/d	Negative
	14	30.7	n/d	n/d	n/d	25.2	n/d	n/d	n/d	n/d	26.6
	15	Negative	n/d	n/d	n/d	26.3	n/d	n/d	n/d	n/d	25.6
	16	Negative	n/d	n/d	n/d	33.4	n/d	n/d	n/d	n/d	Negative
	17	26.3	n/d	n/d	n/d	23.7	n/d	n/d	n/d	n/d	36.4
	18	24.2	n/d	n/d	n/d	23.2	n/d	n/d	n/d	n/d	Negative
	19	22.9	n/d	n/d	n/d	20.1	n/d	n/d	n/d	n/d	Negative
	20	26.7	n/d	n/d	n/d	26.7	n/d	n/d	n/d	n/d	Negative

n/d: not done. Sampling and qPCR tests were limited to what was required to inform cohabitation experiments.

3.3 KHV challenge by immersion

Carp were challenged by immersion in a series of experiments to better understand the role of contact time and concentration of KHV on water-borne transmission. Infection was reliably established with a high dose that was derived from cell culture amplified KHV. Aquarium water contaminated with KHV by carp with clinical disease and high loads of KHV in tissues and skin mucus was not infectious to carp with a 4h contact time. The concentration of KHV in the experiment was consistent with the amount that was frequently observed in aquaria housing infected carp with clinical signs of disease. Transmission of KHV from contaminated aquarium water did occur in an experiment with the highest concentration observed. Specific details are as follows:

(i) Immersion challenge, KHV contaminated aquarium water

Initially, carp were challenged in aquarium water that was contaminated by individually housed, IP injected donor fish (dose 1 x 10^4 $TCID_{50}$/fish). The donor carp were used at 6 days post-challenge, had clinical signs of disease and skin swabs tested positive for KHV (Ct range 23.7 – 33.5). The water was prepared as pools from the 5 x 40 L aquaria containing the most KHV as determined by qPCR on water (Table 2, Experiment 2, Day 6 am) and was mixed at the time of collection to include settled debris. The load of KHV detected by qPCR did not change after 6 naïve carp had been held for 4 h in each 40 L volume (Ct 31.64 and 31.70 for Replicates 1 and 2, respectively). Infectious KHV was not detected in these water samples by virus isolation. KHV DNA was not detected in the water of either aquarium that previously housed carp injected with cell culture medium and were used for the negative control immersion.

At the end of the challenge period, the naive carp were moved out of the contaminated aquaria and housed individually in 40 L aquaria and provided with clean aquarium water. There were no signs of disease in these carp through 20 days of individual housing. Negative PCR results were obtained for the skin swabs collected 7 days after challenge and KHV DNA was not detected in the tissues (gill and spleen) when carp were sampled on Day 20 (n=12). There were no clinical signs for the negative control carp, and they also gave negative results in the KHV qPCR.

(ii) High dose immersion challenge, KHV from cell culture

As the concentration of KHV excreted into the aquarium water was not sufficient to achieve infection after 4 h immersion, a concentrated cell culture preparation of KHV was used to generate 2 higher dose immersion challenges. The titre of the aquarium water after mixing with the concentrated TCF (O680) was predicted to be in the order of 6 x 10^2 and 6 x 10^1 $TCID_{50}$/mL for the higher and lower doses, respectively. There was more KHV DNA in the challenge aquaria at the time of adding 6 carp (high dose Ct 21.5 and lower dose 23.6), compared to the amounts observed in aquaria contaminated by infected carp (no Ct values less than 25 observed in any experiment). At the completion of the 1 h exposure period the Ct value of the water was 25.1 in both aquaria and challenged carp were moved to individual aquaria for observation.

KHV DNA was detected in the skin swabs of all carp for each immersion dose when tested on Days 4 and 7 after immersion. The mortality was 2/6 for each dose (1 carp each on Days 8 – 11) with very large quantities of KHV detected in gills at the time of death (Ct range 14.4 – 21.9). At Day 20, only 4 of 8 surviving fish gave positive results by qPCR and the viral load was low (average Ct for positive samples > 33.9). The negative control carp (immersed for 1h in aquarium water containing the cell culture medium) remained free from signs of disease and skin swabs (Day 4 and 7) and tissues collected at the end of the trial (n=6) each gave negative PCR results.

(iii) Immersion challenge, KHV contaminated aquarium water, maximum dose

Monitoring excretion dynamics for KHV in water identified temporal variation in KHV concentrations (Tables 2,3). It was considered that this variation was not just the result of probable sampling variability due to heterogenous distribution of virus in aquarium water. Therefore, an additional immersion challenge was undertaken using the highest possible concentration of KHV detected by monitoring contamination of aquarium water. In Experiment 3, 12 carp were injected IP with 10^5 or 10^4 $TCID_{50}$/fish. At Day 7 post challenge, the water of 2 aquaria with the highest concentrations of KHV (Ct of 25.56 and 26.64) was pooled and used to challenge 6 fish for 1 h in a single aquarium, 40 L volume. The donor carp that were removed from these tanks had severe clinical signs at this time and gave strong positive KHV PCR results for gill tissue (Ct 17.73, 26.74).

Transmission of KHV in this challenge was confirmed with clinical signs observed in 5/6 fish and positive KHV qPCR results were obtained for gill (6/6) and spleen (5/6) when sampled at Day 10 (completion of the experiment). This immersion challenge dose was lower (KHV DNA concentration) than the lower dilution of cell culture derived KHV used previously and used the same contact time (1h). This represented the most concentrated KHV detected in aquarium water from contamination by infected carp.

3.4 KHV challenge by cohabitation

There were 8 cohabitation challenges in which naïve carp were housed in aquaria for a defined period with donors that were confirmed to be shedding KHV by qPCR on skin swabs. Observation of these challenges indicated that all carp were actively swimming for the duration of the cohabitation period and that close contact between individual carp was frequently observed. These interactions were not measured or quantified.

The outcome of each challenge was determined by holding the 6 challenged carp separately; 41/48 were shown to have been infected with KHV (Table 3). There was a dose response, in which the carp that were not infected by these challenges were those from a challenge of the shortest duration (1h) and when donors were early in the course of infection, prior to the development of clinical signs.

Mild clinical signs including loss of appetite and mild skin lesions were present in the same proportion of fish that had evidence of infection. However, severe clinical signs occurred at a lower frequency and only 2 carp died in the observation period. The amount of KHV at the time of death was high for both fish (Ct for gill tissue: 19.8 and 17.7) and clinical signs were consistent, confirming the mortality was related to KHV infection.

The challenge in Experiment 2 used cohabitation of infected donor carp with 6 naïve carp in 40L of the host aquarium water (i.e., challenge by cohabitation and immersion). The amount of KHV in water was equivalent to the concentration measured for the concurrent immersion challenge in aquarium water (Section 3.3, Table 2). i.e., aquaria with a single donor carp (water Ct 33.2, 32.7 at the start of cohabitation and Ct 33.0 and 33.9 at the completion of cohabitation); aquaria with 2 donors (water Ct 31.6, 31.7 at the start of cohabitation and Ct 32.0 and negative at the end of the cohabitation period). The donor fish gave positive results for KHV on skin swabs prior to use as donors (Table 2; Ct range for selected carp: 22.8 – 29.5).

Cohabitation challenged carp were then housed separately after the 4 h contact period, and infection with KHV was confirmed with skin swabs of all carp positive on Days 7 and 10 with a virus load. The proportion of KHV positive swabs was lower at Day 14 and not all carp tested positive for KHV on tissue samples collected at the completion of the observation period (Day 20, Table 4). Transmission of KHV was confirmed for all carp (n=24, Table 3) with high loads and at least mild clinical disease occurred in all 24 carp (Table 4). The proportion with moderate or severe clinical signs was lower (5/12) and there was a single mortality (Day 11).

For Experiment 3, the contact time was reduced from 4h to 1h and a single infected donor carp was used in all challenge aquaria (Table 3). Two of these challenges used donor carp 4 days after IP injection, prior to development of clinical signs but with KHV detected on skin swabs (Ct 26.2 and 26.7). The carp used as donors with moderate/severe disease on Day 8 after challenge had high loads of KHV on skin swabs (Ct 21.4 and 23.1). With the shorter observation period, KHV infection was assessed using tissue samples alone at Days 9 or 13 (Table 5). Transmission of KHV infection occurred for 8/12 carp with 1 mortality by Day 13 in carp challenged by cohabitation with pre-clinical donors (Table 3). The 1 h cohabitation with clinical donors resulted in transmission of KHV to 10/12 carp and at least mild clinical disease was observed for 7 of these when the trial was ceased on Day 9.

The transmission factor in the cohabitation trials was calculated and is presented in Table 6. There were no signs of disease or detections of KHV in carp challenged by cohabitation with donors that had been injected with cell culture medium i.e., negative control cohabitation in Experiments 2 and 3 (n=12, Table 3).

Table 3: Summary of the outcome for KHV transmission by cohabitation with the possibility for direct contact between fish.

Experiment	Cohabitation challenge	Replicate	Donor			Outcome (carp housed individually after challenge)			
			Clinical signs	KHV qPCR		Duration (days)	Number affected (n= 6)		
				Skin swab	Water		Clinical signs*	Mortality	KHV infection
2	4h with 1 infected donor	1	Yes	27.8	33.2	21	6	0	6
		2	Yes	25.0	32.7	21	6	1 (Day 9)	6
	4h with 2 infected donors	1	Yes Yes	31.4 26.6	31.6	21	6	0	6
		2	Yes Yes	26.6 29.5	31.7	21	6	0	6
	4h with 1 negative donor	1	No	Negative	Negative	21	6	0	0
3	1h with 1 infected donor, early infection	1	Early	26.2	n/d	13	6	1 (Day 11)	4
		2	No	26.7	n/d	13	4	0	4
	1h with 1 infected donor, advanced disease	1	Yes	23.7	n/d	9	4	0	5
		2	Yes	23.2	n/d	9	3	0	5
	1h with 1 negative donor	1	No	Negative	n/d	13	0	0	0

* Number of carp which had at least mild clinical signs at some time during the trial.

Table 4: Detection of KHV to determine if transmission of KHV occurred during cohabitation in Experiment 2. Samples were taken from carp during individual housing to assess if infection occurred during the defined contact period. Data are Ct values for qPCR indicating the relative quantity of KHV DNA.

Cohabitation	4h with 1 infected donor, Replicate 1					4h with 1 infected donor, Replicate 2				
Day (after challenge)	7	10	14	21	21	7	10	14	21	21
Sample type	skin swab	skin swab	skin swab	gill	spleen	skin swab	skin swab	skin swab	gill	spleen
Proportion Positive	6/6	6/6	5/5	5/5	4/5	6/6	6/6	6/6	5/6	6/6
Carp 1	21.2	21.3	24.5	27.4	27.6	20.7	19.4	27.8	29.1	20.4
Carp 2	21.7	26.2	30.7	31.4	28.8	30.3	28.5	32.6	Negative	35.1
Carp 3	22.4	24.0	29.4	29.4	33.7	23.3	26.5	30.9	29.1	31.0
Carp 4	21.3	22.9	29.3	29.1	33.9	28.7	27.2	26.8	18.3	25.2
Carp 5	28.8	33.1	Negative	33.9	Negative	24.7	34.4	30.7	26.5	25.7
Carp 6	30.8	32.7	Dead (n/d)	Dead (+ve)	Dead (n/d)	29.7	23.0	26.4	29.6	25.0

Cohabitation	4h with 2 infected donors, Replicate 1					4h with 2 infected donors, Replicate 2				
Day (after challenge)	7	10	14	21	21	7	10	14	21	21
Sample type	skin swab	skin swab	skin swab	gill	spleen	skin swab	skin swab	skin swab	gill	spleen
Proportion Positive	6/6	6/6	3/6	5/6	4/6	6/6	6/6	4/6	4/6	4/6
Carp 1	23.1	29.9	Negative	29.6	30.0	33.7	35.2	34.7	Negative	Negative
Carp 2	24.6	33.8	29.8	32.5	32.8	31.4	22.8	24.6	29.8	30.0
Carp 3	33.0	32.7	Negative	29.6	32.4	24.8	30.5	Negative	31.2	33.1
Carp 4	32.4	19.9	Negative	Negative	Negative	33.5	34.3	Negative	Negative	Negative
Carp 5	25.9	26.4	36.1	37.0	36.2	32.7	32.0	26.5	37.3	30.4
Carp 6	35.7	27.4	31.0	35.9	Negative	31.0	20.0	21.5	30.6	31.2

Table 5: Detection of KHV to determine if transmission of KHV occurred during 1 h of cohabitation in Experiment 3. Samples were taken from carp at the conclusion of individual housing to assess if infection occurred during the defined contact period. Data are Ct values for qPCR indicating the relative quantity of KHV DNA.

Cohabitation	1h with 1 infected donor, early infection (tested Day 13 after cohabitation)				1h with 1 infected donor, advanced disease (tested Day 9 after cohabitation)			
	Replicate 1		Replicate 2		Replicate 1		Replicate 2	
Sample	Gill	Spleen	Gill	Spleen	Gill	Spleen	Gill	Spleen
Proportion Positive	3/6	3/5	3/6	3/6	5/6	4/6	3/6	5/6
Carp 1	Mort (positive)	Mort (n/d)	Negative	25.4	23.9	27.5	27.1	27.0
Carp 2	29.2	32.6	29.6	Negative	28.2	30.0	Negative	35.1
Carp 3	19.6	29.4	Negative	Negative	Negative	Negative	26.4	32.5
Carp 4	Negative	Negative	Negative	Negative	21.6	24.6	Negative	Negative
Carp 5	Negative	Negative	22.0	28.7	32.3	30.9	30.5	29.4
Carp 6	Negative	32.8	24.6	32.7	36.6	Negative	Negative	38.6

Table 6. Transmission coefficient (β) for all experiments in which carp were challenged by cohabitation with the potential for contact or by immersion in the water that housed infected carp but in which contact was no longer possible due to removal of the infected carp.

Exp. ID	Challenge type	Method	Duration (h)	Dose	Replicate	Number of carp			β
						I_0	S_0	S_i	
2	Immersion	40L aquarium water, KHV donor carp removed	4	low (KHV Ct >30)	1	1*	6	6	0
				low (KHV Ct >30)	2	1*	6	6	0
	Cohabitation with potential for contact	KHV infected donor carp / 40L aquaria	4	1 donor, clinical signs	1	1	6	0 **	1.00
					2	1	6	0**	1.00
				2 donors, clinical signs	1	2	6	0**	0.99
					2	2	6	0**	0.99
3	Immersion	40L aquarium water, KHV donor carp removed	1	highest water (KHV Ct 25-26)	1	1*	6	0**	1.00
	Cohabitation with potential for contact	KHV infected donor carp / 40L aquaria	1	1 donor, prior to clinical signs	1	1	6	2	0.67
					2	1	6	2	0.67
				1 donor, advanced clinical signs	1	1	6	1	0.83
					2	1	6	1	0.83

I_0 is the number of KHV infected carp at the start of the trial (*the donors were removed from the immersion only treatment groups to prevent contact)
S_0 is the number of susceptible carp at the start of the trial
S_i is the number of susceptible carp that remained uninfected at the completion of the experiment (a nominal value of 0.001 was added when all carp where infected with KHV to facilitate the equation.

Discussion

The series of experiments completed in this project provide strong evidence that direct contact between carp provides for efficient transmission of KHV. This is highly relevant for models of carp biocontrol which are sensitive to assumptions about the relative importance of indirect water-borne transmission (whereby naïve carp can be infected by KHV shed into the water) compared to direct transmission (requiring direct fish-to-fish contact) (Durr et al., 2019). The efficacy of biocontrol and the magnitude of disease outbreaks will thus be influenced by carp density and impacted by aggregation events such as breeding (Todd et al., 2019).

Horizontal transmission of KHV between carp can occur through exposure to water-borne virus and with contact between infected and naïve carp (Bergmann et al., 2020; Boutier et al., 2015). Whilst the horizontal transmission of infection through water has been described, the relative importance of virus free in water, aggregates of virus associated with particulates in water, and contact between carp has not been established. The experiments designed for this study were able to identify the outcome of carefully defined KHV infection challenges with control of the KHV concentration, contact time and stage of infection for donors in cohabitation. This required individual housing of carp to assess the outcome of the challenge, limiting the number of fish which could be studied in a single experiment without changing the infection challenge. These experiments were facilitated by the transfer of virus and cell cultures that have been used in previous Australian studies and the establishment and utilisation of laboratory assays that could quantify KHV, either as an infectious dose (virus isolation and titration) or number of genome copies (qPCR). The capacity to undertake qPCR on a large number of samples to provide results within hours of collection adds an important dimension to studies such as these and made it possible to monitor the progression of infection in 'real time'. The close alignment of quantitative measurements for the KHV isolate with those of the laboratory from which it was sourced confirms that data from *in vivo* studies in different locations can be reliably interpreted (McColl & Crane, 2013; Sunarto et al., 2021). The present study identified a clear dose response for establishing infection which was consistent with these previous studies. Although artificial, intraperitoneal injection provided a very reliable method of infecting a high proportion of carp with a measured dose of KHV. Immersion challenge was equally reliable at a high dose and is more applicable to understanding the disease due to the more natural mechanisms of infection. However, a key finding in these experiments was the more efficient transmission when there was contact between carp in cohabitation. The focus of this study was to evaluate transmission under natural conditions whereby KHV contaminated water was generated by infected carp and infected carp were used for contact transmission in a limited cohabitation period so that the dose was not distorted by progression of the disease in the 'donor' or other exposed fish.

Transmission of KHV is influenced by release of virus from infected carp, persistence in the environment and effective contact with another host to initiate infection. Acute KHV infection is systemic resulting in high viral load in a wide variety of tissues including the gill and skin mucus which provide the most likely point of shedding of infective virus (Gilad et al., 2004). Primary establishment of KHV infection can occur through the skin (Costes et al., 2009) or the gill and gastrointestinal tract (Monaghan et al., 2015), specifically the pharynx on ingestion of contaminated material (Fournier et al., 2012). The effectiveness of transmission of infection will be influenced by the dynamics of KHV excretion more than the quantity in visceral tissues. The present study generated new data showing the amount of KHV excreted in water and skin mucus throughout the course of infection. KHV was excreted before and after clinical signs and was highly variable for individual fish and over time. Nevertheless, the amount of virus was much higher when lesions were present and was concentrated in skin mucus compared to relatively low concentrations in water, even in confined aquaria with controlled exchange of water. Additionally, the observations of KHV excretion after death did not identify any peaks that would indicate that high loads of virus in tissues are released after death or

able to persist as a potential source of infection. These data strongly support the observation of contact between fish being needed for efficient KHV transmission. This suggests that water-borne spread would be limited to the peak of an outbreak where many fish are infected concurrently, and circumstances where carp are confined, such as aquaculture systems rather than natural water bodies such as rapidly flowing rivers where there is constant exchange of water and potential dilution of virus.

The minimum dose of KHV required to establish infection through immersion challenge ranged from 1 - 10 $TCID_{50}$/mL for juvenile carp (McColl & Crane, 2013). Experimental infection of carp with KHV by immersion in contaminated water has been achieved with a broad range in doses reported, probably reflecting variation in the host, virus strain and environment. For example, 200 KHV plaque forming units/ml aquarium water (Boutier et al., 2015). The lowest dose of cell culture derived KHV used to generate infection by immersion in this study was 60 $TCID_{50}$/mL and qPCR data suggested that immersion in contaminated aquarium water with approximately 10 $TCID_{50}$/mL established infection. It is likely that the minimum infective dose is influenced by multiple factors relating to the environment and the host, particularly the age with higher susceptibility in young juveniles. Detection of KHV in the water where wild carp are infected has used concentration techniques due to the dilution factor of the virus in vast water bodies (Matsui et al., 2008). At these low concentrations, waterborne transmission is unlikely to occur simply by immersion. The present study clearly demonstrated that the concentration of KHV contamination in aquarium water was frequently below the quantity estimated to establish infection by immersion in a short period of time and KHV was not detected when tested by virus isolation. This included times when clinically affected carp with high loads of KHV in skin mucus and tissues were confined in relatively small water volumes. Even if KHV was heterogeneously distributed in water by virtue of attachment to particulate matter, this would have been identified when carp were placed in the contaminated aquaria for 4h. Furthermore, the absence of detectable KHV in water after death of carp suggested that dead bodies are a hostile location for KHV persistence and bodies are not a key source for virus transmission compared via contamination of water. Therefore, the potential for an infectious dose of KHV for water-borne infection in field conditions is unlikely to very low due to dilution in much larger volumes and adverse conditions for the persistence of infectious KHV.

Koi herpesvirus disease can result in high mortality including in wild carp (Garver et al., 2010; Hedrick et al., 2000). However, the disease is multifactorial and the outcome is influenced by environmental factors such as water temperature (Cano et al., 2020) and host factors including the age and genotype of the carp (Palaiokostas et al., 2018). In the present study there was a low mortality but high morbidity for infected carp. It is likely that this reflected the unique methodology in which carp were infected by a defined dose of virus and then housed individually with high quality aquarium conditions. Recovery and survival were observed for a large proportion of carp that developed moderate clinical signs of disease. Many of these carp may have died under field conditions due to adverse water quality, secondary pathogens, and predators. The pathogenesis of disease caused by KHV includes primary disruption to the mucous and skin barrier to enable secondary bacterial invasion (Adamek et al., 2013), with environmental bacteria that are reported in many natural disease outbreaks, e.g. (Hedrick et al., 2000).The present study was not designed to predict mortality from KHV in a biocontrol scenario and its management excluded or minimised the impact of many of these secondary aspects of the disease pathogenesis. It should be noted that the adverse conditions that impact mortality in the field might also impact transmission KHV dynamics due to the alteration of the clinical course of the disease and impact on persistence of KHV. Similarly, for carp that survive there is an adaptive immune response including production of neutralising antibodies (Cabon et al., 2017) as well as the potential for persistent or latent infection which can recrudesce (St-Hilaire et al., 2005). Each state is likely to alter the transmission of KHV compared to the naïve carp used in this study.

The impact of reduced mobility of fish in the wild on transmission by contact is also unknown and could either enhance or reduce large scale transmission. The higher importance of fish contact for effective transmission can be investigated under different conditions for water temperature, carp genotype and the age/health/life history of the fish.

The present work identified values for β approaching the extremes of 1 and 0. This reflected the primary objective of demonstrating the relatively high importance of close contact cohabitation between carp for transmission of KHV compared to water borne exposure. Variability in β was identified despite small group sizes, indicting the large effects of conditions that impacted the dose of KHV i.e. concentration of KHV in water, number of infected carp, stage of infection of donor carp and duration of potential contact time. It is noteworthy that the experimental conditions used relatively short exposures, high density (8 or 9 juvenile carp in 40 L) or targeted the highest concentration of contaminated aquarium water that could be achieved for one of the immersion-without-contact experiments. These experimental conditions were informed by initial experiments using immersion or injection with cell culture derived KHV and monitoring of KHV quantities by qPCR which provided a result within hours to guide the timing of experimental exposures.

It is recognised that experiments to estimate transmission rates are difficult and time consuming (Kirkeby et al., 2017). Furthermore, the conditions in an experimental system are different to the field, leading to the expectation that transmission parameters might be scenario specific. Nevertheless, experimental animal studies have been useful to determine the significance of different host and environmental factors when undertaking studies of the transmission of viruses (Butler et al., 2008) and to compare the efficacy of disease control measures (Dekker et al., 2020). A recent study using a different approach to experiment design and calculation of transmissibility of KHV has confirmed the relative importance of contact between carp for effective transmission of KHV (Tolo et al., 2022). Interestingly, this study also found that the mechanism of transmission was dependant on the stage of infection with direct transmission occurring more readily in the incubation stage while indirect transmission was more likely during the clinical stages when there was a higher concentration of KHV and reduced opportunity for fish contact. Knowledge of the factors that influence β for KHV are useful to inform epidemiologic models of biocontrol scenarios.

The present study included carp that were juveniles (< 6 months of age) and some approaching the size at which sexual maturity might be expected. Infection was established by each transmission mechanism for carp across the size range studied (10 – 30 cm). This spans young-of the-year and fish approaching sexual maturity, which for carp in the Murray River occurs at a minimum length of 150 mm (male) and 250 mm (female), with the majority of the fish over 350 mm being mature (Smith & Walker, 2004). Previous observations confirm increased disease severity occurs in younger compared to older carp e.g. (Perelberg et al., 2003). Susceptibility to severe disease and mortality would influence the epidemiology of KHV in Australia. Consequently, the population structure has been considered by the NCCP (Todd et al., 2019). The present study and previous work have identified the susceptibility of carp nearing sexual maturity to infection with KHV (Sunarto et al., 2021). Sourcing carp across this age range from the areas where carp control is required provided a study population that was representative of the target of a biological control programme. There is, however, merit in confirming the genotype of carp included in trials such as these.

Conclusion

Koi herpesvirus (KHV) is being considered as a biocontrol agent to assist in management of feral carp (*Cyprinus carpio*) in Australia. The main objectives of this project were to generate a data set that would contribute to epidemiological modelling so that strategies for release of virus into the field and

subsequent management of spread of this virus can be controlled for optimal impact. These data included a determination of the concentrations of virus that were needed to infect fish by different methods of exposure and the subsequent outcomes including the clinical impact and shedding of virus. The study methods were designed to assess the efficiency of virus infection and spread at the level of individual fish. Specific host and environmental risk factors were investigated and it is acknowledged that the findings are likely to be influenced by a range of host and environmental factors including the age and genotype of carp and the water quality and temperature.

Wild Australian carp that were obtained from a number of waterways in NSW and Victoria were initially infected with known concentrations of KHV by immersion in contaminated water or by injection. Samples of skin mucus and the water in which individual fish were collected daily to determine the levels of virus excretion. Infected fish that were showing signs of disease were held alive to monitor the progression of disease and changes in virus excretion. Fish that were considered to be terminally affected were humanely euthanised. Levels of KHV excretion were monitored throughout the course of infection whether fish were sub-clinically affected or during clinical disease up to near death and for 24 hours after death when fish died or were euthanised.

Subsequently, as most published studies have utilised virus produced in cell culture, the transmission efficiency of KHV derived from cell culture was compared with virus excreted from an infected fish, particularly as there are high concentrations of virus in mucus excreted from the skin. This was achieved by immersing fish in water contaminated with cell culture amplified KHV or by using water that had housed 'donor' fish at the peak of infection. Looking at the role of mucus in skin further, either 1 or 2 fish at the peak of infection were housed in very close contact with a number of naive fish for short periods of time. The naive fish were then housed separately to assess the outcome of a short period of contact to compare the significance of virus being present free in water with infection arising from the transfer of mucus between fish.

Collectively, from these experiments it was shown that direct contact between carp was necessary for efficient transmission of KHV. Conversely, water-borne transmission of KHV was inefficient under these experimental conditions. However, infection by immersion could be achieved by using high concentrations of cell culture amplified KHV. Nevertheless, a concentration of KHV that was sufficiently high to establish infection by immersion was rarely generated by infected carp, even when aquarium water was contaminated by carp with severe clinical signs of disease and very high viral loads. The quantity of KHV in tissues, skin swabs and water was monitored throughout the course of infection from pre-clinical stages, through the development of lesions and after death or recovery. Despite very high loads of KHV in tissues and skin mucus, the concentration of virus released into water rarely reached a concentration that was needed to establish infection.

These results confirm that epidemiological models for KHV transmission should focus on transmission by fish-to-fish contact. The role of water-borne transmission under field conditions is unlikely to contribute significantly to the spread of KHV where the large volume of natural waters result in a high dilution of virus and there are adverse conditions for the maintenance of infective KHV

While in these studies there were many fish that showed clinical signs ranging from mild through to fish being found in extremis and requiring euthanasia, the level of mortality was in general lower than expected. A number of fish experiencing moderately severe disease showed signs of recovering. Despite these observations, where fish were held under optimal conditions in regard to water quality, temperature and availability of feed, it is possible that many of these fish would have died in the wild. Finally, although these fish were collected from the wild be a very experienced operator who excluded any fish that may have been goldfish hybrids based on visual inspection, there is merit in checking the genetic composition of a proportion of the fish used in this project.

Implications

These studies have clearly demonstrated the need for close contact between carp to optimise KHV transmission. There can be KHV transmission when fish are held in contaminated water, but the virus load needs to be high and is not always achieved by the presence of infected fish. When incorporated into suitable epidemiological models, this knowledge should provide greater certainty about predictions of the course and efficacy of KHV epidemics in wild carp and the efficacy of this virus as a biocontrol agent.

If KHV is to be used as a biocontrol agent, for maximum impact it will be necessary to release the virus under circumstances where close contact between fish is occurring or can be “engineered”.

Recommendations

The outcomes of this project should be incorporated in models of carp biocontrol using KHV. The study identified that transmission of this virus occurs efficiently when acutely infected carp are in direct contact with naïve carp and inefficiently by the water-borne route. Combined with knowledge of carp density and aggregation behaviour it will be possible to better understand the extent of disease expected in various freshwater environments in south-eastern Australia. The time course for excretion of KHV from infected fish will also inform disease models, including the potential role of intraperitoneal injection of trojan carp to initiate infection in a population.

Further development

The experiments in this project were not intended and did not provide an indication of the levels of mortality that would occur from KHV infection of carp in field conditions. The clinical course of disease is expected to be different for wild feral carp where sub-optimal water quality and nutrition, together with secondary pathogens and predators may result in more rapid progression of disease and disease and possibly different mortality to that seen in the experimental model. The objectives of the present study required carp to be individually housed in aquaria with optimised water quality to assess the outcome of very specific KHV transmission events defined by dose and contact time. Wide variation in contact time, KHV concentration and the proportion of infected carp would be expected over the course of a natural disease epidemic. Transmission of KHV is likely to be sensitive to environmental conditions (particularly water temperature) and host factors (including age and genotype of carp). Each of these factors should be considered experimentally to determine if KHV disease will be altered under specific conditions such as a large population of young juvenile carp or weather events that cause variations or extreme water temperatures.

This study was also limited to the acute and immediate post-recovery stages of KHV infection in carp that were naïve to KHV. Transmission of KHV from carp with latent infection and the impact on carp that have been previously exposed and developed an immune response needs further consideration.

Extension and Adoption

The NCCP will be the primary beneficiary of the data and knowledge derived from this work. This will assist evaluating the options and efficacy of different approaches to biocontrol of carp using KHV. The data generated in this project are available as a report and for presentation to the scientific

advisory committee. A presentation from the research team should be considered to ensure all aspects of this project are fully understood.

Project coverage

NA

Project materials developed

After review by the relevant NCCP committees, the outcomes of this project will be considered for publication in an appropriate scientific journal.

Appendices

Appendix 1. Monitoring sheet used to record and score clinical signs.

Trial:	**KHV Transmission by Cohabitation, Exp #:**			
Date: **Time:**		**Tank/Fish ID**	**Observer initials:**	
Disease severity		**Description**	**Observed (Y/N)**	**Comments**
Mild	Mucous	Excessive (skin)		
		Excessive (foamy water surface)		
	Skin	dark lor light colouration		
		Focal reddening		
		Focal 'sandpaper lesions'		
	Other observation:			
Moderate	Appetite	Reduced or absent		
	Mucous	Excessive (gill)		
	Swimming	Reduced		
	Skin	Erosion or ulceration		
	Eyes	Sunken		
	Opercula	increased rate		
	Other observations:			
Severe	Swimming	No swimming activity		
	Opercula	Gasping, rapid shallow		
	Nervous	twitching, erratic movements		
		Non-responsive to stimuli		
		Altered buoyancy (recumbency)		
	Gills	Discolouration, gross lesions		
	Fins	Damaged margins		
	Other observations:			
	Classification of disease severity:			

Appendix 2. Temporal monitoring of KHV excretion in skin mucus and water after ip injection of carp. Data are Ct values for KHV qPCR.

Fish ID	Size	weight (g)	length (mm)	Dose ($TCID_{50}$/fish)	Sample	Day 0 pm	Day 1 am	Day 1 pm	Day 2 am	Day 2 pm	Day 3 am	Day 3 pm	Day 4 am	Day 4 pm
2	Large	130	195	Neg control	skin swab	Negative	n/d	Negative	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
4	Small	34	120	Neg control	skin swab	Negative	n/d	Negative	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
5	Large	75	155	10^1	skin swab	Negative	n/d	Negative	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
7	Large	116	180	10^1	skin swab	Negative	n/d	Negative	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
8	Small	14	90	10^1	skin swab	Negative	n/d	Negative	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
9	Large	222	230	10^2	skin swab	Negative	n/d	Negative	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
10	Large	223	250	10^2	skin swab	Negative	n/d	Negative	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
11	Large	94	175	10^2	skin swab	Negative	n/d	Negative	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
12	Small	17	90	10^2	skin swab	Negative	n/d	Negative	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
13	Large	459	300	10^3	skin swab	Negative	n/d	Negative	Negative	n/d	Negative	n/d	36.29	n/d
					water	Negative	Negative	Negative	Negative	34.45	Negative	Negative	31.49	35.1
14	Large	371	280	10^3	skin swab	Negative	n/d	Negative	n/d	n/d	36.13	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
15	Large	230	240	10^3	skin swab	Negative	n/d	Negative	Negative	n/d	32.62	n/d	29.22	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	28.13	34.4
16	Small	41	135	10^3	skin swab	Negative	n/d	Negative	Negative	n/d	Negative	n/d	33.19	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
17	Large	150	205	10^4	skin swab	Negative	n/d	Negative	Negative	n/d	30.64	n/d	29.18	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	34.81	33.1
18	Large	250	240	10^4	skin swab	Negative	n/d	Negative	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
19	Large	132	200	10^4	skin swab	Negative	n/d	Negative	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
20	Small	15	95	10^4	skin swab	Negative	n/d	Negative	37.06	n/d	27.52	n/d	23.96	n/d
					water	Negative	Negative	Negative	Negative	34.43	32.92	30.88	Negative	33.8

Appendix 2 (continued). Temporal monitoring of KHV excretion in skin mucus and water after IP injection of carp. Data are Ct values for KHV qPCR.

Fish ID	Size	weight (g)	length (mm)	Dose ($TCID_{50}$/fish)	Sample	Day 5 am	Day 5 pm	Day 6 am	Day 6 pm	Day 7 am	Day 7 pm	Day 8 am	Day 8 pm	Day 9 am	Day 9 pm	Day 10 am	Day 10 pm
2	Large	130	195	Neg control	skin swab	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
4	Small	34	120	Neg control	skin swab	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
5	Large	75	155	10^1	skin swab	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	35.33	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
7	Large	116	180	10^1	skin swab	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
8	Small	14	90	10^1	skin swab	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
9	Large	222	230	10^2	skin swab	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
10	Large	223	250	10^2	skin swab	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
11	Large	94	175	10^2	skin swab	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	29.9	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
12	Small	17	90	10^2	skin swab	35.1	n/d	Negative	n/d	Negative	n/d	32.96	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
13	Large	459	300	10^3	skin swab	36.3	n/d	35.9	n/d	25.24	n/d	26.88	n/d	26.14	n/d	24.7	n/d
					water	31.3	30.5	31.1	31.04	31.68	31.86	32.59	31.98	30.87	30.18	30.66	30.4
14	Large	371	280	10^3	skin swab	33.8	n/d	34.4	n/d	Negative	n/d	30.80	n/d	30.82	n/d	29.2	n/d
					water	Negative	34.8	Negative	Negative	Negative	Negative	Negative	Negative	Negative	33.62	Negative	Negative
15	Large	230	240	10^3	skin swab	29.4	n/d	29.5	n/d	26.25	n/d	23.18	n/d	23.45	n/d	25.60	n/d
					water	31.3	34.5	30.1	32.78	32.63	32.12	28.45	31.49	35.36	32.77	35.84	Negative
16	Small	41	135	10^3	skin swab	Negative	n/d	35.5	n/d	31.24	n/d	28.33	n/d	26.63	n/d	26.7	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	31.77	Negative	Negative	33.0	Negative
17	Large	150	205	10^4	skin swab	26.8	n/d	28.3	n/d	24.4	n/d	21.19	n/d	23.44	n/d	25.6	n/d
					water	35.1	31.3	31.8	33.71	30.95	32.15	33.98	30.47	31.22	30.63	34.37	35
18	Large	250	240	10^4	skin swab	Negative	n/d	Negative	n/d	Negative	n/d	35.67	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
19	Large	132	200	10^4	skin swab	Negative	n/d	Negative	n/d	35.28	n/d	Negative	n/d	Negative	n/d	31.2	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
20	Small	15	95	10^4	skin swab	29.5	n/d	27.6	n/d	24.7	n/d	19.61	n/d	21.15	n/d	20.4	n/d
					water	Negative	Negative	36.3	34.17	33.66	33.80	29.85	31.61	31.31	29.78	31.69	30.9

Appendix 2 (continued). Temporal monitoring of KHV excretion in skin mucus and water after IP injection of carp. Data are Ct values for KHV qPCR.

Fish ID	Size	weight (g)	length (mm)	Dose ($TCID_{50}$/fish)	Sample	Day 11 am	Day 11 pm	Day 12 am	Day 12 pm	Day 13 am	Day 13 pm	Day 14 am	Day 14 pm	Day 15 am	Day 15 pm	Day 16 am	Day 16 pm
2	Large	130	195	Neg control	skin swab	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
4	Small	34	120	Neg control	skin swab	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
5	Large	75	155	10^1	skin swab	Negative	n/d	Negative	n/d	34.7	n/d	28.53	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	35.14	Negative	Negative	Negative	Negative	Negative
7	Large	116	180	10^1	skin swab	Negative	n/d	Negative	n/d	Negative	n/d	34.68	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
8	Small	14	90	10^1	skin swab	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
9	Large	222	230	10^2	skin swab	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
10	Large	223	250	10^2	skin swab	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
11	Large	94	175	10^2	skin swab	Negative	n/d	Negative	n/d	34.8	n/d	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
12	Small	17	90	10^2	skin swab	38.3	n/d	Negative	n/d	Negative	n/d	34.89	n/d	29.85	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
13	Large	459	300	10^3	skin swab	25.9	n/d	25.98	n/d	27.4	n/d	32.01	n/d	33.63	n/d	35.4	n/d
					water	31.3	31	29.38	29.9	28.9	37.61	34.01	35.08	34.94	37.96	34.84	Negative
14	Large	371	280	10^3	skin swab	32.7	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
15	Large	230	240	10^3	skin swab	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
					water	Negative	Negative	36.52	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
16	Small	41	135	10^3	skin swab	33.3	n/d	37.18	n/d	34.2	n/d	35.37	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
17	Large	150	205	10^4	skin swab	n/d	n/d		n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
					water	Negative	Negative	Negative	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
18	Large	250	240	10^4	skin swab	Negative	n/d	35.76	n/d	Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
19	Large	132	200	10^4	skin swab	34.9	n/d	Negative	n/d	38.4	n/d	Negative	n/d	Negative	n/d	Negative	n/d
					water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
20	Small	15	95	10^4	skin swab	22.9	n/d	23.19	n/d	28.1	n/d	n/d	n/d	n/d	n/d	n/d	n/d
					water	30.8	32.3	33.46	32.8	Negative	Negative	Negative	n/d	n/d	n/d	n/d	n/d

Appendix 2 (continued). Temporal monitoring of KHV excretion in skin mucus and water after IP injection of carp. Data are Ct values for KHV qPCR.

Day 17 am	Day 17 pm	Day 18 am	Day 18 pm	Day 19 am	Day 19 pm	Day 20 am	Day 20 pm	Day 21 am		Gill	Spleen	Post. Kid	Ant. Kid
Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	n/d		Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative					
Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	n/d		Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative					
33.74	n/d	Negative	n/d	n/d	n/d	n/d	n/d	n/d		34.76	29.55	29.85	29.37
Negative	Negative	Negative	Negative	Negative	Negative	Negative	n/d	n/d					
Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	n/d		33.7	34.57	28.47	33.7
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative					
Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	n/d		Negative	34.96	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative					
Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	n/d		Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative					
Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	n/d		Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative					
Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	n/d		34.11	31.74	32.79	33.9
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative					
33.55	n/d	Negative	n/d	Negative	n/d	37.84	n/d	n/d		28.03	32.25	32.81	33.41
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative					
Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	n/d		28.75	29.15	21.77	33.5
Negative	Negative	Negative	35.66	Negative	Negative	Negative	Negative	Negative					
Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	n/d		Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative					
n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d		21.74	n/d	n/d	n/d
n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d					
Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	n/d		32.59	31.29	26.36	33.73
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative					
n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d		18.74	n/d	n/d	n/d
n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d					
Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	n/d		Negative	34.76	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative					
Negative	n/d	Negative	n/d	Negative	n/d	Negative	n/d	n/d		36.44	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative					
n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d		21.77	23.05	22.57	22.46
n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d					

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