

**Towards a quantitative risk assessment for Koi  
Herpesvirus (KHV) transmission via anglers nets**

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## **Executive Summary**

A quantitative risk assessment was designed to assess the probability of Koi Herpesvirus (KHV) transmission occurring on angling nets with a view to including the data in an existing epidemic model. Steps in the risk pathway pertaining to initial contamination of angling nets and subsequent survival of viable virus during storage were identified for quantification with experimental techniques.

A study monitoring the feeding behaviour and virus shedding in mucus of KHV-challenged carp revealed that carp shedding higher titres of virus eat a smaller proportion of food and are therefore less likely to be caught by an angler than uninfected carp or those shedding lower titres of KHV. Throughout the trial, however, infected carp were simultaneously shedding virus and feeding, thus presenting a risk of being caught and contaminating angling equipment.

A second study, to assess how different storage conditions affect KHV survival, found that dark and damp environments presented the highest risk of preserving viable KHV for transmission to a naïve water. Conversely, light and dry environments reduced rates of KHV survival and sufficient exposure to UV light caused complete inactivation of the virus.

To enable detection of viable KHV, existing culture and titration techniques were modified and improved to enhance sensitivity of the methods and ensure maximum viral titre was achieved quickly. Also, a novel technique for isolating KHV from spiked mucus on cell culture was developed, using centrifugation and filtration to remove bacterial and fungal contaminants.

This project has succeeded in designing a risk assessment and experimental techniques and has initiated quantification of the steps necessary for assessing the risk of transmitting KHV on angling equipment. It has also broadened prospect for future studies using viable KHV for epidemiological studies.

## **1. Introduction**

Koi Herpesvirus (KHV) is an emerging pathogen that is threatening the welfare of wild and commercial populations of common carp (*Cyprinus carpio*) both in the UK and globally. KHV is a listed notifiable disease under European Community Directive (2006/88/EC). Consequently member countries which declare themselves KHV-free or in a control and eradication programme can impose restrictions on the introduction of susceptible fish species from countries with a lower health status, which may have substantial economic impact on exporting countries. The UK has declared itself positive for KHV and thus cannot restrict imports from other infected countries. Efforts to control and limit the spread of the pathogen must be applied at the national level. To do this it is crucial to have a clear knowledge of the pathogen's biology and modes by which it is transmitted between waters. This section of the report therefore aims to review the current scientific literature pertaining to KHV, with a view to identifying knowledge gaps and highlighting information that could aid our understanding of pathogen spread and dynamics.

### **1.1 Clinical signs and pathology**

KHV is the aetiological agent of a highly infectious and lethal disease of common (*Cyprinus carpio carpio*) and koi carp (*Cyprinus carpio koi*). Carp are economically important in global aquaculture, fishery and ornamental trades and are highly susceptible to KHV disease. Way and Dixon, (2007) established that a five minute exposure to 10-40 virus particles ml<sup>-1</sup> can cause 70% mortality in a population within 15 days. They also demonstrated a rapid onset of disease with mortalities, depending on water temperature, which begin to occur seven to 21 days post-exposure to KHV. Temperatures conducive to viral replication are restricted to a range between 18 and 28°C and this has great implications for the virulence of the virus (Gilad *et al.*, 2003; Hedrick *et al.*, 2000; Omori & Adams, 2011). Temperatures above 18 °C usually result in 100 % infection of a naïve carp population, with 90 % succumbing to clinical signs and, inevitably, death (Gilad *et al.*, 2003). Transmission of the

virus and mass mortalities caused by KHVD are therefore limited by seasonal fluctuations in water temperature (Minamoto *et al.*, 2009a; Omori & Adams, 2011). Clinical Koi Herpesvirus disease (KHVD) is characterised by necrosis of the gills and skin lesions. Other clinical signs manifest as fin rot, mucus sloughing, enophthalmia, reduced or lack of appetite, erratic swimming behaviours and oedema of the internal organs (Hedrick *et al.*, 2000; Pikarsky *et al.*, 2004). Microscopic examination of the internal organs confirm systemic spread of the virus which has been detected in the brain, spleen, liver, gastrointestinal tract, kidney and blood leucocytes of diseased carp (Gray *et al.*, 2002; Gilad *et al.*, 2004; Ilouze *et al.*, 2006; Bergmann *et al.*, 2010). Pathology caused by the virus at a cellular level can be observed by microscopy of infected common carp brain (CCB) and koi fin (KF-1) cell lines (Davidovich *et al.*, 2007). Cytopathic effect (CPE) is observable as cell enlargement and elongation, syncytia formation and vacuolation, with affects developing 5-11 days post infection (Hedrick *et al.*, 2000).

## **1.2 Classification of Koi Herpesvirus**

Disease signs and host range of the virus were reflected in the initial nomenclature, KHV and Carp Interstitial Nephritis and Gill Necrosis Virus (CNGV), which were used interchangeably. Early classification of KHV as a Herpesvirus was due to morphological similarities such as a core diameter of 100-110 nm and an overall diameter of 170-230 nm (Miwa *et al.*, 2007), supportive teguments in the virus core, an envelope derived from the host cell wall upon entry and an icosadeltahedral capsid which are known to be well-conserved among Herpesviruses (Hedrick *et al.*, 2000; Hutoran *et al.*, 2005; Waltzek *et al.*, 2005). Further molecular examination of KHV revealed a double stranded DNA genome which is 295 kbp in size - considerably larger than usual for Herpesviruses (Aoki *et al.*, 2007). Though mostly well conserved, the genome was demonstrated to contain divergent DNA sequences that are similar to a range of other large DNA viruses such as poxviruses (Ilouze *et al.*, 2006), and to encode a variety of polypeptides not found in Herpesviruses (Costes *et al.*, 2009). Subsequently KHV was designated Cyprinid Herpesvirus-3 (CyHv-3) due to its close genetic and

morphologic relationship with Cyprinid Herpesvirus-1 (CyHV-1) and Cyprinid Herpesvirus-2 (CyHV-2), which are the aetiological agents of carp pox and goldfish haematopoietic necrosis respectively (Waltzek *et al.*, 2005). Genetic homology between the three Cyprinid Herpesviruses suggests transfer of genomic DNA occurred between these viruses and also shows a high level of genetic conservation (Waltzek *et al.*, 2005). These viruses were reclassified to a newly created family, *Alloherpesviridae*, which contains other aquatic herpesviruses such as Ictalurid Herpesvirus-1 (Davison *et al.*, 2009).

### **1.3 Techniques for detecting Koi Herpesvirus in hosts and the environment**

A generic polymerase chain reaction method for detecting all three Cyprinid Herpesviruses is available (Jeffery *et al.*, 2007). There are a variety of techniques available for specific detection of KHV in fish including a standard PCR (Gilad *et al.*, 2002), tissue culture (Hedrick *et al.*, 2000; Davidovich *et al.*, 2007), Enzyme-linked Immunosorbent Assay (ELISA) (Adkison *et al.*, 2005), gene sequencing (Ishioka *et al.*, 2005), immunocapture (Soliman & El-Matbouli, 2005) and loop mediated isothermal amplification (LAMP) (Soliman & El-Matbouli, 2005 & 2009). The recommended method of KHV detection is nested PCR (Bercovier *et al.*, 2005; El-Matbouli *et al.*, 2007) which provides a higher sensitivity than standard PCR. Real time PCR can also be used (Gilad *et al.*, 2004) and has the additional benefit of quantifying the number of copies of genomic DNA detected in a sample, thereby providing a viral titre. However, PCR is less effective than serological test methods, such as ELISA which detects anti-KHV antibody, at identifying carriers of KHV due to the low amount of virus present. ELISA therefore indicates whether a fish has been exposed to KHV and, like PCR which only detects viral genomic DNA, does not detect live, infectious virus. Culture of KHV in CCB and KF-1 cells is therefore important for identifying active, infectious KHV. Growth of the KHV can be observed in the progression and increased severity of characteristic CPE and virus identity can be confirmed with a molecular tool. However, tissue culture has a lower sensitivity than molecular tools and the virus is fastidious and slow growing, which made initial identification of the virus difficult.

In addition to diagnosing KHV in live fish, quantification of KHV in environmental water is important, though has proven difficult due to high dilution factors. Initially methods for recovering virus from spiked water were ineffective, with the most efficient elution process resulting in a 5.5 % yield (Haramoto *et al.*, 2009). Recently developed techniques have been more successful, achieving 50 % virus recovery and a sensitivity of 60 copies of KHV DNA per litre of water (Honjo *et al.*, 2010).

#### **1.4 Environmental survival**

Degradation of KHV in environmental water has also been an area of interest and a study by Shimizu *et al.*, (2006) established that KHV infectivity is lost within three days in environmental water and within seven days in sterile, filtered water. The study further suggested that the presence of environmental bacteria with anti-viral properties could hasten KHV inactivation. In addition to bacterial concentration Minamoto *et al.* (2009b) demonstrated factors such as water turbidity, redox potentials and chlorophyll  $\alpha$  concentration reduce KHV infectivity. Similarly, KHV has exhibited inactivation when exposed to  $4.0 \times 10^3 \mu\text{W cm}^{-2}$  UV irradiation, pH below three or above 11 and disinfectants such as chloroform, lipid solvents, oxidising agents and temperatures above 60 °C or consistent 35 °C for two days (Kasai *et al.*, 2005) which can be utilised from a biosecurity perspective. KHV is able to persist in the environment and remain viable during the colder months. Suggestions for how this is achieved include virions shed in significant quantities in carp faeces settling into sediment on water beds where they are protected by a more reductive atmosphere (Dishon *et al.*, 2005) or by developing persistent or latent infection in susceptible fish species, including carp and goldfish (Bergmann *et al.*, 2010; El-Matbouli & Soliman, 2010).

#### **1.5 Temperature manipulation and latent infection**

The ability to establish latent infections in immunocompetent hosts is a common characteristic of other Herpesviruses and it is possible KHV could do the same. Currently, there is mounting evidence for lengthy survival of the virus in host cells (Dishon *et al.*, 2007), persistent infection of carrier

species (Bergmann *et al.*, 2010) and of latent infection in koi (Eide, 2011). There is also evidence for development of KHVD in naïve fish cohabited with carp persistently infected but exhibiting no signs of clinical disease using temperature manipulation (St-Hilaire *et al.*, 2005) and reactivation of KHV in carp that produce high titres of anti-KHV antibodies following a move to permissive temperatures (St-Hilaire *et al.*, 2009). Finally, a study by Eide *et al.*, (2011) has begun genomic analysis of latency, which has identified KHV DNA in infected carp but not mRNA, which would be expected in a lytic (acute) infection, thus suggesting there may be latent KHV present. Again, temperature stress was used to induce reactivation of the virus and cause KHVD in the carp. Latency has the potential to facilitate the rapid spread of KHV as latently infected fish show no clinical signs and are therefore likely to be moved, posing a threat to naïve carp if the virus becomes reactivated.

### **1.6 Host range and carrier species**

Clinical KHVD has currently been observed only in varieties of the *C. carpio* species; however there is mounting evidence to suggest that the virus can also infect carrier species without causing signs of disease. These proposed carriers include grass carp (*Ctenopharyngodon idella*) and the common goldfish (*Carassius auratus auratus*) (Davidovich *et al.*, 2007). KHV has been shown to persist for over 60 days in goldfish (Bergmann *et al.*, 2010) and to successfully transmit from infected goldfish to naïve common carp when cohabited (El-Matbouli & Soliman., 2011). Fisheries that stock carrier species alongside common or koi carp could be unknowingly increasing the risk of introducing KHV to their site, as carriers may shed infective virus whilst appearing healthy, as they are not susceptible to clinical KHVD.

### **1.7 Hybridisation programmes for improving resistance to Koi Herpesvirus disease**

The discovery of carrier species and less susceptible carp strains for KHV has resulted in investigative work to determine whether hybridisation breeding programmes could be used to produce



commercially viable carp populations with high resistance to KHV infection. An initial study by Shapira *et al.*, (2005) demonstrated that crossing wild, more resistant carp varieties with greater resistance such as *Cyprinus carpio hamatopterus* with susceptible domesticated strains created less susceptible hybrids. These results suggest it may be possible to develop a selective breeding programme for more robust, healthy carp stocks especially as the hybrid strains showed increased fry productivity. However for commercial validity a number of problems need to be addressed, particularly the increased tendency for the hybrids to escape, full scaled progeny as opposed to mirror or leather varieties (Shapira *et al.*, 2005) and the development of resistant ornamental koi as well as common carp. Hedrick *et al.* (2006) continued this work by exploring the prospect of crossing common carp with goldfish to generate KHV resistance. Male goldfish crossed with female common carp resulted in moderate resistance to KHV with 75% of the population surviving the infection, though the sample sizes were small and the common carp used appeared to have a greater than usual resistance to KHVD. The most recent work exploring hybridisation, a study by Bergmann *et al.* (2010), focused on koi rather than common carp, crossing them with crucian carp and goldfish. The koi hybrids showed no reduction in mortality rates when crossed with crucian carp and suffered significant losses when hybridised with goldfish. These three studies clearly demonstrate that although there may be scope to produce KHV resistant carp hybrids, greater research is clearly required to achieve an economically, commercially and epidemiologically sound solution.

### **1.8 Developing an effective vaccine against Koi Herpesvirus**

With successful hybridisation programmes seemingly still out of reach, vaccination may provide a preventative method that could be used to protect carp populations from KHVD and is also the subject of much research. Live attenuated virus vaccines have been tested in two studies and resulted in full resistance to KHVD when injected, though can induce KHVD in carp if they are exposed by immersion to the vaccine for too long (Ronen *et al.*, 2003; Perelberg *et al.*, 2005). These tests also concluded that a high frequency of passages in cell culture were required to attenuate the

virus enough to stimulate a significant level of immunity in the challenged carp. They also demonstrated that for successful immunisation to occur the carp had to be challenged at a temperature permissive to viral replication and that the attenuated virus only remained active in water for between two and four hours. This obviously has practical implications for potential vaccine use on an industrial scale. Antibody response to infection with attenuated virus has been demonstratively high, though antibody titre in sera reduces almost to that of a naïve carp 280 days post infection (Perelberg *et al.*, 2008). Another problem with the use of attenuated virus as a tool for vaccination is the potential reversion to pathogenic strain of virus following *in vivo* replication. No experiments have thus far rigorously tested this possibility and until the likelihood of the attenuated virus reverting to an infectious pathogen is fully analysed then use of such vaccines would be dangerous and unethical. Therefore recent attempts to manufacture a vaccine against KHV have focused on genetic recombinants of attenuated virus with gene knockouts encoding enzymes required for nucleotide metabolism (Fuchs *et al.*, 2011). This is a novel area of research for KHV vaccines which has only been made possible due to ongoing research into the KHV genome. Other methods of vaccination have also been explored, including formalin-inactivated KHV encased in a liposomal membrane (Yasumoto *et al.*, 2006). Omori and Adams, (2011), have also used thermal treatment by disrupting usual water temperature changes caused by seasonal differences to generate an immune response to KHV in carp. Safety, efficacy, economy and practicality of vaccination schemes are areas that sorely need to be addressed before vaccination against KHV can become a reality. However, vaccinated fish have the potential to become carriers of the virus and therefore, if not properly utilised and regulated, vaccination could result in increased dissemination of the disease.

## **1.9 Transmission and global distribution of Koi Herpesvirus**

It has been proposed that horizontal transfer of KHV between carp occurs via the skin and is followed by systemic dissemination (Costes *et al.*, 2009). This mode of entry provides an explanation

for the rapidity of transmission and the ability of the virus to establish infection in a host at high dilutions of virus in environmental water. It has also been observed that the quantity of viable KHV, shed in the faeces of infected fish is sufficient to subsequently infect fish experimentally, and could increase the rate of transmission through a carp population (Dishon *et al.*, 2005). The effectiveness of KHV transmission has become evident in the rapid global dissemination of KHVD. Since the first outbreaks in Israel, Germany and the US (Bretzinger *et al.*, 1999; Hedrick *et al.*, 2000) KHV has been detected in European countries including Austria (Marek *et al.*, 2010), Poland (Antychowicz *et al.*, 2005; Bergmann *et al.*, 2006), Czech Republic (Pokorova *et al.*, 2007), and the UK (Taylor *et al.*, 2009a); Asian countries including Indonesia (Sunarto *et al.*, 2011), Japan (Sano *et al.*, 2004), South Korea (Gomez *et al.*, 2011) and Taiwan (Tu *et al.*, 2004). Additionally, detection of KHV in Belgium, China, Denmark, France, Italy, Luxembourg, Malaysia, The Netherlands, Singapore, Switzerland, Thailand and South Africa has been reported (Haenen *et al.*, 2004). Subsequent analysis of genomic data from virus isolates found that two discrete genotypes exist - European (including USA and Israel isolates) and Asian, and within these genotypes are seven and two variants respectively (Kurita *et al.*, 2009). This suggests either independent emergence of diverse strains of KHV or else introduction of unique genotypes of KHV to the different locations (Kurita *et al.*, 2009).

A study of the situation in the UK (England and Wales) by Taylor *et al.*, (2010a) demonstrated infection of both farms and fisheries, though fisheries are more likely to be exposed (37 % tested antibody positive compared to 4 % of farms). Distribution of the disease throughout England and Wales was found to be widespread, a result of effective transmission and a microcosm of the situation worldwide. Match fisheries, where large numbers of carp may be held in keepnets for a considerable length of time, were found to be particularly at risk of KHV introduction. Taylor *et al.*, (2010b) later reported that 34 % of KHV-positive sites could not identify the original source of infection. Further modelling of KHV transmission identified consented live fish movements of either stock or ornamental fish posed the highest threat, though also confirmed suspicions that live fish movements alone could not account for the prevalence of anti-KHV antibody positive sites in the UK

(Taylor *et al.*, 2011). However for sites where no source of introduction could be identified, natural water connections, fomites such as angling equipment and vectors are possible alternative routes of infection (Taylor *et al.*, 2010a).

### **1.10 Scope for limiting spread of Koi Herpesvirus between carp populations**

With KHV characterised and classified and many of the recent studies and published literature focusing on hybridisation schemes, vaccination development and potential establishment of latent and persistent infections, a gap was evidently widening in research of the quickening global dissemination of the virus between populations. As previously stated, for many infected waters the source of contamination is not known. Preventing transmission of KHV may be an effective, practical and economic method of reducing the threat of mass carp mortalities induced by the disease. This project was devised to investigate the possible routes of transmission of KHV that have so far been neglected or overlooked. The aim of this study was to determine whether there is a risk of transmitting KHV from an infected water to a naïve water either by contaminated angling equipment. Ultimately the aim was to quantify the risk of transmission and to add this information to existing models of transmission to determine whether infected waters with no apparent source of contamination can be accounted for. Finally this project aimed to provide practical advice to limit the spread of the virus between waters that could be enforced by recreationalists, industrialists and hobbyists that deal with carp, based on the conclusions drawn from this study.

## **2. Risk Assessment**

Many pathogens, including viruses, can be spread to new populations by several routes and identifying which carry the highest risk of successful transmission is important when devising preventative measures. Taylor *et al*, 2010b demonstrated live fish movements and addition of ornamental species as high risk routes of KHV introduction and dissemination. Other possible routes of transmission are via natural or man-made water connections, however proof that KHV rapidly loses virulence when in environmental water for extended periods of time (Shimizu *et al*, 2006) renders transfer of KHV via water a negligible risk. Additionally, several stillwater fisheries, with no record of live fish movements for KHV positive sites and lacking water connections have tested positive for anti-KHV antibodies (Taylor *et al*, 2010b). In such sites, transmission via fomites such as aquatic birds or angling equipment are possible routes of introduction. Fomites and possibly vectors, such as crustaceans and non-Cyprinid fish species (European Food Safety Association, 2007), that move unrestricted between waters could conceivably pose a significant risk of transmitting viable KHV but with little prospect of controlling such movement. This study focused on assessing the likelihood of transmission of KHV occurring between carp populations via anthropogenic movement of fomites. Fisheries are at greater risk of introducing KHV than farms (Taylor *et al*, 2010a), and angling equipment may be considered a high risk fomite as it is used in direct and prolonged contact with fish which may be shedding viable virus. Netting equipment (keepnets and landing nets) potentially constitutes the highest risk category of angling equipment due to its direct contact with fish. Frequent use and storage in stink sacks (sealed, watertight bags used to prevent odour and fish-tainted water escaping) may provide a suitable environment for KHV to survive and remain viable. Angling is a common pastime and it is likely that anglers visit many different sites, which, especially in summer months, is when disease progression and viral titres in environmental water are at their peak (Omori & Adams, 2011; Minamoto *et al*., 2009a). This high frequency of occurrence provides the potential to cause infection events even if the risk posed by a single transfer via netting is

extremely low. Transfer via angling equipment has the potential to be minimised through disinfection regimes, educating anglers to be more conscious of biosecurity and fisheries providing anglers with nets to prevent the use of nets that may have been used on other sites.

The aim of this study was to work towards quantifying the risk of transmission of KHV via angling equipment for inclusion in a model of transmission created by Taylor *et al.* (2011). The first step was to create a risk pathway (see figures 1, 2, 3 and appendix 1) chronicling the events required for successful transmission from an infected water to a naïve population of carp.

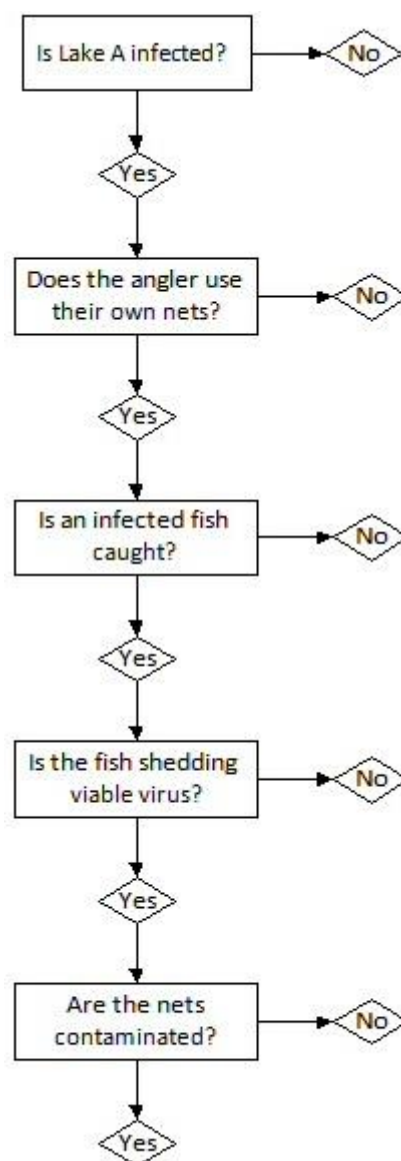
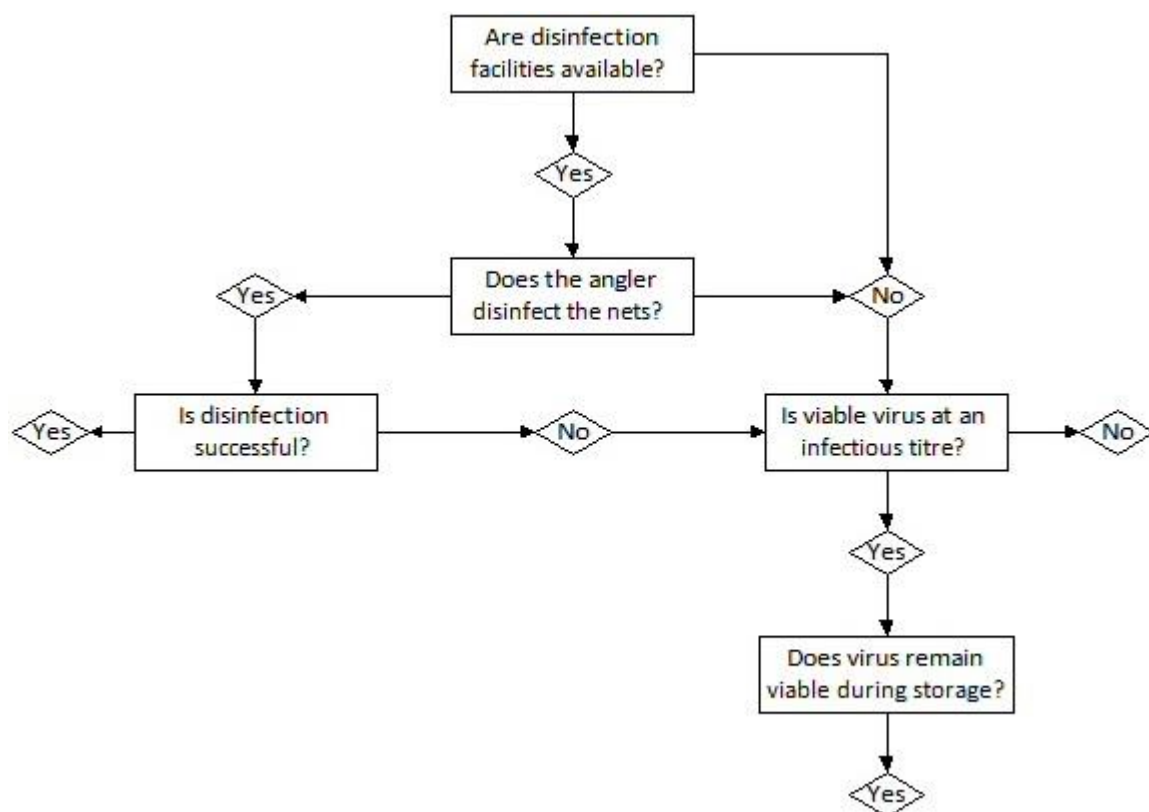


Figure 1: Steps of the risk pathway detailing the initial contamination of angling nets with KHV

The first section of the risk pathway (see Figure 1) involves the initial contamination of angling equipment caused by contact with an infected fish shedding viable virus. The likelihood of catching an infected fish is dependent on the prevalence of KHV infection and fish feeding habits; lack or loss of appetite are a clinical signs commonly observed in carp with clinical KHVD (Hedrick *et al*, 2000; Pikarsky *et al*, 2004). The captured fish must also be shedding viable virus to contaminate the equipment with a titre capable of infecting a naïve carp, preferably at a non-clinical stage of infection so the angler remains unaware of the disease and is therefore less likely to disinfect their equipment. Finally the angler must be using their own equipment; a review of angling magazines suggests some fisheries insist on providing keepnets for onsite use, particularly in matches involving carp, and this removes the risk of transferring the virus to another site.

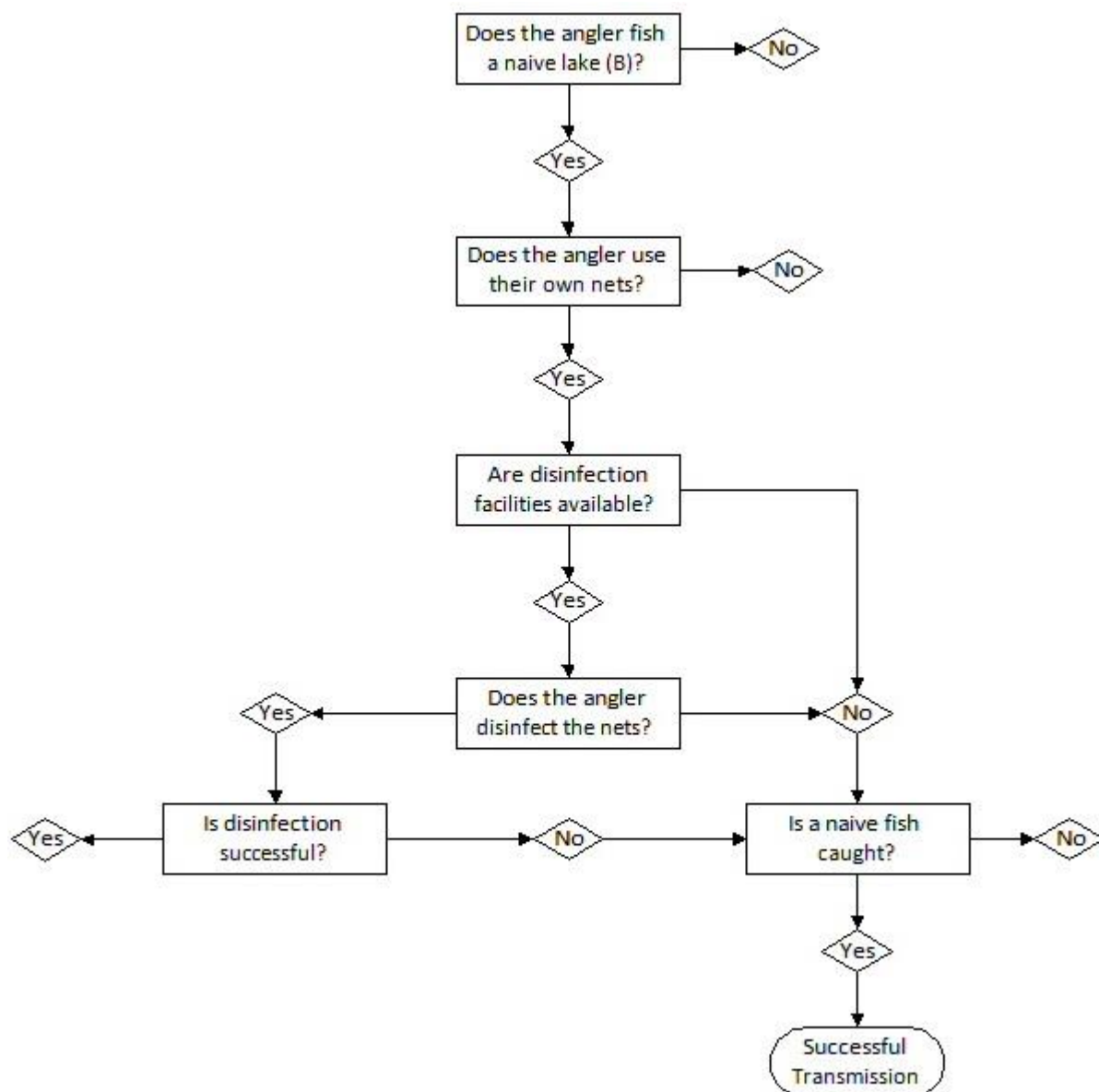


**Figure 2: Steps of the risk pathway showing KHV surviving disinfection and storage to remain viable**

Following contamination of angling equipment with viable KHV, the virus must persist and remain viable until contact with a naïve lake occurs. Figure 2 shows that the virus must survive any

disinfection processes and storage *ex vivo* before being transmitted to another population of susceptible fish. Disinfection stations are present at some fisheries, though it is not known what proportion offer these facilities. Disinfection stations are not required under the fisheries registration scheme (by law all fisheries in England and Wales must register with CEFAS), and for those fisheries that do provide disinfectant there are no regulations to ensure a suitable, effective standard is maintained. Successful disinfection of equipment is reliant on the correct type and dilution of an effective disinfectant being used and for a sufficient duration. A study by Kasai *et al.* (2005), concluded that 3 mg chlorine per litre of water was the best method for inactivating KHV. Storage of angling equipment is also important and is a topic that can be discussed with anglers to achieve best practice when limiting the spread of KHV. As previously stated, a common method of storing nets is to place them in sealed stink sacks and it is hypothesised that this provides an optimum dark and damp environment to provide the best chance for virus survival. Survival of the KHV is also dependent on temperature (Gilad *et al.*, 2003; Kasai *et al.*, 2005) and duration of time spent in the environment (Shimizu *et al.*, 2006).





**Figure 3: Final steps of the risk pathway showing KHV transmission to a naïve lake**

Finally, virus surviving storage must be transported to a naïve lake (Figure 3). Once at the site, the angler must not effectively disinfect their equipment and must catch a naïve carp which is susceptible to KHV infection.

This study aimed to quantify steps in the risk assessment pertaining to the contamination of netting equipment and the subsequent survival of KHV under different storage conditions. In particular, tank experiments were designed to scrutinise the feeding habits of KHV-infected fish and to correlate this behaviour with virus shedding to establish a probability that an angler will catch an infected fish and

to compare the survival rates of virus exposed to different external conditions. This study also developed new methods and enhanced existing techniques for culturing and isolating KHV, therefore broadening the scope for further research.

### **3. Materials and Methods**

#### **3.1 Study 1: Relationship between viral shedding and feeding rate**

This study was designed to simultaneously monitor feeding behaviour and virus shedding in carp challenged with KHV. Feeding behaviour is indicative of the probability an angler will catch a fish and virus shedding in mucus is a risk posed by infected fish which could cause subsequent contamination of angling equipment. This quantitative data can be fed directly into the risk pathway (see figure 1). The KHV isolate UK N076 (recovered from clinical samples at CEFAS) at concentration  $10^5$  TCID<sub>50</sub> was used in all the experiments, unless otherwise indicated. Twenty five 40 g carp (*Cyprinus carpio*) were obtained from a farm in the UK with no prior exposure to KHV for use in this study.

Twenty carp were bath challenged with KHV for four hours at 23 °C. Simultaneously, 5 carp were mock challenged with media under the same conditions. The fish were then moved to individual 10 L static tanks, maintained at 23 °C and 80 % aeration, with negative controls kept in a separate room. On a daily basis all debris (uneaten food and faeces) was removed and each fish was fed 40 pellets of food. The number of uneaten pellets were counted every day to calculate the percentage of food eaten by the fish and thus to define feeding behaviours over the course of the infection. Water samples were taken daily to measure virus accumulation over time. Each day 30 % of the tank water was exchanged for fresh water, to prevent stagnation. Mucus samples were also taken daily by netting each fish and holding for 30 s, returning the fish to the tank whilst preventing the net from contact with the water and finally cutting the net and placing in a centrifuge tube. The samples were centrifuged at 3,000 rpm at 4 °C for 15 min to recover the mucus and water from the net. These samples were then stored at -20 °C for later DNA extraction and analysis with real time PCR. Observations were made of the progression and clinical signs of the disease. The fish were held until they either succumbed to the disease or were terminally anaesthetised when moribund. Blood samples were taken from survivors at 30 days post infection and sera analysed by the Taylor *et al.*

(2009) ELISA method for presence of anti-KHV antibodies. Survivors were then terminally anaesthetised with benzocaine.

### **3.1.1 DNA extraction of mucus samples from nets in preparation for real time PCR**

A 100 µl aliquot of mucus sample and 60 µl AVE buffer were manually added, along with other pre-packaged reagents, to the Quiagen Biorobot EZ1 workstation. The process was fully automated using the EZ1 Virus programme. The condensed 60 µl extracted DNA was then stored at -20 °C until required.

### **3.1.2 Quantitative real time PCR**

Real time PCR was run according to the Gilad *et al.* (2004) method. The Gilad TaqMan probe (KHV-109p) and primers (Gilad -86 forward and Gilad -163 reverse) were used (sequences shown in figure 4). The reaction mastermix was purchased from Roche. Carp DNA negative for any Cyprinid herpesvirus contamination (including KHV) was prepared at CEFAS following the method below.

Gilad KHV -86F = 5' GACGCCGAGACCTTGTG 3'

Gilad KHV -163r = 5' CGGGTTCTTATTTTGTCTTGT 3'

GILAD KHV-109P Probe = 5' CTCCTCTGCTCGGCGAGCACG 3'

**Figure 4: Sequences of the primers and probe used for real time PCR**

The standard curve created ranged from 10 to 10<sup>5</sup> copies of DNA and the reaction was performed on a StepOne Plus machine, running version 2.2 of the StepOne Software.

### **3.1.3 Preparation of carp DNA negative for Cyprinid herpesvirus contamination for use in real time PCR**

Carp DNA negative for Cyprinid herpesvirus contamination was extracted according to the Cefas Molecular Biology protocol 'Extraction of total DNA from fish tissue homogenates using the DNAzol method.' The homogenate used was derived from the kidney, liver and spleen harvested from

common carp with no prior exposure to KHV. A 0.1 g sample of tissue was added to 900 µl of TM and homogenised for 2 min using a Fastprep (MPBio) machine. Nested PCR was used to confirm absence of any Cyprinid herpesvirus-derived nucleic acids. The primers and internal primers used were described by Jeffery *et al.* (2007) and were designed to amplify a sequence of the DNA polymerase gene from all cyprinid herpesviruses and were not KHV-specific. The sequences can be seen in figure 4.

CyHV Reverse primer (1<sup>ST</sup> round PCR) = 5' CCGTARTGAGAGTTGGCGCA 3'

CyHV Forward primer (1<sup>ST</sup> round PCR) = 5' CCAGCAACATGTGCGACGG 3'

CyHV Forward primer (2<sup>nd</sup> round PCR) Internal = 5' CGACGGAGGCATCAGCCC 3'

CyHV Reverse primer (2<sup>nd</sup> round PCR) Internal = 5' GAGTTGGCGCAYACYTTCATC 3'

**Figure 4: sequences of the Cyprinid Herpesvirus primers used in Nested PCR**

All the PCR reagents were from Promega, apart from the primers (Sigma), molecular grade water (VWR) and oil (Sigma). For the gel electrophoresis step the Agarose was from Bioline, Ethidium Bromide from Fisher and the 100 bp ladder and TAE buffer from Promega.

#### **3.1.4 Statistical analysis**

The proportion of feed fish eaten by KHV exposed and control fish, and the relationship between feed uptake and viral shedding were examined graphically and analysed using Generalised Linear Mixed Models (GLMM). In these analyses, to account for the lack of independence of samples taken within fish at different time points, fish were treated as a random factor. Exposure to KHV and viral titres detected in mucous were treated as fixed effects. As the outcome variable, feed uptake, was a proportion, logistic regression models were used that assumed a binomial error distribution (log-link function). Analysis was

conducted in R version 2.13.0 (R Development Core Team 2011), and the GLMMs were performed using the glmmADMB library (Skaug, Fournier and Nielsen, 2008).

## **3.2 Study 2: Development of culture methods to allow assessment of viral survival**

Presence of viable KHV was determined by *in vitro* tissue culture and titration by monitoring for cytopathic effect (CPE) caused by viral proliferation. Virus isolation on cell monolayers was performed in 25 cm<sup>2</sup> flasks containing 7.0 ml culture medium, Minimum Essential Medium Eagle's (EMEM). Titrations were performed on 24-well plates with 1.0 ml EMEM: 1.0 ml L-15, unless otherwise defined and the TCID<sub>50</sub> calculated according to the Spearman-Kärber method (Spearman, 1908). Both flask cultures and titrations were incubated at 20 °C for 7-14 days. The common carp brain (CCB) cell line and were used in all experiments, except where stated. Leibovitz (L-15), EMEM, Transport Medium (TM) and any additional reagents were purchased from Sigma.

During the course of these experiments, the methods for culturing and titrating KHV were tested with different conditions to improve the techniques. For each of these variances, either cultures in flasks were monitored for CPE or titrations were incubated for 10 days, fixed and stained (see below), checked for CPE and a TCID<sub>50</sub> calculated.

### **3.2.1 Virus isolate and passage number**

Different passage numbers of the isolates UKH361, UKM083, UKN071, UKN076 and UKN099 were cultured in flasks and titrated with EMEM on 24-well plates.

### **3.2.2 Media**

Titrations of UKM083 and UKN076 were repeated using L-15, EMEM or an L-15/EMEM (1:1) mixture of media in 24- and 48-well plates.

### **3.2.3 Inoculum dilution**

Samples of UKN076 were used to inoculate three cell cultures. The first with 500 µl KHV, the second with 500 µl KHV at  $10^{-1}$  TCID<sub>50</sub> and the third with 50 µl virus diluted to  $10^{-1}$  TCID<sub>50</sub>. Further to this, four more flasks were inoculated with 50 µl UKN076 at  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$  TCID<sub>50</sub> dilutions. A fifth flask was inoculated with 50 µl undiluted KHV as a control.

### **3.2.4 Time point for harvest of virus**

Ten flasks were inoculated with 500 µl KHV diluted to  $10^{-1}$  TCID<sub>50</sub>. They were incubated at 20°C and then harvested in duplicate at five time points, regardless of presence or absence of CPE. The cells were harvested on days five, seven, nine, 11 and 13 and titrated. A further eight flasks were inoculated with 500 µl UKN076 diluted to  $10^{-1}$  TCID<sub>50</sub>, to be harvested and titrated in duplicate on days four, five, six and seven of incubation at 20°C.

### **3.2.5 Storage conditions**

Two strains of KHV, UKN076 and UKM083, were stored in cryotubes at 4°C and -80°C for two weeks and then titrated in L-15 onto 48-well plates with the EMEM removed.

### **3.2.6 Fixing and Staining**

Fixing and staining cells improves visualisation for early identification of CPE. Cells were fixed by adding approximately 2.5 ml 10% Neutral Buffered Formalin (NBF) to the media in the wells. After 1 hour the fixative was removed and 1% crystal violet added to the wells (0.5 ml for 24 well plates and 0.3 ml for 48 well plates). The stain was left for 20 minutes before being removed and the cells washed with distilled water. The plates were allowed to dry overnight before being examined under a light microscope at 100x magnification. Stained plates were kept in dark conditions at room temperature to test the longevity of the stain.



### **3.2.7 Plaque titration**

Plaque titration is a variation on standard titration which prevents even spread across the cell monolayer and isolates virus to create well defined plaques, not unlike bacterial colony counts. KHV strains UKM083 and UKN076 were titrated in duplicate onto four six-well plates. A fifth plate was used as a negative control. Serial dilutions of virus from  $10^{-1}$  to  $10^{-6}$  were made in L-15 medium. The EMEM culture medium was removed from the wells before 250  $\mu$ l of each dilution was pipetted onto the cells. The plates were then gently swirled to ensure the surface of the cell monolayer was fully covered with the virus suspension. The plates were kept at room temperature for an hour, gently swirling the plates at 10 minute intervals, to allow the virus to adsorb to the monolayer and infect the cells.

The overlay, which was prepared during the adsorption step, consisted of low gelling temperature (LGT) agarose and L-15 medium. 50.0 ml L-15 was warmed in a water bath to 34°C, while the agarose was prepared. To make a 2% concentrated gel, 1.0 g powdered LGT agarose was added to 50.0 ml distilled water. This was then heated in a microwave, mixing at regular intervals, until all the powder was fully dissolved. The 50.0 ml of L-15 at 34°C was then added to the 50.0 ml 2% LGT agarose and thoroughly mixed. The gel was used immediately and stored in the 34°C water bath throughout usage to prevent it from solidifying.

Following 1 hr incubation to allow adsorption to the cell monolayer, the virus dilutions were aspirated from the wells and 2.5 ml of gel was carefully pipetted into the wells, ensuring no air bubbles were formed in the process. The plates were then allowed to set at room temperature. Once set, they were placed into a humidified box to prevent the gel from drying and incubated at 20°C for 14 days.

On the day 14 of incubation the plates were removed for fixing and staining. The cells were fixed by covering the overlay with 3.0 ml 10% NBF for 20 minutes. The NBF was then removed along with the

gel overlays which were carefully extracted from the wells using a metal spatula, with a rapid flicking action, to avoid damaging the cell monolayer. The cells were then covered with 2.0 ml 1% crystal violet and allowed to stain for 20 minutes before being removed and the cells washed with distilled water. The plates were then allowed to dry overnight.

### **3.2.8 Recovering and titrating virus from frozen fish**

Isolating virus from frozen fish has proven difficult and the improved titration method was tested to see if it made virus recovery from archived samples possible. For this, whole frozen common carp from a 2007 cohabitation study at CEFAS were used as the source for isolating virus. Two fish were used initially and the test was repeated later with three more fish from the same previous experiment. 1.0 g of tissue from the gills was removed from each fish as they defrosted. This was homogenised in a pestle and mortar with coarse sterile sand. 9.0 ml TM was added to the homogenised tissue. The virus suspensions were titrated and incubated for 14 days.

### **3.3 Study 3: Developing methods to recover viable KHV from carp mucus**

Although real time PCR is extremely useful for detecting and quantifying genomic copies of KHV DNA, it does not indicate the presence of viable, infectious virus. In order to assess viral survival and determine the risk of infection from KHV-infected angling equipment could not be tested without a method for recovering viable virus from mucus. Therefore it was necessary to develop new techniques for isolating live virus. All tissue culture techniques used in these experiments were carried out according to the methods previously described in Study 2 unless otherwise stated.

#### **3.3.1 Re-isolating KHV from spiked mucus**

Mucus was harvested from the skin of carp non-lethally anaesthetised with benzocaine. The mucus was gently scraped from the skin with a razor blade and collected with a 2.0 ml syringe. The mucus was refrigerated at 4 °C and used within an hour after harvesting.

Two preparations were made with the mucus, with four replicates of each. The first was a mixture of 100 µl mucus and 10 µl of KHV and the second contained 100 µl mucus and 10 µl KHV but with the addition of 50 µl water from the tank containing the carp. Four controls were prepared with 100 µl TM and 10 µl of KHV. All of the samples were thoroughly mixed by vortexing. Two samples of each preparation were chilled overnight at 4°C with the other two remaining in 4°C storage for seven days.

After storage, 500 µl TM was added to each sample (450 µl to the samples containing tank water) and vortex mixed. This created a  $10^{-1}$  dilution of virus and from this five serial dilutions of 50 µl of sample into 450 µl L-15 were made to  $10^{-3}$ . Before titration, 500 µl EMEM (half of the culture medium) was first removed from the wells. The dilutions were then pipetted into the wells, 500 µl in each, thus halving the concentration and creating a titration of  $10^{-2}$  to  $10^{-6}$ . This process was repeated seven days later with the remaining samples.

### **3.3.2 Removing contaminants from carp mucus by UV sterilisation for use on cell culture**

Carp mucus is naturally contaminated with pathogens, and sterilisation to remove bacteria and fungi that compete with KHV in cell culture may help increase viral titre and development of CPE caused by KHV. Mucus was exposed to UV light for varying periods of time to remove fungal and bacterial contaminants prior to adding KHV. Initially, 2.0 ml aliquots of mucus were exposed to UV light for 11 hours. 300 µl aliquots of a 1 in 5 mucus dilution in 1.0 ml EMEM and of a 1 in 3 mucus dilution in molecular grade water were exposed to UV for 1 hr, 2 hr, 3 hr and 4 hr periods. Finally, using molecular grade water, 1 in 2 and 1 in 3 dilutions of mucus were exposed to UV light for 6 hr and 15 hr respectively. All of the samples were rehydrated with 500 µl of EMEM, incubated at 26 °C and monitored for signs of bacterial and fungal contamination.

### **3.3.3 Removing contaminants from carp mucus by filtration for use on cell culture**

As an alternative to sterilising mucus, attempts were made to physically remove contaminants. Three variations using centrifugation and filtration to remove pathogen contaminants were trialled. Firstly, a 1:1 dilution of mucus to KHV was incubated at 15 °C for 30 min. The mixture was separated into 200 µl aliquots in centrifuge tubes, to which 800 µl TM with extra antibiotic-antimycotic solution (AAS) was added. The samples were returned to 15 °C for a further 4 hr incubation. They were then thoroughly vortexed before being centrifuged at 2,000 rpm for 10 min at 15 °C. The supernatant was removed using a needle and syringe, taking care not to extract the settled mucus, before being filtered at 0.45 µm. The lowest yield recovered was 400 µl and so this quantity was used from each sample to inoculate cell cultures.

Secondly, two centrifuge tubes containing 1.4 ml mucus and one with 1.4 ml TM were mixed with 1.05 ml KHV and incubated at 15 °C for 2 hr. The samples were then diluted with 2.55 ml TM with added AAS and vortexed. One sample containing mucus was incubated at 4 °C before processing; the remaining samples were immediately centrifuged at 2,500 rpm for 15 min at 15 °C. The supernatant

was extracted and filtered as previously described. A yield of 1.6 ml was achieved and this was used to inoculate CCB cells on 75 cm<sup>2</sup> flasks. For the tube incubated at 4 °C, 5.0 ml TM was added to the tube and vortexed before processing. 3.2 ml of the filtrate recovered was then used to inoculate a 75 cm<sup>2</sup> flask of CCB cells.

Finally, four tubes were prepared with 500 µl mucus, 50 µl KHV and 3.45 ml L-15 plus AAS and two with 500 µl mucus, 500 µl KHV and 3.0 ml L-15 plus AAS. Half of the tubes were incubated at 15 °C for 2 hr and the others processed immediately by centrifugation at 3,000 rpm for 10 min at 15 °C. The supernatant was extracted and filtered, yielding 1.2 ml recovered substrate. A tube with 3.5 ml L-15 plus extra AAS and 500 µl KHV acted as a positive control. Two flasks of CCB cells were inoculated with 500 µl of the filtrate per sample.

#### **3.3.4 Re-isolating KHV from mucus spiked with different concentrations of virus**

To identify the lowest concentration of virus detectable from spiked mucus using the cell culture technique, different viral dilutions were used to spike carp mucus. 1.1 ml samples of four concentrations of KHV ( $10^5$ ,  $10^4$ ,  $10^3$  and  $10^2$  TCID<sub>50</sub>) were mixed in centrifuge tubes with 1.1 ml mucus. A positive control containing 1.1 ml  $10^5$  TCID<sub>50</sub> KHV and 1.1 ml L-15 was also prepared. A 100 µl aliquot was removed from each sample for analysis with real time PCR, with the remainder processed by centrifugation (3,000 rpm for 10 min at 15 °C) and filtration of the substrate at 0.45 µm. Between 300 – 500 µl filtrate was recovered for each sample, from which a 100 µl aliquot was removed and stored at -20 °C for later analysis with real time PCR. The remaining filtrate was used to inoculate CCB cells (100 µl per flask). A total of test six flasks, two each for mucus with  $10^5$  and  $10^4$  TCID<sub>50</sub> KHV and one each for mucus with  $10^3$  and  $10^2$  TCID<sub>50</sub> KHV, were incubated at 20 °C along with two flasks inoculated with the positive control.

### **3.3.5 Re-isolating KHV from mucus after different durations of incubation**

Trail A: To establish the decline in viral titre in carp mucus over time, KHV spiked mucus samples were incubated and subsamples tested by real time PCR and titration at different time points. Two centrifuge tubes were prepared with 4.0 ml mucus, 4.0 ml KHV and 200 µl AAS. A third tube contained 4.0 ml KHV, 200 µl AAS and 4.0 ml L-15 as a positive control. Samples were thoroughly mixed with a vortex and 2.0 ml aliquots were removed. The original tubes were incubated at 15 °C. Mixed into each subsample was 1.5 ml L-15 before immediate processing (centrifuged at 3,000 rpm for 10 min at 15 °C, substrate removed and filtered at 0.45 µm). The lowest yield of filtrate was 900 µl so two 100 µl aliquots were taken for analysis with real time PCR and the remainder was titrated onto 24-well plates containing 300 µl EMEM. Further 2.0 ml subsamples were processed as described after 24, 48 and 72 hour incubations at 15 °C. A titration and aliquots for real time PCR were taken for the virus stock on the first day (at time 0) for comparison with the recovered samples.

Trial B: This trial was repeated with the following adaptations; the test sample was prepared with 5.0 ml KHV and 5.0 ml mucus and for the positive control, 5.0 ml KHV diluted in 5.0 ml L-15. Larger subsamples (2.5 ml) were removed at each time point and added to 3.75 ml L-15. Titrations were carried out on plates containing 500 µl EMEM.

### **3.3.6 Analysing viral titre in mucus spiked with KHV preserved with dithiothreitol**

This experiment tested 0.14g dithiothreitol (DTT) diluted in 10.0 ml molecular grade water as a method of preserving KHV in mucus samples. A 1:1 mixture of KHV plus carp mucus was incubated at 15 °C for 30 min. This was used to prepare two samples which were set up in centrifuge tubes in duplicate. The first contained 4.0 ml L-15, 200 µl AAS and 4.0 ml of the mucus-virus mixture and the second contained 1.0 ml dithiothreitol (DTT) plus 1.0 ml of the mucus-virus mixture. The tubes containing DTT were incubated at 26 °C for 1 hr whilst the other tubes were processed

(centrifugation at 3,000 rpm for 10 min at 15 °C then filtered at 0.45 µm). 100 µl aliquots were taken from all of the tubes for real time PCR analysis and the remaining filtrate from the samples containing media was used to inoculate CCB cell cultures (two flasks inoculated with 500 µl).

### **3.3.7 Re-isolating KHV from mucus of carp with clinical KHVD signs**

The ability to isolate viable virus from live, clinically infected fish would be a useful tool for establishing the infectiousness of an individual fish. Mucus was harvested, as previously detailed, from mirror carp with clinical KHVD signs. For each of the four fish tested, 2.0 ml mucus was vortexed in a centrifuge tube with 4.0 ml L-15 and processed by centrifugation at 3,000 rpm for 10 min at 20 °C and filtered at 0.45 µm. The lowest yield of filtrate was 2.4 ml so this amount was taken from each sample and mixed with 1.6 ml L-15. Serial dilutions from  $10^1$  to  $10^3$  were prepared and titrated in duplicate onto 12-well plates and incubated at 20 °C.

### **3.4 Study 4: Assessing virus survival in mucus under different storage**

#### **conditions**

This experiment was designed to give a proportional indication of the risk posed by different storage conditions on allowing virus survival and thus ability to transmit KHV. The aim is to establish the probability that viable virus will survive storage on contaminated equipment which is necessary for transmission of the virus to a naïve population (see figure 2).

#### **3.4.1 Test using artificial UV light and nets rolled up in plastic tubes**

Segments of keepnet were cut into 5 cm x 10 cm strips and placed in clear plastic centrifuge tubes. Mucus was harvested from carp as previously described. 20.0 ml mucus was diluted with 20.0 ml molecular grade water and was added to 20.0 ml KHV and thoroughly vortexed. 1.5 ml of the mucus/virus mixture, sufficient to coat the net, was then pipetted into each of the tubes. Four conditions, each with 10 replicates, were created; dark and damp, dark and dry, light and damp, light and dry. Each of the 'dark' tubes were wrapped in foil to prevent penetration of UV light. 'Damp' and 'dry' conditions were created by either leaving the lid of the tube on or off respectively. The tubes were incubated at room temperature for 12 hr and the temperature was monitored for the duration with a TinyTag thermal recorder. The 'light' tubes were placed in a UV cabinet and exposed to four hours of UVA ( $1.7 \mu\text{W cm}^{-2}$ ) and UVB ( $0.4 \mu\text{W cm}^{-2}$ ). After incubation, 4.5 ml EMEM was added to each tube to wash the nets, which were compressed with forceps, during removal from the tube, to recover as much sample as possible. The tubes were then centrifuged at 3,000 rpm for 10 minutes at 15°C. The supernatant was removed and filtered at 0.45  $\mu\text{m}$ . The entire filtrate from each tube (ranging from 2.7 to 3.3 ml) was used to inoculate 25 cm<sup>2</sup> flasks of CCB cells that contained a reduced amount of culture medium (4.0 ml EMEM). The flasks were then incubated at 20 °C for 14 days and monitored for CPE. Each of the flasks had a full change of media after 14 days and returned to 20 °C incubation for a further seven days. Controls for this experiment were; 10.0 ml 50 % mucus diluted



in molecular grade water mixed with 5.0 ml  $10^5$  TCID<sub>50</sub> UK N076 KHV processed by centrifugation and filtration and used to inoculate ten 25 cm<sup>2</sup> CCB flasks at time 0, the same mixture kept at room temperature and processed after 12 hr incubation to inoculate ten 25 cm<sup>2</sup> CCB flasks and a mixture of 5.0 ml KHV with 10.0 ml EMEM kept in the dark at 4 °C and used to inoculate two flasks at time 0 and eight flasks after 12 hr incubation. The latter control was also centrifuged and filtered under the same conditions as the samples containing mucus. Two flasks with medium were used as negative controls.

### **3.4.2 Test of nets exposed to sunlight and contained in clear plastic bags**

The test described above was repeated using the same four conditions, dark and damp, dark and dry, light and damp, light and dry, with modifications. The strips of nets were placed in clear zip-locked bags instead of tubes and by sealing the bags a damp atmosphere was achieved. To create dry conditions the bags were split open to increase aeration. 'Light' samples were exposed to direct sunlight in the evening and morning which peaked at UVA 790  $\mu\text{W cm}^{-2}$  and UVB 199  $\mu\text{W cm}^{-2}$  for 'light and dry' samples and UVA 725  $\mu\text{W cm}^{-2}$  and UVB 187  $\mu\text{W cm}^{-2}$  for 'light and damp' samples. Dark conditions were simulated by placing the bags of nets in opaque plastic boxes. Temperature was monitored with a TinyTag thermal recorder for the 18 hr incubation (of which, approximately 7-8 were daylight hours). Before processing, the 'dry' samples had to be rehydrated by immersing the nets in separate, intact bags containing 1.5 ml molecular grade water for 90 min at 4 °C. All the nets were compressed with forceps during removal from the bags and more than 2.0 ml of substrate was recovered each time. The recovered samples were vortexed and 2.0 ml of each was transferred to centrifuge tubes containing 3.0 ml EMEM. These were centrifuged and filtered as previously described. The lowest amount of supernatant recovered was 3.5 ml and so this was the quantity used from each sample to inoculate 25 cm<sup>2</sup> flasks of CCB cells that contained a reduced amount of culture medium (4.0 ml EMEM). The dark and damp conditions were adopted as the positive control

for optimal conditions. Three flasks were inoculated with the filtrate from 4.5 ml diluted mucus and virus mixture which was processed at time 0.

### **3.5 Study 5: Generating persistent, lysogenic KHV infection in CCB cells**

Establishing if KHV can cause persistent, and potentially latent, infection is important for accurately calculating the prevalence of KHV infection in carp populations which can be related to the likelihood of an angler catching an infected fish. Flasks of CCB cells with a persistent KHV infection were created by exploiting the inhibitory temperature range that restricts KHV replication. Cells were inoculated with KHV and monitored until the appearance of very early CPE. The flasks were then moved to a 30 °C incubator and stored until the monolayer repaired and CPE was no longer visible. Decreasing the temperature to 12 or 15 °C was also tested. The culture medium was then removed from the flasks and cell monolayers washed with 4.0 ml Dulbecco's phosphate buffered saline (PBS). The cells were then exposed to 1.0 ml trypsin for 40 s or until translucent in appearance. To re-suspend the infected cells in medium, 21.0 ml EMEM was then added to the flasks and the cells scraped from the surface. The suspension was then pipetted into three new 25 cm<sup>2</sup> flasks (7.0 ml into each). The flasks were then incubated at 30 °C to allow new cell growth, a confluent monolayer usually being achieved 2-4 days after passaging. Some flasks were kept at 30 °C and passaged approximately once per month (or more frequently if the cells began to appear unhealthy) to determine how long CCB cells can remain persistently infected and to ensure a fully lysogenic infection was achieved. Periodically, some flasks were returned to normal 20 °C incubation to induce lytic infection and verify that the cells were still infected and the virus retained pathogenicity.

### **3.6 Study 6: Surveying carp stocking and keepnet use in UK fisheries.**

An analysis of 168 copies of the Angling Times newspaper was conducted to determine what type of fisheries exist in the UK, what proportion stock carp and what proportion allow the use of keepnets on site. This was to compile data on what percentage of UK fisheries are most at risk of becoming infected with KHV (those which stock carp and allow use of keepnets).

## **4.0 Results**

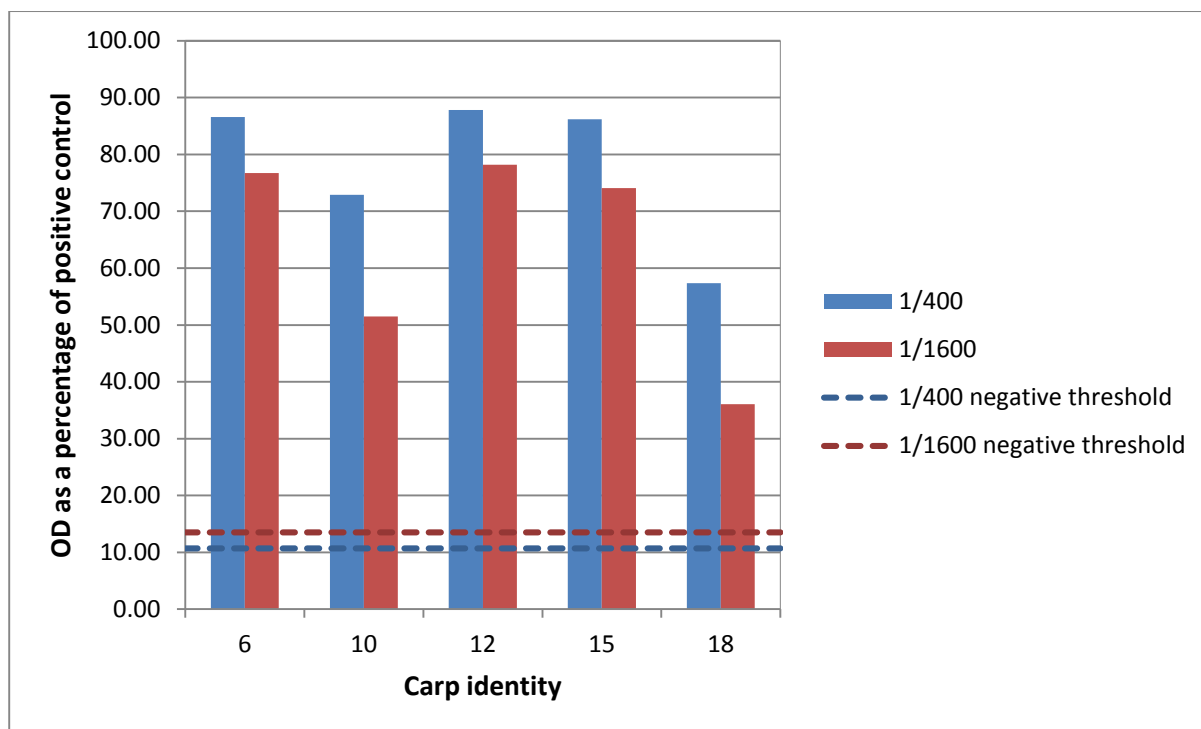
### **4.1 Study 1: Relationship between viral shedding and feeding rate**

Of the 20 carp bath challenged with KHV, 15 (75 %) succumbed to clinical KHVD and either died or were terminated when moribund, whereas the remaining five (25%) did not develop any clinical signs and survived for the duration of the trial (30 days). All five control carp remained healthy and survived the duration of the trial.

**Table 1: The frequency of mortalities per day post KHV challenge, with mortality first occurring on day 7**

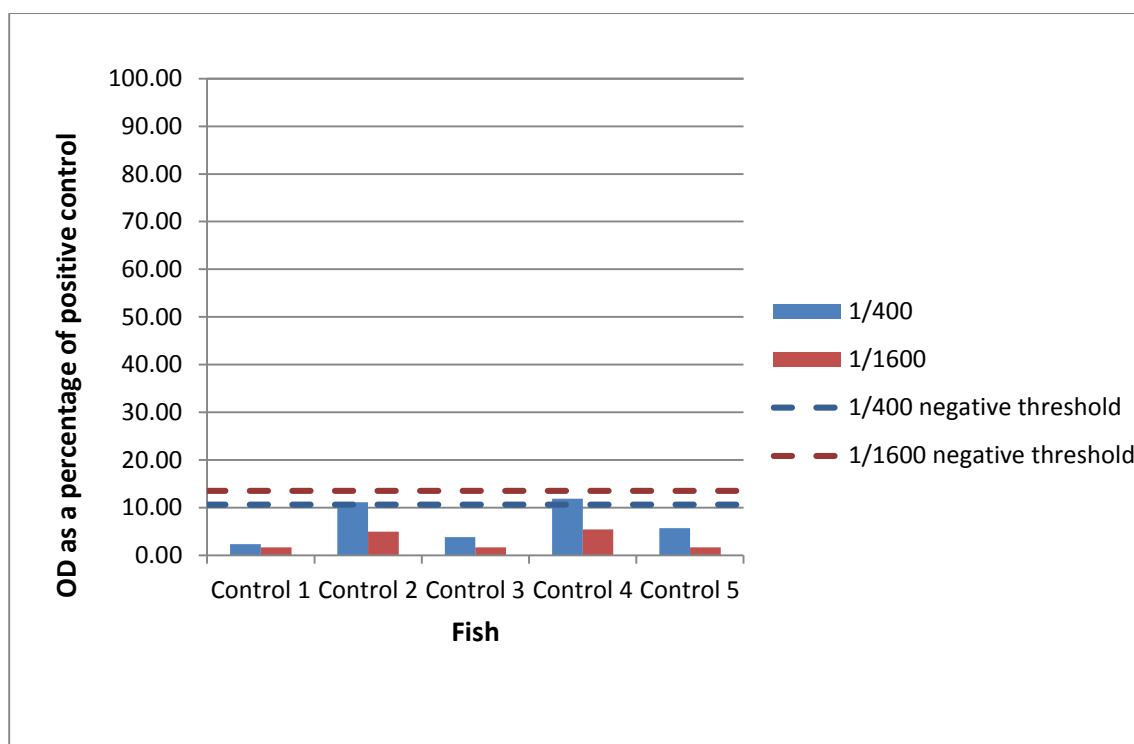
<b>Day (post infection)</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>	<b>13</b>	<b>14</b>	<b>15</b>	<b>16</b>	<b>17</b>	<b>18</b>	<b>19</b>	<b>20</b>
<b>No. mortalities</b>	1	0	1	2	0	1	7	0	0	1	0	1	0	1

All 15 of the clinically infected fish developed severe disease signs, particularly mucus sloughing and dryness of the skin. The first clinical signs were observed on day 5 post-challenge. The first mortality occurred on day 7 and continued until day 20 (Table 1). The mean and median day for mortalities was day 13, with the final mortality occurring on day 20.



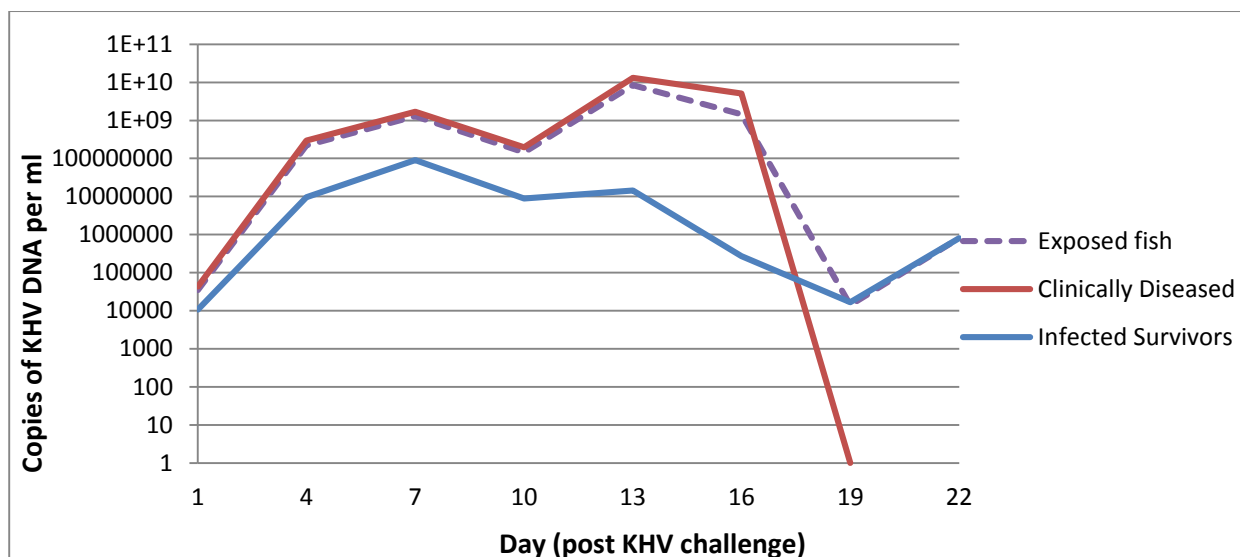
**Figure 1a: presence of antibody in KHV-infected carp 30 days post challenge at 1/400 and 1/1600 sera dilutions**

Blood samples were taken from the five surviving carp on day 30 for ELISA analysis for anti-KHV antibodies. High antibody titres far exceeding the negative threshold were observed for all of the survivors in both sera dilutions (Figure 1a). This shows that the carp were non-clinically infected with KHV and successfully mounted an immune response to the virus. In comparison the antibody titres



**Figure 1b: presence of antibody in control carp 30 days post mock-challenge at 1/400 and 1/1600 sera dilutions**

of control carp were not sufficient to exceed the negative threshold at 1/1600 sera dilution (Figure 1b). Two of the carp had titres slightly above the negative threshold at 1/400 dilution but the levels were insubstantial and probably indicative of exposure to carp pox, as anti-carp pox antibody displays low-level cross reaction with KHV. This indicates antibody levels in the exposed fish were a result of infection from the KHV-challenge.



**Figure 2: average copies of viral DNA per ml mucus transferred to nets from carp challenged with KHV**

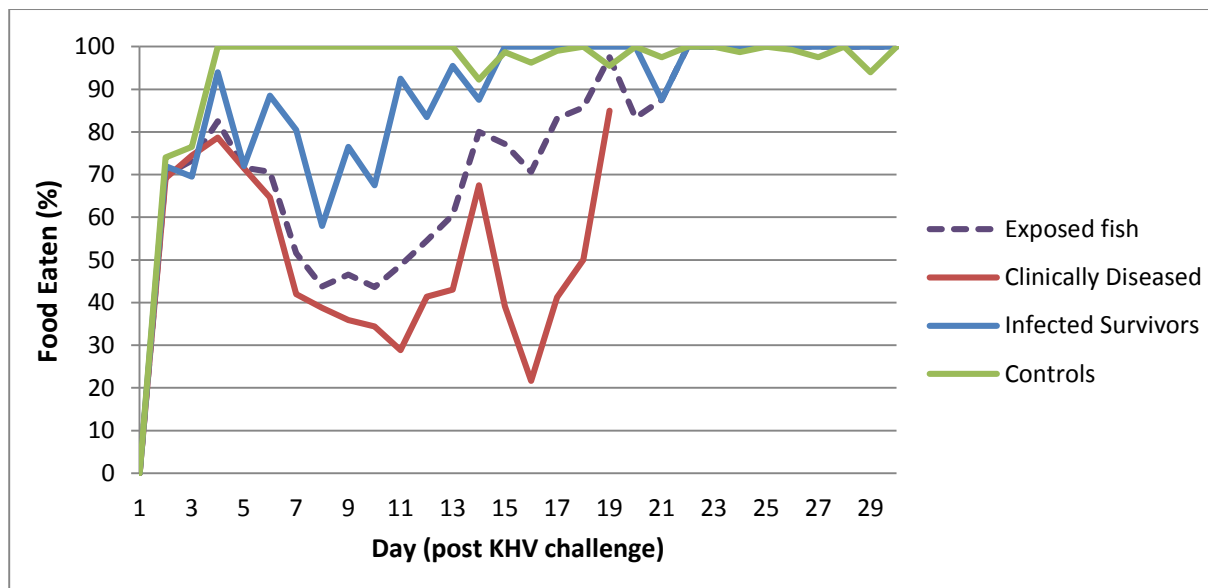
Monitoring the dynamics of viral shedding (Figure 2) in the carp confirmed that, whether they succumbed to clinical disease or not, all became infected with KHV and successfully transferred virus onto netting equipment. All of the carp, except two infected survivors, began shedding virus on day 1 post challenge. The two which did not shed KHV on day 1 were both shedding virus by day 4 (see Appendix 1).

The viral titre shed by the 15 clinically diseased carp increased steadily throughout the trial from  $4.3 \times 10^4$  copies KHV DNA ml<sup>-1</sup> mucus on day 1 to a peak of  $1.3 \times 10^{10}$  copies KHV DNA ml<sup>-1</sup> mucus on day 13 (Figure 2). Though it looks like the viral titre in clinically infected fish reduces to zero on day 19, only one clinically infected fish (fish 5) was still alive at this time point. This fish died on day 20, suggesting moribund carp continue shedding virus almost to the point of death.

For the infected survivors, viral titre shed in the mucus increased from  $1.1 \times 10^4$  copies KHV DNA ml<sup>-1</sup> mucus on day 1 to a peak of  $9.0 \times 10^7$  copies KHV DNA ml<sup>-1</sup> mucus on day 7. This peak was substantially lower than in those fish succumbing to clinical disease. Finally, there was an increase in viral shedding recorded between days 19 and 22 (Figure 2). Two of the infected survivors tested negative for two or more consecutive sampling points at the end of the trial; days 16, 19 and 22 for fish 15 and days 19 and 22 for fish 12 (see Appendix 1). Throughout progression of the infection,



viral titre shed by the infected survivors remained consistently lower than that of the clinically diseased carp.



**Figure 3: proportion of food eaten by carp for 30 days post challenge with KHV.**

Observation of feeding rates for the KHV-exposed and mock challenged controls (Figure 3) shows that on day 1 post-challenge none of the exposed or control carp were feeding, however by day 2 the feeding rates (average percentage of food eaten) for the clinically diseased, infected survivor and control groups had increased to a similar level. By day 3 differences became apparent in the feeding behaviours of the separate groups.

For the clinically diseased carp, initial feeding rates increased to a peak of 79 % average proportion of food eaten on day 4 before steadily declining to an average 29 % on day 11 (Figure 3). A peak of 65.7 % on day 14 followed mass mortality of the most severely diseased fish on day 13 (Table 1) and a peak of 85 % food eaten on day 19 can be attributed to a single surviving fish (Appendix 2).

Average feeding rates of the clinically diseased carp remained lower than the infected survivors and control groups throughout the trial.

In contrast, Figure 3 shows the feeding behaviour of the five infected survivors steadily increased from 72 % average proportion food eaten on day 2 to 100 % on day 15. There was a dip in the increase in feeding rates between day 7 and 11, with a low of 58 % on day eight. The infected survivors continued to eat at a rate of 100 % food eaten for the remainder of the trial with the exception of day 21, which dropped to 87.5 % average proportion of food eaten.

Finally, the control fish reached an average 100 % feeding rate on day 4 and remained above 90 % until the end of the trial (Figure 3).

**Table 2: GLLM results, comparing the proportion of feed ration eaten by KHV exposed carp that did or did not develop clinical disease to unexposed control carp.**

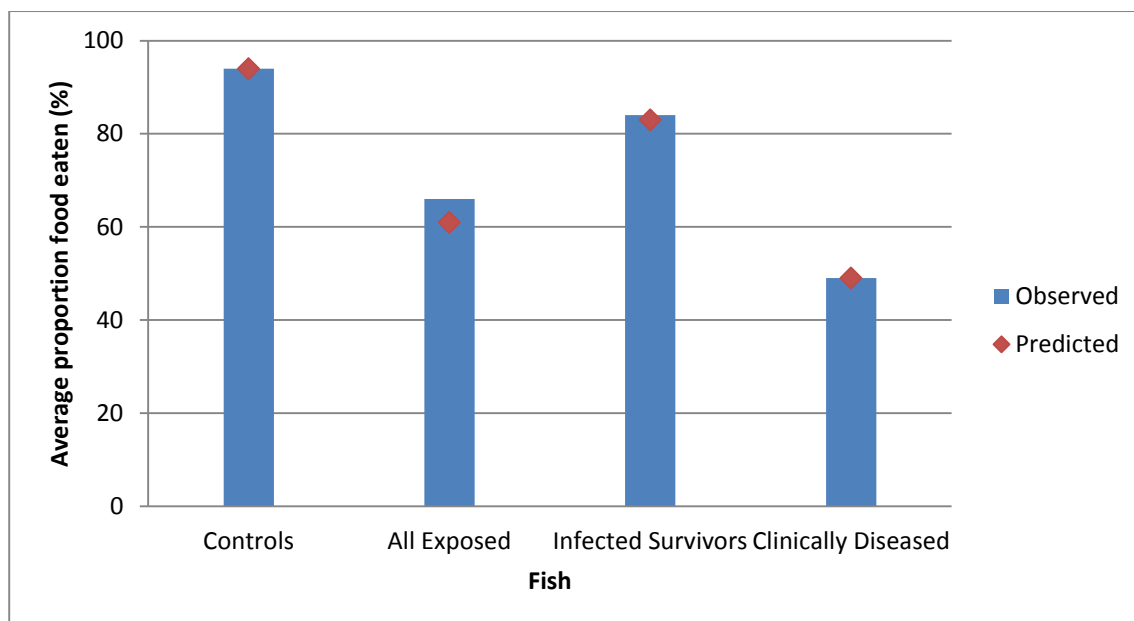
	Estimate	Std. Error	z value	Pr(> z )
(Intercept)	2.7733	0.2808	9.878	< 2e-16 ***
Infected Survivors	-1.1774	0.3789	-3.108	0.00189 **
Clinically Diseased	-2.8197	0.3263	-8.641	< 2e-16 ***
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1				

Analysis by logistic regression demonstrated that there was a significant reduction in the average feeding rate of infected survivors, and an even greater significant reduction in the average feeding rate of the clinically diseased carp when compared with the mock-challenged control carp (Table 2).

**Table 3:**GLLM results, comparing the proportion of feed ration eaten by all KHV exposed carp to unexposed control carp.

	Estimate	Std. Error	z value	Pr(> z )
(Intercept)	2.7746	0.4122	6.731	1.69e-11 ***
All Exposed fish	-2.3290	0.4605	-5.057	4.25e-07 ***
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1				

Pooling of the average feeding rates of the clinically diseased and infected survivor groups creates an overall average for all KHV-exposed fish. Table 3 shows that the average proportion of food eaten for all exposed fish is significantly lower than that of the control group.



**Figure 4: the observed and predicted average proportion of food eaten by the mock and KHV-challenged carp for the total duration of the trial**

Observed average feeding rates were close to the predicted feeding rates for the different groups of fish which were 94 % for the control fish, 61 % for all exposed fish, 84 % for infected survivors and 49 % for the clinically diseased (Figure 4).

**Table 4: showing the quartiles used for logistical regression for viral shedding in carp**

Quartile	Low	Medium	High	Very High
Average titre KHV DNA ml <sup>-1</sup>	< 6.0 x 10 <sup>6</sup>	6.0 x 10 <sup>6</sup> to < 1.1 x 10 <sup>8</sup>	1.1 x 10 <sup>8</sup> to < 5.9 x 10 <sup>8</sup>	> 5.9 x 10 <sup>8</sup>

To compare the effect of virus shedding on the proportion of food eaten by KHV infected carp, it was useful to separate the titres of virus shed into low, medium, high and very high groups. The quartile boundaries can be seen in Table 4.

**Table 5: GLLM results, examining the association between shedding of KHV from exposed fish and the proportion of feed eaten.**

	Estimate	Std. Error	z value	Pr(> z )
(Intercept)	3.6339	0.3920	9.270	<2e-16 ***
Medium Titre	-2.8134	0.2836	-9.921	<2e-16 ***
High Titre	-3.0738	0.3082	-9.974	<2e-16 ***
Very High Titre	-3.7361	0.3124	-11.961	<2e-16 ***
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1				

Table 5 demonstrates that there is a significant difference in the average proportion of food eaten by KHV infected carp shedding medium, high and very high titres of virus compared to those shedding low titres. It also shows that the higher the average titre of virus shed  $\text{ml}^{-1}$  the lower the average proportion of food eaten by a KHV-infected carp.

## **4.2 Study 2: Development of culture methods to allow assessment of viral survival**

### **4.2.1 Virus isolate and passage number**

The titres of four different isolates of KHV were  $8.89 \times 10^3$  for UK N071,  $8.89 \times 10^4$  for UK M083,  $2.32 \times 10^5$  for UK N076 and  $3.41 \times 10^5$  TCID<sub>50</sub> ml<sup>-1</sup> for UK H361. From observations of cell cultures of these isolates, CPE developed first in cultures of UK N076 on day five of incubation and not until day eight for UK M083 and day 13 for UK H361 and UK N071.

**Table 13: TCID<sub>50</sub> ml<sup>-1</sup> of different passage number of KHV isolate UK N076**

<b>UK N076 passage no.</b>	<b>P1</b>	<b>P2</b>	<b>P3</b>	<b>P4</b>
<b>TCID<sub>50</sub> per ml</b>	$2.32 \times 10^5$	$5.0 \times 10^4$	$8.89 \times 10^4$	$2.81 \times 10^3$

Different passage numbers of UK N076 were titrated on day 13 post infection. Table 13 shows there is a reduction in viral infectivity each time it is passaged to new cells.

### **4.2.2 Media**

Titration of UK N076 using three different media EMEM, L-15 and a 1:1 mixture of EMEM and L-15 resulted in  $2.81 \times 10^5$ ,  $7.34 \times 10^3$  and  $1.58 \times 10^5$  TCID<sub>50</sub> ml<sup>-1</sup> respectively. Titrations using L-15 alone caused deterioration and detachment of the monolayer and confidently identifying CPE on these wells was difficult. Titrations using EMEM as the sole culture medium required the culture plates to be incubated in a CO<sub>2</sub> rich atmosphere in order to buffer the medium at the correct pH.

### **4.2.3 Inoculum dilution**

All flasks inoculated with different dilutions of KHV (500 µl  $10^5$ , 500 µl  $10^4$  and 50 µl  $10^4$  KHV TCID<sub>50</sub> per ml) had developed extensive CPE by day ten post inoculation. Cell cultures inoculated with 50 µl of  $10^5$  and  $10^4$  TCID<sub>50</sub> KHV per ml developed CPE six days post inoculation and ten days post

inoculation for  $10^3$  TCID<sub>50</sub> KHV per ml. Flasks inoculated with  $10^2$  and  $10^1$  TCID<sub>50</sub> KHV per ml remained negative for CPE.

#### **4.2.4 Time point for harvest of virus**

KHV was harvested at different time points from cell cultures and titrated to determine the optimal time point for harvesting the virus.

**Table13: TCID<sub>50</sub> ml<sup>-1</sup> KHV achieved after harvesting the virus at different time points before titration**

<b>Harvest point</b>	<b>Day 7</b>	<b>Day 9</b>	<b>Day 14</b>
<b>CPE at harvest</b>	Moderate CPE	Severe CPE	Severe CPE
<b>TCID<sub>50</sub> per ml</b>	$8.89 \times 10^4$	$8.89 \times 10^4$	$8.89 \times 10^4$

Table 14 shows no difference in viral titre achieved between KHV harvested on days 7, 9 or 14 post inoculation when CPE was moderate or severe.

**Table14: repeat of TCID<sub>50</sub> ml<sup>-1</sup> KHV achieved after harvesting the virus at different time points before titration**

<b>Harvest point</b>	<b>Day 4</b>	<b>Day 5</b>	<b>Day 6</b>	<b>Day 7</b>
<b>CPE at harvest</b>	No CPE	Early CPE	Mild CPE	Moderate CPE
<b>TCID<sub>50</sub> per ml</b>	$2.81 \times 10$	$2.81 \times 10^2$	$2.81 \times 10^3$	$5.0 \times 10^3$

To optimise harvesting KHV for highest titre, earlier time points were considered. Table 15 shows that there is no difference in titre of virus harvested on days 4, 5 and 6 when CPE ranges in severity from not visible to mild. However on day 7, when a moderate level of CPE has developed, the viral titre almost doubles.

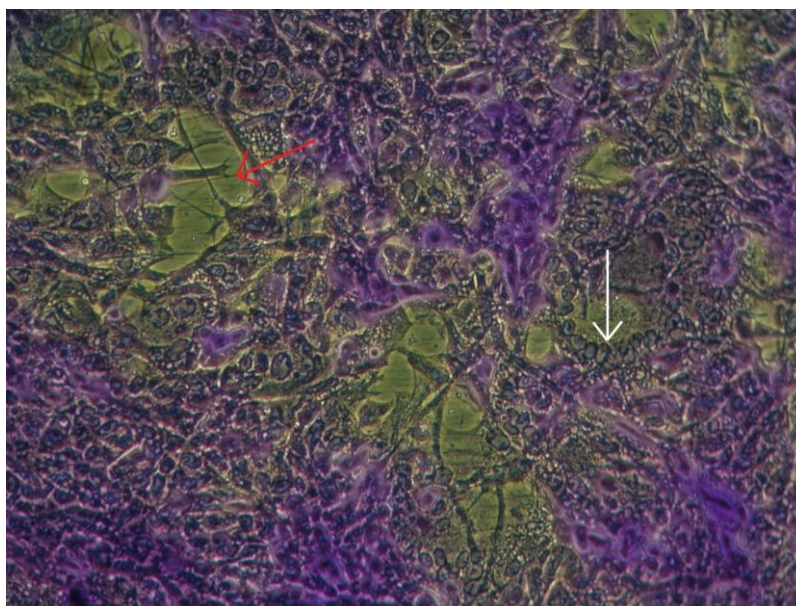
#### **4.2.5 Storage conditions**

Initial growth on of the UK N076 and UK M083 samples from -80 °C storage was slow with CPE only appearing in the  $10^{-1}$  to  $10^{-3}$  wells on day 13 of incubation compared CPE being evident in  $10^{-5}$  wells for those from 4 °C storage. However by day 20 the final TCID<sub>50</sub> achieved was  $5.0 \times 10^4$  for both UK N076 and UK M083 stored at -80 °C and  $1.58 \times 10^5$  for UK N076 stored at 4 °C. It was not possible to

calculate a TCID<sub>50</sub> for UK M083 from 4 °C storage as the monolayers had become badly damaged and detached in all the wells. There was a large amount of cytotoxicity and some detachment on the edges of the monolayers on most of the wells on the plate.

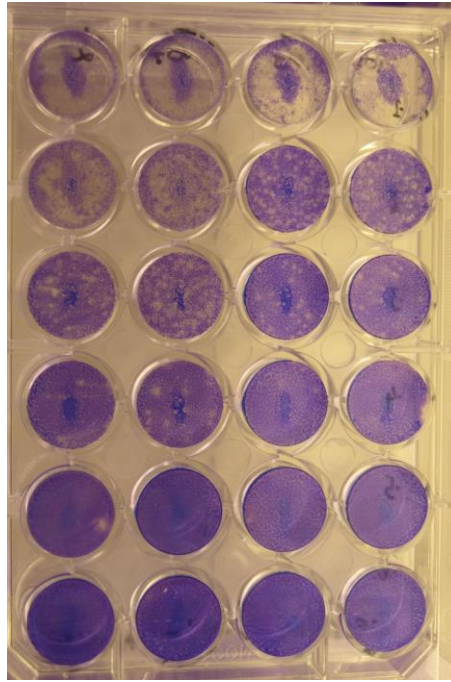
#### **4.2.6 Fixing and Staining**

The fixing and staining process caused no damage to the cell monolayer and prepared plates remained readable after 10 months in storage in cool, dry, dark conditions.



**Figure 8: 1 % crystal violet stained CCB cells with CPE from KHV infection showing syncytia (white arrow) and vacuolation (red arrow)**

Crystal violet stained cells improved visualisation of characteristic CPE, such as syncytia formation, caused by KHV (see figure 8).



**Figure 9: Stained titration plate showing vacuolation as a result of KHV infection decreasing in severity from a low dilution (top row) to a high dilution (bottom row)**

Another benefit achieved by the fixing and staining process was the creation of pseudo-plaque titration plates (Figure 9) which provide a quick estimation of viral titre (TCID<sub>50</sub>) without the need of microscopy.

#### **4.4.7 Plaque titrations**

There was no plaque formation on any of the wells for M083. A small amount of vacuolation appeared on the 12<sup>th</sup> day of incubation in both 10<sup>-1</sup> dilution wells for N076 but there was no further development. Overlay removal and staining of the cells did not reveal any additional CPE.

#### **4.4.8 Recovering and titrating virus from frozen fish**

For all titrations, bacterial contamination of the 10<sup>-1</sup> dilution wells caused a severe drop in pH and caused cytotoxicity. Some 10<sup>-2</sup> dilution wells also showed bacterial contamination. There was no CPE observed on any of the plates.



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### **4.3 Study 3: Developing methods to recover viable KHV from carp mucus**

To enable viable virus recovery from contaminated nets after storage, a technique for re-isolating KHV from spiked carp mucus for inoculating cell cultures had to be developed.

#### **4.3.1 Re-isolating KHV on cell culture from unprocessed spiked mucus**

All the titrations of KHV spiked mucus were contaminated with fungi and bacteria. Media turned yellow, indicating a drop in pH. Severe cytotoxicity was also observed in most wells. No definite CPE caused by KHV was identifiable.

#### **4.3.2 Removing contaminants from carp mucus by UV sterilisation for use on cell culture**

Aliquots of mucus without additional water were completely desiccated and could not be successfully rehydrated. All of the UV irradiated samples remained contaminated with bacteria, including samples exposed for 11 and 15 hours. Fungal contamination was not evident in any of the samples following UV irradiation, irrespective of the duration of exposure

#### **4.3.3 Removing contaminants from carp mucus by filtration for use on cell culture**

For the first attempt at removing pathogen contaminants from mucus by filtration, all of the flasks which contained the unprocessed or centrifuged but unfiltered samples succumbed to fungal growth. No contamination or CPE developed on the flasks inoculated with the samples which were both centrifuged and filtered.

In the second trial for filtering mucus, CPE developed on the positive control flask on day 6 post-inoculation. In comparison, first signs of CPE were observed on day 8 post-inoculation in the flask containing the virus and mucus mixture incubated for two hours. The flasks inoculated with the mixture of virus and mucus which was incubated for 24 hours before processing became contaminated with fungus.

**Table 6: progression of CPE on CCB cells inoculated with KHV spiked mucus at different dilutions, with or without an incubation step**

	Flask	Day 8 CPE	Day 12 CPE	Day 18 CPE
Mucus +10 <sup>5</sup> TCID <sub>50</sub> ml <sup>-1</sup> KHV Immediate	1	-	- (mild cytotoxicity)	+
	2	-	- (mild cytotoxicity)	+
Mucus +10 <sup>5</sup> TCID <sub>50</sub> ml <sup>-1</sup> KHV 2hr incubation	1	-	-	+
	2	-	+	+
Mucus +10 <sup>4</sup> TCID <sub>50</sub> ml <sup>-1</sup> KHV Immediate	1	-	-	+
	2	-	+	+
Mucus +10 <sup>4</sup> TCID <sub>50</sub> ml <sup>-1</sup> KHV 2hr incubation	1	-	- (mild cytotoxicity)	+
	2	+	+	+
Positive control	1	+	+	+
	2	+	+	+

For the final adaptation two different virus dilutions were used to spike mucus and were either processed immediately or incubated for two hours beforehand. Progression of CPE on the flasks can be seen in table 1. Any cytotoxicity observed was mild and did not prevent development of CPE.

#### **4.3.4 Re-isolating KHV from processed spiked mucus and determining the effect of processing on viral titre**

Different concentrations of KHV were used to seed mucus to establish the sensitivity of the cell culture method for re-isolating infectious virus from centrifuged and filtered spiked mucus. No CPE developed on any of the flasks after 23 days incubation. CPE was observed on all positive control flasks without mucus.

**Table 7: showing the average copies of KHV DNA detected by real time PCR per ml sample and average cycle number at which the cycle threshold was crossed.**

Sample	KHV TCID <sub>50</sub> ml <sup>-1</sup>	C <sub>T</sub> Mean	Mean copies KHV DNA ml <sup>-1</sup>
Virus Stock	10 <sup>5</sup>	14.2	1.1 x 10 <sup>16</sup>
Unprocessed Control	10 <sup>5</sup>	18.4	2.5 x 10 <sup>13</sup>
Processed Control	10 <sup>5</sup>	19.8	3.2 x 10 <sup>12</sup>
Unprocessed Neat	10 <sup>5</sup>	15.3	2.3 x 10 <sup>15</sup>
Processed Neat	10 <sup>5</sup>	17.6	1.6 x 10 <sup>14</sup>
Unprocessed 1/10	10 <sup>4</sup>	21.4	3.5 x 10 <sup>11</sup>
Processed 1/10	10 <sup>4</sup>	21.1	6.5 x 10 <sup>11</sup>
Unprocessed 1/100	10 <sup>3</sup>	22.6	5.8 x 10 <sup>10</sup>
Processed 1/100	10 <sup>3</sup>	23.4	1.9 x 10 <sup>10</sup>
Unprocessed 1/1000	10 <sup>2</sup>	19.5	5.5 x 10 <sup>12</sup>
Processed 1/1000	10 <sup>2</sup>	22.0	3.4 x 10 <sup>11</sup>

Samples were taken before and after processing (centrifugation and filtration) and analysed with real time PCR (Table 2). Unprocessed neat samples had a ten-fold higher titre of KHV DNA per ml compared with corresponding processed samples. At 1/10 dilution processing made no difference to the titre of KHV DNA and at 1/100 and 1/1,000 dilutions, the processed samples had higher titres than the subsamples taken before processing.

#### **4.3.5 Re-isolating KHV from mucus after different durations of incubation**

Subsamples of two replicates, A and B, of KHV-spiked mucus and positive control with media (C), were incubated for different periods of time and were titrated and analysed by real time PCR.

**Table 8: average copies of KHV DNA ml<sup>-1</sup> detected by real time PCR (Q) and average cycle number at which the cycle threshold was crossed (C<sub>T</sub>) for samples of spiked incubated for different periods of time**

	Time 0		24 hr		48 hr		72 hr	
Sample	C <sub>T</sub>	Q	C <sub>T</sub>	Q	C <sub>T</sub>	Q	C <sub>T</sub>	Q
A	17	3.3 x 10 <sup>9</sup>	16.9	3.4 x 10 <sup>9</sup>	16.6	4.4 x 10 <sup>9</sup>	17	3.3 x 10 <sup>9</sup>
B	17.1	3.1 x 10 <sup>9</sup>	17	3.4 x 10 <sup>9</sup>	16.7	4.2 x 10 <sup>9</sup>	17.1	3.0 x 10 <sup>9</sup>
C	15.7	8.9 x 10 <sup>9</sup>	15.4	1.1 x 10 <sup>10</sup>	15.6	9.3 x 10 <sup>9</sup>	15.4	1.1 x 10 <sup>10</sup>

Copies of KHV DNA ml<sup>-1</sup> in each sample remained relatively consistent with no apparent reduction or increase in the samples over time (Table 8). The titre in the control sample was higher than in the samples containing mucus, though this was less than a tenfold difference. Titrations for the processed (centrifuged and filtered) mucus samples were unsuccessful as CPE failed to develop. Titres achieved by the control were 5.0 x 10<sup>3</sup>, 1.58 x 10<sup>3</sup>, 5.0 x 10<sup>2</sup> and 1.58 x 10<sup>2</sup> TCID<sub>50</sub> ml<sup>-1</sup> for time points 0, 24 hours, 48 hours and 72 hours respectively. This showed a drop in viral infectivity over time when incubated in media at 15 °C.

**Table 9: repeat of average copies of KHV DNA ml<sup>-1</sup> detected by real time PCR (Q) and average cycle number at which the cycle threshold was crossed (C<sub>T</sub>) for samples of spiked incubated for different periods of time**

	Time 0		24 hour		48 hour		72 hour	
Sample	CT	Q	CT	Q	CT	Q	CT	Q
A	16.8	2.1 x 10 <sup>10</sup>	17.0	1.8 x 10 <sup>10</sup>	16.6	2.4 x 10 <sup>10</sup>	18.3	6.0 x 10 <sup>9</sup>
B	16.6	2.4 x 10 <sup>10</sup>	16.8	2.0 x 10 <sup>10</sup>	16.7	2.3 x 10 <sup>10</sup>	17.0	1.7 x 10 <sup>10</sup>
C	15.5	6.2 x 10 <sup>10</sup>	15.4	6.9 x 10 <sup>10</sup>	15.5	6.1 x 10 <sup>10</sup>	15.2	7.7 x 10 <sup>10</sup>

A repeat of the experiment also showed no decline in titre of KHV DNA when stored in mucus for different lengths of time (Table 9). Once again, titrations of the processed mucus samples failed to produce any CPE, indicating no replication of KHV. The control titrations resulted in titres of 1.58 x 10<sup>5</sup>, 1.58 x 10<sup>5</sup>, 5.0 x 10<sup>4</sup> and 1.58 x 10<sup>4</sup> TCID<sub>50</sub> ml<sup>-1</sup> for time points 0, 24 hours, 48 hours and 72 hours respectively, confirming the decay of KHV when held at 15 °C in media without mucus over time.

#### **4.3.6 Analysing viral titre in mucus spiked with KHV preserved with dithiothreitol**

KHV-spiked mucus in dithiothreitol (DTT) and in media were compared by real time PCR to determine whether DTT could be used as a preservative for sampling KHV when isolating viable virus was not required and to improve the PCR method by reducing the viscosity of mucus.

**Table 10: average copies of KHV DNA ml<sup>-1</sup> in mucus samples diluted with media or DTT**

<b>Sub-samples</b>	<b>Mean CT</b>	<b>Mean Q</b>
<b>KHV Spiked Mucus + Media A</b>	17.7	1.0 x 10 <sup>10</sup>
<b>KHV Spiked Mucus + Media B</b>	17.1	1.6 x 10 <sup>10</sup>
<b>Average</b>	<b>17.4</b>	<b>1.3 x 10<sup>10</sup></b>
<b>KHV Spiked Mucus + DTT A</b>	16.5	2.6 x 10 <sup>10</sup>
<b>KHV Spiked Mucus + DTT B</b>	16.6	2.5 x 10 <sup>10</sup>
<b>Average</b>	<b>16.5</b>	<b>2.6 x 10<sup>10</sup></b>

Table 10 shows that the samples containing DTT achieved more consistent results and maintained a higher viral titre than the samples with media.

#### **4.3.7 Re-isolating KHV from mucus of carp with clinical KHVD signs**

No CPE developed on titrations of mucus from carp with clinical KHVD signs following incubation for 20 days. There were cytotoxic effects in the wells containing the 10<sup>-1</sup> dilution.

## **4.4 Study 4: Assessing virus survival in mucus under different storage**

### **conditions**

Strips of net were inoculated with KHV-spiked mucus and incubated under different conditions to simulate storage of angling equipment. KHV was recovered on cell culture to assess remaining levels of infectivity.

#### **4.4.1 Effect of artificial UV light and drying on KHV survival**

Nets artificially contaminated with KHV-spiked mucus and incubated under different conditions showed differences in the proportion of KHV that survived storage and successfully infected CCB cell cultures.

**Table 11: showing the number and proportion of flasks positive for KHV-induced CPE on days 14 and 21 of incubation. Cytotoxic flasks are included in the proportion positive for KHV, but excluded from the adjusted proportion.**

<b>Conditions</b>	<b>Dark and Damp</b>		<b>Dark and Dry</b>		<b>Light and Damp</b>		<b>Light and Dry</b>	
<b>Result</b>	Day 14	Day 21	Day 14	Day 21	Day 14	Day 21	Day 14	Day 21
KHV positive	3	5	4	6	1	2	1	1
KHV negative	5	2	6	4	7	6	8	8
Cytotoxic	2	3	0	0	2	2	1	1
Proportion positive for KHV	<b>30%</b>	<b>50%</b>	<b>40%</b>	<b>60%</b>	<b>10%</b>	<b>20%</b>	<b>10%</b>	<b>10%</b>
Adjusted proportion positive for KHV	37.5%	71.4%	40%	60%	12.5%	25.0%	11.1 %	11.1 %

Table 4 shows the number and percentage of KHV positive CCB cell cultures on day 14 of incubation and on day 21, after replacement of the culture medium. CPE emerged on five flasks, on three of the conditions, between days 14 and 21. Only samples exposed to the light and dry conditions did not yield more positive results after 14 days incubation.

Cytotoxicity of such severity that prevented confirmation of the presence or absence of CPE was observed on six of the 40 test flasks. When these flasks were included in the results dark and dry (60

%) and dark and damp (50%) had the highest proportion of KHV positive flasks compared to light and damp (20 %) and light and dry (10 %). However if cytotoxic flasks are discounted, because confirmation of the presence or absence of KHV is not possible, then dark and damp has a higher percentage of KHV positive flasks (71 %) than dark and dry (60 %). Irrespective of the inclusion or exclusion of cytotoxic flasks, dark conditions averaged either 55.5 or 65.7 % KHV positive compared to 15 or 18.05 % for light conditions. Conversely damp and dry conditions had similar percentages of KHV positive flasks; damp conditions had either a 35 or 48.2 % of KHV positive flasks compared to 35 or 35.55 % for dry conditions.

TinyTag monitoring of the temperature showed an average temperature of 19°C for samples treated with UV light and 21°C for those kept in dark conditions.

#### **4.4.2 Effect of sunlight and drying on KHV survival**

Storage conditions were retested using sunlight and a more effective drying method.

**Table 5: showing the number and proportion of flasks positive for KHV-induced CPE on day 20 incubation**

<b>Conditions</b>	<b>Dark and Damp</b>	<b>Dark and Dry</b>	<b>Light and Damp</b>	<b>Light and Dry</b>
<b>Result</b>				
KHV positive	15	3	0	0
KHV negative	0	12	15	15
Cytotoxic	0	0	0	0
Proportion positive for KHV	100 %	20 %	0 %	0 %

After 14 days incubating, all 'dark and damp' flasks were positive for CPE. None of the samples exposed to sunlight were positive for KHV. 20 % of the dark and dry flasks turned positive for KHV after medium replacement between days 14 and 20 of incubation.

TinyTag monitoring of the temperature showed a peak temperature of 36°C for samples treated with UV light and 25°C for those kept in dark conditions.



All of the flasks displayed some level of bacterial contamination in all of the flasks. However, flasks inoculated with the 'wet' samples had a higher level of contamination than the 'dry' samples and so had their media changed on day 11, compared to day 15 for the 'dry' flasks. Some mild cytotoxicity was observed on the cell cultures inoculated with 'light' samples.

#### **4.5 Study 5: Generating persistent, lysogenic KHV infection in CCB cells**

To determine whether there is the possibility of latent KHV infection in carp, which could have implications for estimates on prevalence in carp populations, persistently KHV-infected CCB cultures have been successfully maintained for eight months. Initial CPE was repaired when the cultures were moved from 20 to 30 °C and the CCB cells remained healthy, though divided rapidly. Cultures moved to 15 °C from 20 °C experienced a ten day lag in CPE development, during which time CCB cells improved in condition before CPE continued to increase in severity. Moving cells from 20 to 12 °C appeared to cease the spread of CPE, however the severity of CPE did steadily increase at localised foci, though no new CPE developed at other sites on the monolayer.

**Table 16: copies of KHV DNA per ml in persistently infected CCB cells at different stages of the passaging process.**

<b>Sample</b>	<b>Sample Type</b>	<b>Conditions</b>	<b>Average KHV DNA copies per ml</b>	<b>Mean C<sub>T</sub></b>
<b>15S</b>	Supernatant	At 30°C for 21 days before passage	$3.1 \times 10^4$	31.82
<b>15C</b>	Cell Suspension	At 30°C for 21 days before passage	$2.6 \times 10^3$	35.30
<b>15a S</b>	Supernatant	At 30°C for 7 days after passage	$1.1 \times 10^3$	36.44
<b>15a C</b>	Cell Suspension	At 30°C for 7 days after passage	$2.8 \times 10^3$	35.39
<b>15b S</b>	Supernatant	At 15°C for 6 days after temperature change	$1.4 \times 10^4$	33.04
<b>15b C</b>	Cell Suspension	At 15°C for 6 days after temperature change	$2.0 \times 10^5$	29.17

After six passages over a fourth month period viral titres in persistent cell culture remained between  $1.1 \times 10^3$  and  $2.0 \times 10^5$  copies KHV DNA per ml (see table 16) with little difference in titre at the different stages of passaging.

## **4.6 Study 6: Surveying carp stocking and keepnet use in UK fisheries**

To establish the current status of carp distribution and prevalence of keepnet use in KHV fisheries a review of the Angling Times was conducted.

**Table 17: Regulations in different fishery types based on 168 samples from the Angling Times**

<b>Fishery Type</b>	<b>Stock Carp (%)</b>	<b>Allow keepnets (%)</b>	<b>Stock carp and allow keepnets (%)</b>
<b>Lake / Pond</b>	97.6 %	47.6 %	45.2 %
<b>River / Canal</b>	59.1 %	86.4 %	50.0 %
<b>All</b>	87.5 %	57.7 %	45.2 %

The survey revealed that, in the UK, 73.8 % of fisheries are lakes or ponds and 26.2% are rivers or canals. The stocking of carp by fisheries and the allowance of some form of keep net use can be seen in Table 17. Lake and pond-style fisheries are more likely to stock carp than rivers or canals but are less likely to allow the use of keepnets. Almost half of all fisheries with carp in their waters allow the use of keepnets.

Of the fisheries that stock carp (147/168), 47.6% prohibit the use of keepnets entirely, whereas 52.4% allow some form of keepnet use. 39.5% allow unrestricted use of keepnets with carp and a further 15.6% allow the use of keepnets but impose restrictions which may or may not be carp-related. 14.5% allow use of keepnets but ban holding carp, regardless of size, in the keepnets

Of the fisheries that stock carp and ban the use of keepnets (70/147), or prohibit carp in keepnets, 30% do not hold matches and 70% do hold matches and allow the use of keepnets during matches.

## **5.0 Discussion**

Koi Herpesvirus (KHV) is an emerging pathogen which causes a highly contagious and fatal disease of valuable carp populations. Mass mortalities associated with Koi Herpesvirus disease (KHVD) have continued to occur globally due to the rapid spread of the virus between waters, which has been attributed mainly to the movement of live infected stock and ornamental fish (Taylor *et al.*, 2010b). With much of the recent research focusing on vaccination and hybridisation programmes to promote genetic resistance to KHVD, gaps have emerged in the knowledge of routes of transmission other than live fish movements. It was hypothesised that, due to the higher prevalence of anti-KHV antibody positive fish in fisheries in comparison with farms in the UK (Taylor *et al.*, 2010a), angling equipment could pose a significant risk of transmitting the virus between angling waters. A risk assessment (Figures 1, 2, 3) was devised to chronicle the events that must occur in order for the successful transmission. The aim of this study was to begin quantifying steps in the pathway which will ultimately be used to calculate the overall risk of transmitting KHV between waters on angling equipment for incorporation into the epidemic model by Taylor *et al.*, (2011).

The likelihood an angler will catch an infected fish was the first step in the pathway that could be quantified. An *in vivo* study was designed to monitor feeding rates and viral shedding to obtain the probability of catching an infected carp. The results demonstrated that Koi Herpesvirus is extremely virulent, establishing infection in 100 % and causing clinical disease in 75 % of the carp from a single exposure. This correlates with previous studies where challenged fish were cohabited also found that the virus can infect 100 % of a naïve population (Gilad *et al.*, 2003). The proportion of a population that develops clinical disease ranges from 70 to greater than 90 % depending on water temperature and the viral titre used in the challenge (Gilad *et al.*, 2003; Way and Dixon, 2007), however this was the first study to isolate carp after a single exposure. Whether clinical signs developed or not, all of the carp exposed to KHV were simultaneously feeding and shedding virus during the trial, suggesting that there is the potential for an angler to catch a KHV-infected fish. The

carp were also shedding virus for extended periods, beginning on day 1 or 2 of infection and lasting until around the time of death for the clinically infected carp and for at least 22 days in three of the five infected survivors. Similar extended periods of viral shedding was observed in KHV-infected carp by Gilad *et al.*, (2004). Feeding rates correlated with the titre of virus shed, the greater the virus shedding the lower the feeding rate, therefore reducing the probability an angler will catch a fish shedding very high titres of infective KHV. Overall feeding rates for the exposed fish was 61 % compared to 94 % of mock-challenged controls, demonstrating that an angler is around a third less likely to catch a KHV-infected carp.

The second quantifiable step was the probability of KHV surviving storage in mucus on contaminated equipment. An initial trial, with cytotoxic flasks omitted from the results, showed that dark and damp environments were most conducive to virus survival whereas light and dry environments were least able to preserve viral infectivity. UV light has been shown to deactivate many viruses that cause disease of fish, including white spot syndrome baculovirus (Yoshimizu *et al.*, 2005; Chen *et al.*, 1998) which corresponds with the findings of this study. In the second trial the highest proportion of virus survival again occurred in dark and damp conditions. It can therefore be concluded that storing angling equipment in self-contained stink sacks that are either opaque or kept in a dark environment (such as a shed or car boot) poses the highest risk of enabling viable virus to survive storage. Other pathogenic viruses, such as alphaviruses, have been shown to persist and maintain virulence on fomites in dark conditions (Sagripanti *et al.*, 2010). Dark and dry conditions yielded a small proportion of infectious virus suggesting that drying alone is not sufficient to negate the risk of transmitting KHV between waters on angling equipment. however when compared with three days survival in environmental water (Shimizu *et al.*, 2005) drying is evidently a factor in deactivation of KHV. Both light and damp and light and dry conditions inactivated all of the KHV. The virucidal effect of UV on KHV was tested by Kasai *et al.*, (2005) and found a dose of  $4.0 \times 10^3 \mu\text{W cm}^{-2}$  was required for inactivating 100% of the virus. In contrast, this study observed complete inactivation of KHV when exposed to much lower levels of UV irradiation (UVA  $725 \mu\text{W cm}^{-2}$  and UVB  $187 \mu\text{W cm}^{-2}$ ) for a

period of time (7-8 hours). Therefore, anglers should be advised to expose their nets to sunlight for the maximum duration possible when their equipment is not in use. Transparent stink sacks or drying the nets in sunlight in between use are cheap and practical suggestions for reducing the risk of KHV surviving storage.

Finally, the survey of angling literature revealed that less than 50 % of fisheries stock carp and allow the use of keepnets, thus reducing the number of fisheries most at risk of introducing KHV to their waters on angling equipment by half. However, of these fisheries, 70 % allow the use of keepnets during matches, which provides the opportunity of introducing KHV to the site if anglers are allowed to use their own nets.

To assess survival rates of KHV in mucus under different storage conditions, culture and titration techniques for KHV had to be improved and a method for isolating viable virus from mucus developed. KHV isolate UK N076 was identified as being quick to grow in culture and to consistently achieve higher titres when compared to other isolates. It was also found to be beneficial to use low passage numbers of KHV as virulence was lost with each passage. This was comparable with vaccine trials that attenuate KHV by passaging to create an avirulent strain (Perelberg *et al.*, 2005 & 2008). Sensitivity of the cell culture technique was limited to inoculation with  $50 \mu\text{l } 10^4 \text{ TCID}_{50} \text{ ml}^{-1}$  or higher onto CCB cells in  $25 \text{ cm}^2$  flasks with 7.0 ml EMEM for CPE to develop. The optimal point for harvesting KHV from cultures was on or after day 7 when moderate CPE and the best conditions for storing stock virus was  $4^\circ\text{C}$  for short periods of time or  $-80^\circ\text{C}$  for longer durations. For titrations a 1:1 mixture of EMEM:L-15 media enabled maximum titre of KHV to be achieved whilst maintaining healthy CCB cells without the need for a  $\text{CO}_2$ -enriched atmosphere, which limited the number of titration plates that could be used at any one time. Fixing and staining titration plates successfully enhanced visualisation of mild CPE and provided a quick method for estimating viral titre in the form of pseudo-plaque formations. However, plaque titrations were inefficient because of the slow growth of KHV. Titrating live virus from frozen fish also failed, possibly due to the large amount of

pathogen contamination competing with any remaining viable KHV. Future attempts may be more successful if the sample is used to inoculate CCB cells in flasks as the media can be easily changed to dilute the contaminants and, if necessary, the cells can be harvested and re-passaged to help encourage viral growth (though this of course would not provide a quantitative result.)

Re-isolation from spiked mucus was successful when samples were processed by centrifugation at 3,000 rpm for 10 minutes at 15 °C. UV sterilisation failed to remove bacterial contaminants, which are possibly less susceptible to UV degradation than KHV. As mucus is found on the skin of fish, one hypothesis is that mucus filters out harmful UV light to protect the skin from damage by exposure to sunlight. It was possible to culture KHV isolated from spiked mucus in flasks, but every attempt at titration failed. This suggests that titration on plates is not as sensitive as culture in flasks. Processing samples for culture with high TCID<sub>50</sub> ml<sup>-1</sup> KHV DNA caused a tenfold reduction in the titre which could be due to loss of the virus in the mucus pellet during centrifugation or from becoming trapped in the filter. However at lower concentrations processing caused an increase in the titre of KHV DNA which could be the result of inaccurate DNA extractions due to the viscosity of unprocessed mucus hindering extraction of low levels of KHV DNA. High levels of protein contamination or inaccuracies in aliquoting the mucus could also be factors in these results. Incubating the spiked mucus at 15 °C for short periods of time (2 hours or less) had no detrimental effect on the ability for KHV to infect cell cultures indicating that any damage caused to KHV by the mucus is slow and possibly indicating mucus has no damaging effect on KHV. Longer durations of storage (up to 72 hours) did not cause substantial reduction in the titre of KHV DNA, however there were fluctuations in the real time PCR results which again could be due to inaccurate DNA extractions or variance in the real time PCR method. To combat the problems caused by analysing unprocessed spiked mucus with real time PCR, DTT was used to preserve KHV spiked mucus samples and to reduce mucus viscosity before the DNA extraction step. For these samples, real time PCR results were more consistent than the control samples containing spiked mucus and medium thus suggesting that inaccuracies in previous experiments were in fact due to problems with the DNA extraction. Although useful for preserving

viral titre and reducing viscosity of mucus, DTT is unsuitable for use in experiments where re-isolating viable KHV is desired. Re-isolating viable virus from diseased carp was unsuccessful and, as with the frozen samples, this was probably due to high contamination of competing pathogens. Likewise, future attempts to isolate viable virus from live, KHV- infected fish could focus on isolating KHV in cell culture flasks first to determine whether it is possible before attempting titrations.

The persistence flasks were maintained for many months and KHV retained pathogenicity, implying that KHV can establish persistent and possibly latent infection. Real time PCR analysis of persistently infected cells revealed low levels of KHV DNA, suggesting replication of the virus had greatly reduced. Extensive genetic analysis is required for absolute confirmation of a latent infection and is a subject of current KHV research. CPE development stopped entirely, allowing repair of the CCB monolayer, at 30°C showing that viral replication had ceased. Slow progression of CPE at 12 and 15 °C suggests that KHV can continue replicating at lower temperatures, which has implications for the timeframe for transmission of the virus.

## **6.0 Conclusion**

In conclusion, this project has made substantial progress in assessing the risk posed by angling equipment as a route for KHV transmission. It has clearly demonstrated that fish exposed to KHV are less likely to be caught by an angler, but that they do shed virus and feed simultaneously and can contaminate the netting equipment with high titres of KHV which is likely to be sufficient to infect a naïve fish. The study has also revealed that simple advice can be given to the angling community, such as drying nets in sunlight, to reduce the risk of transmitting KHV to a naïve water. This project has also successfully improved existing culture techniques and developed a novel method for isolating viable KHV from mucus which could facilitate future studies on the survival and transmission of KHV.



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Appendix 1: Titres of KHV shed in mock and KHV-challenged carp

Fish \ Day	1	4	7	10	13	16	19	22
1	31419.65	74736275	2.89E+09	1.5E+08	555415000	-	-	-
2	3149.457	85955338	6.36E+08	1.31E+08	897945100	-	-	-
3	100050.8	1.19E+08	1.32E+09	1.49E+08	4.128E+09	-	-	-
4	59647.68	1.34E+08	2.42E+09	4.24E+08	7.78E+09	-	-	-
5	11258.04	55248675	3.58E+08	1.89E+08	1.32E+09	1.25E+08	0	-
6	11553.17	18372553	1.74E+08	17629491	39246891	925864.5	83755.62	0
7	366.5395	28527438	5.02E+08	1.06E+08	-	-	-	-
8	7520.714	2.71E+08	7.48E+08	1.22E+08	-	-	-	-
9	16216.49	1.44E+08	4.66E+08	45391000	3.195E+09	-	-	-
10	0	2465388	41210709	736377.3	0	94180.91	0	752261.9
11	2327.451	87366600	6.78E+08	95522431	6.232E+09	1.01E+10	-	-
12	26728.52	1154839	54595006	5744066	11819642	247569.1	0	0
13	29223.78	1.49E+08	4.38E+09	2.75E+08	9.433E+10	-	-	-
14	4610.691	6.07E+08	3.55E+09	21231120	-	-	-	-
15	0	2418361	91259738	6909517	20569309	0	0	0
16	298352.9	8.73E+08	8.9E+08	-	-	-	-	-
17	11599.26	3.41E+08	2.75E+09	-	-	-	-	-
18	15193.24	23365736	91162900	13442463	595375.78	82573.13	0	3288062
19	50008.75	1.43E+08	3.03E+08	51372009	232380275	-	-	-
20	22979.01	1.3E+09	3.28E+09	7.75E+08	-	-	-	-

DAILY MEANS	1	4	7	10	13	16	19	22
All fish	35110.31	2.23E+08	1.28E+09	1.43E+08	8.482E+09	1.46E+09	13959.27	808064.7
Clinically Diseased	43248.75	2.94E+08	1.68E+09	1.95E+08	1.319E+10	5.11E+09	0	
Infected Survivors	10694.98	9555376	90481266	8892383	14446244	270037.5	16751.12	808064.7



Appendix 2: Proportion of food eaten by KHV- and mock-challenged carp

Fish \ Day	1	2	3	4	5	6	7	8	9	10
1	0	50	35	62.5	70	77.5	80	67.5	15	35
2	0	75	100	85	50	67.5	0	70	47.5	0
3	0	90	50	70	97.5	50	0	12.5	10	22.5
4	0	50	50	100	100	57.5	0	30	92.5	10
5	0	92.5	92.5	100	62.5	100	95	90	7.5	27.5
6	0	85	50	72.5	47.5	97.5	75	55	90	100
7	0	50	50	37.5	100	40	42.5	60	57.5	25
8	0	92.5	92.5	100	95	50	20	10	0	5
9	0	50	75	100	80	75	100	65	32.5	10
10	0	62.5	50	97.5	12.5	70	27.5	0	0	22.5
11	0	50	100	50	72.5	57.5	70	42.5	37.5	27.5
12	0	50	85	100	100	100	100	100	100	100
13	0	50	50	100	100	37.5	87.5	75	82.5	90
14	0	62.5	75	75	37.5	62.5	70	0	62.5	100
15	0	62.5	62.5	100	100	75	100	100	100	20
16	0	62.5	95	50	80	52.5	5	-	-	-
17	0	82.5	90	50	35	42.5	0	0	0	-
18	0	100	100	100	100	100	100	35	92.5	95
19	0	92.5	100	100	92.5	100	40	20	47.5	95
20	0	87.5	62.5	100	0	100	20	0	10	0
Control A	0	80	82.5	100	100	100	100	100	100	100
Control B	0	95	50	100	100	100	100	100	100	100
Control C	0	50	50	100	100	100	100	100	100	100
Control D	0	62.5	100	100	100	100	100	100	100	100
Control E	0	82.5	100	100	100	100	100	100	100	100

All fish	0	69.9	73.3	82.5	71.6	70.6	51.6	43.8	46.6	43.6
Clinically Diseased	0	69.2	74.5	78.7	71.5	64.7	42.0	38.8	35.9	34.4
Infected Survivors	0	72.0	69.5	94.0	72.0	88.5	80.5	58.0	76.5	67.5
Controls	0	74.0	76.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Fish highlighted in yellow are the infected survivors

Days highlighted in red were sample points for viral shedding

Appendix 2: Proportion of food eaten by KHV- and mock-challenged carp

Fish \ Day	11	12	13	14	15	16	17	18	19	20
1	22.5	25	25	-	-	-	-	-	-	-
2	30	47.5	100	37.5	25	0	-	-	-	-
3	12.5	27.5	50	-	-	-	-	-	-	-
4	0	70	50	-	-	-	-	-	-	-
5	0	35	37.5	72.5	80	57.5	82.5	100	85	0
6	100	90	95	67.5	100	100	100	100	100	100
7	-	-	-	-	-	-	-	-	-	-
8	32.5	60	0	-	-	-	-	-	-	-
9	20	65	0	-	-	-	-	-	-	-
10	62.5	100	100	85	100	100	100	100	100	100
11	0	50	72.5	92.5	12.5	7.5	0	0	-	-
12	100	62.5	87.5	92.5	100	100	100	100	100	100
13	60	50	95	-	-	-	-	-	-	-
14	55	0	-	-	-	-	-	-	-	-
15	100	75	95	92.5	100	100	100	100	100	100
16	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-
18	100	90	100	100	100	100	100	100	100	100
19	85	25	0	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-
Control A	100	100	100	81.25	93.75	81.25	95	100	81.25	100
Control B	100	100	100	92.5	100	100	100	100	100	100
Control C	100	100	100	100	100	100	100	100	100	100
Control D	100	100	100	100	100	100	100	100	100	100
Control E	100	100	100	87.5	100	100	100	100	96.25	100

All fish	48.8	54.5	60.5	80.0	77.2	70.6	83.2	85.7	97.5	83.3
Clinically Diseased	28.9	41.4	43.0	67.5	39.2	21.7	41.3	50.0	85.0	
Infected Survivors	92.5	83.5	95.5	87.5	100.0	100.0	100.0	100.0	100.0	100.0
Controls	100.0	100.0	100.0	92.3	98.8	96.3	99.0	100.0	95.5	100.0

Fish highlighted in yellow are the  
infected survivors

Days highlighted in red were sample  
points for viral shedding

Appendix 2: Proportion of food eaten by KHV- and mock-challenged carp

Fish \ Day	21	22	23	24	25	26	27	28	29	30
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-
6	82.5	100	100	100	100	100	100	100	100	100
7	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
10	90	100	100	100	100	100	100	100	100	100
11	-	-	-	-	-	-	-	-	-	-
12	65	100	100	100	100	100	100	100	100	100
13	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-
15	100	100	100	100	100	100	100	100	100	100
16	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-
18	100	100	100	100	100	100	100	100	100	100
19	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-
Control A	93.75	100	100	93.75	100	96.25	87.5	100	70	100
Control B	100	100	100	100	100	100	100	100	100	100
Control C	100	100	100	100	100	100	100	100	100	100
Control D	100	100	100	100	100	100	100	100	100	100
Control E	93.75	100	100	100	100	100	100	100	100	100

All fish	87.5	100.0	100.0	100.0	100.0	100.0	100.0	100	100	100
Clinically Diseased	-	-	-	-	-	-	-	-	-	-
Infected Survivors	87.5	100.0	100.0	100.0	100.0	100.0	100.0	100	100	100
Controls	97.5	100.0	100.0	98.8	100.0	99.3	97.5	100	94	100

Fish highlighted in yellow are the infected survivors

Days highlighted in red were sample points for viral shedding

