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Cyprinid herpesvirus 3 (CyHV-3): its potential as a biological control agent for carp in Australia

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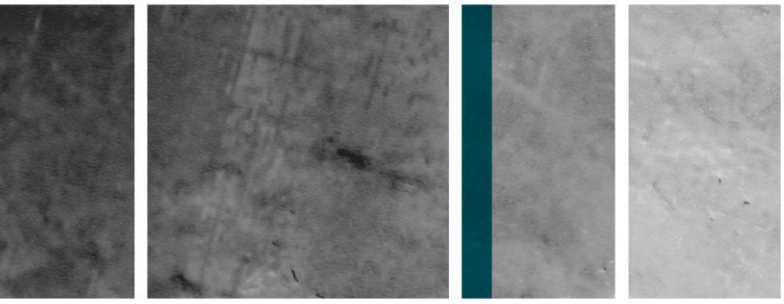

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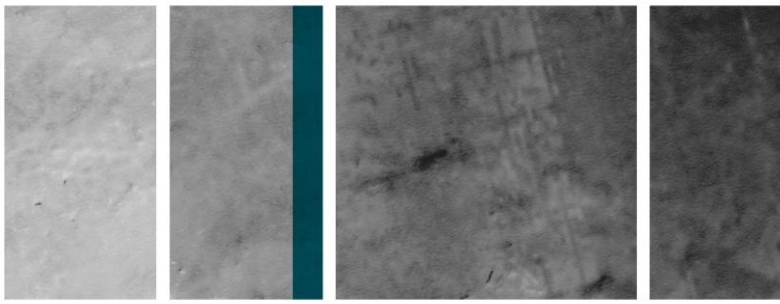

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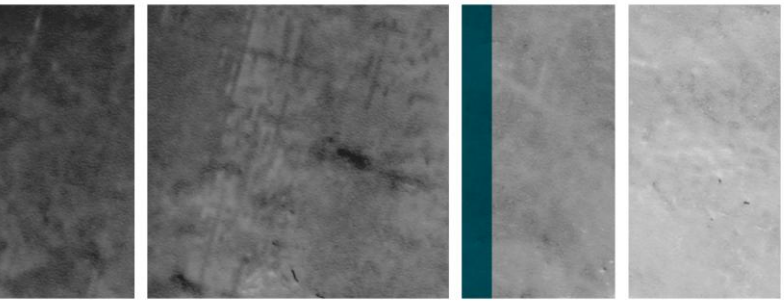

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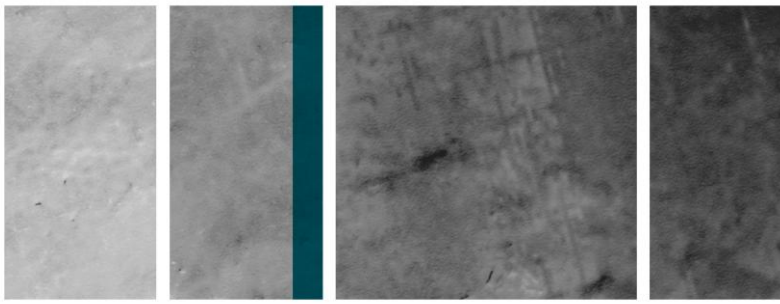

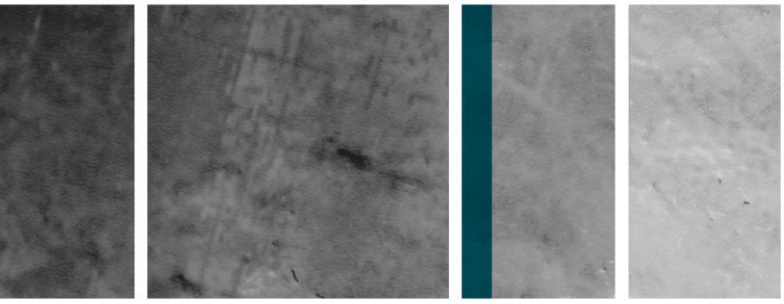

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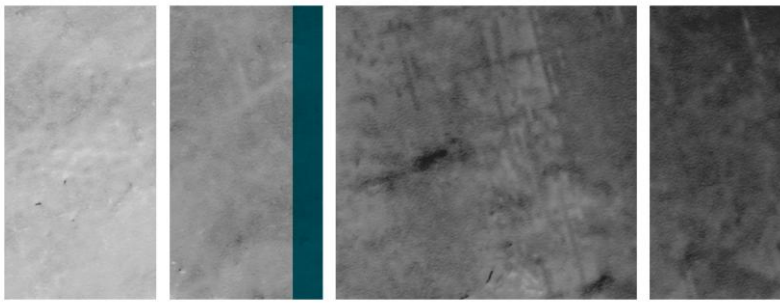


Summary

This report represents the culmination of work conducted in Phases 1 and 2 of a study to investigate the potential of Cyprinid herpesvirus 3 (CyHV-3; also known as koi herpesvirus) as a biological control agent for carp in Australia. Many of the Milestones/Objectives were delayed due to the lack of juvenile carp in the wild (due, in turn, to the drought in south-eastern Australia that persisted for many years of the project). Furthermore, a serious illness affecting one of the Principal Co-investigators (who was on sick leave for 7 months, and part-time duty for another 6 months), caused a major delay in the final stage of Phase 2.

Initially, CyHV-3 reference strains and the KF-1 and CCB cell lines were imported into AAHL from CEFAS Weymouth Laboratory, UK and University of California, Davis, USA. CyHV-3 strains were grown in both cell lines. The CyHV-3 strains were checked for purity, and the cell lines were inocuity-tested. Conditions for isolation and growth of CyHV-3 were established at AAHL. Eventually, an Indonesian strain was also introduced to the lab, and this was chosen for all subsequent experimental work.

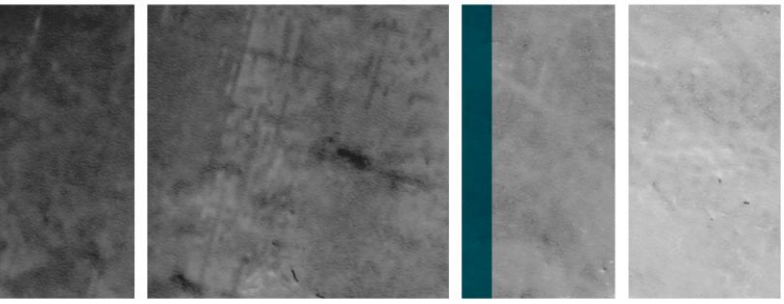
Assays for the detection of CyHV-3 infections of carp were introduced to AAHL. These included: (i) virus isolation and growth of CyHV-3 in carp cell lines; (ii) several PCRs used by international aquatic animal disease diagnostic laboratories; (iii) development of a generic cyprinid herpesvirus PCR with the potential to differentiate CyHV-1 (carp pox virus), CyHV-2 (goldfish haematopoietic necrosis virus) and CyHV-3; and, (iv) immunoassays for localisation of CyHV-3 in infected fish tissues and in cell cultures.

In vivo infectivity trials were completed, and demonstrated that 4-10 cm carp in Australia are not only susceptible to infection by CyHV-3, but that they develop disease and die in a matter of days following infection. Other studies revealed a much lower mortality in older fish. The sensitivity of juvenile carp to infection with different doses of CyHV-3 was determined, although this work may have been compromised by the lack of availability of immature carp at the time. It is likely that many of the fish used in this work were, in fact, carp-goldfish hybrids. An experimental system was devised that enabled detection of excreted virus from CyHV-3-infected immature carp. By this means, a preliminary temporal pattern of excretion of virus was determined.

A number of non-target species (Murray cod, golden perch, silver perch, a galaxiid and rainbow trout) were also tested in susceptibility trials. There was no evidence of virus replication nor of histological lesions in the non-target species. Given that mortality in carp-goldfish hybrids challenged with CyHV-3 is lower than in pure carp, a new bar-coding system, based on Luminex® technology, was introduced to allow differentiation of carp from goldfish and hybrids. Results were compared with a previously published PCR test. An examination of a small sample of carp (99) collected from Victorian waters appeared to detect one hybrid.

An extensive survey of carp collected from eight sites across the Murray-Darling Basin (M-DB) showed no evidence for any cross-reactive cyprinid herpesviruses that might compromise the efficacy of CyHV-3 were it to be released into the M-DB.

Finally, an extensive written review of the literature related to CyHV-3, and the disease caused by the virus, was prepared for use in any future attempts to gain approval for release of CyHV-3 into Australian waterways. This review has been published separately.



1. Introduction

Carp (*Cyprinus carpio*), members of the cyprinid family, were probably introduced to Australia in the 19th Century, but they did not become a problem until the 1960s when a strain adapted for European aquaculture, known in Australia as the Boolara strain, was imported. Floods in the mid 1970s resulted in the escape of these fish from isolated farm dams into the Murray-Darling River system, triggering a major environmental problem (Davidson, 2002). In the mid-2000s, work began on developing an integrated carp control program (Fulton, 2006), including an investigation of cyprinid herpesvirus 3 (CyHV-3; Davison, 2010), also known as koi herpesvirus, as a potential biological control agent.

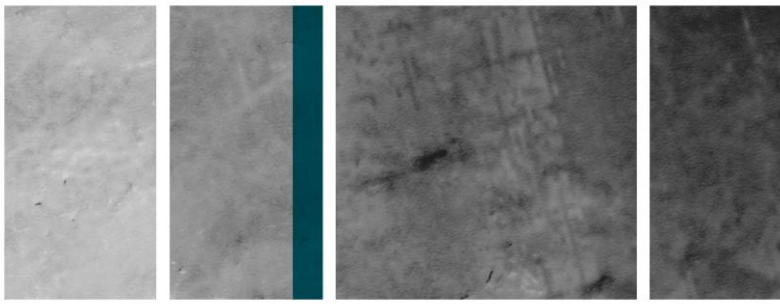
There have been only three instances around the world where viral pathogens have been used successfully to counter vertebrate pest species (Saunders *et al*, 2010). These include two rabbit viruses, the myxoma virus (Fenner, 2010) and the calicivirus known as rabbit haemorrhagic disease virus (Cooke, 2002), both used in Australia, and feline panleukopaenia virus, a DNA parvovirus that was used as part of a program to eradicate feral cats that were devastating wild seabird colonies on sub-Antarctic Marion Island in the southern Indian Ocean (Howell, 1984; van Rensburg *et al*, 1987).

In developing a biological control program with CyHV-3 for carp, it is important to consider what can be learned from both the experience with the three previous viruses used in successful biological control programs of vertebrates, and from the epidemiology of the disease in natural populations of wild carp (McColl *et al*, 2014). Determining the host specificity of a viral biological control agent is clearly important, as is an understanding of the pattern of mortality in the target species. Familiarity with the clinical course of disease in the target species will allow an assessment of the suitability of the agent based on animal welfare considerations, and an understanding of the transmissibility of CyHV-3 from infected to uninfected fish is required in order to assess its suitability as a control agent. The susceptibility of the target species to CyHV-3 must be considered, and it may be dependent on a number of factors, e.g., age-related immunity; passive immunity; the presence of cross-reactive viruses in the target population; ambient temperatures; and the proportion of hybrid individuals in the target population. In summary, the success of a viral biological control agent is likely to depend upon an intimate knowledge of the biology of the target species, and of the epidemiology of the virus.

While the literature contains a great deal of information on many of these topics, this project aimed to address many of the issues, particularly in an Australian context. In the process, we hoped to provide a solid base of knowledge that would allow a rational decision to be made about the potential use of CyHV-3 as a biological control agent for carp in Australia.

To begin the work, CyHV-3 reference strains and the KF-1 and CCB cell lines were imported into AAHL, and CyHV-3 strains were grown in both cell lines. Eventually, an Indonesian strain of CyHV-3 was also introduced to the lab, and this was chosen for all subsequent experimental work.

In order to work with the virus, assays for the detection of CyHV-3 infections of carp were introduced to AAHL. These included: (i) virus isolation and growth of CyHV-3 in carp cell lines; (ii) several PCRs used by international aquatic animal disease diagnostic laboratories; (iii) development of a generic cyprinid herpesvirus PCR with the potential to differentiate

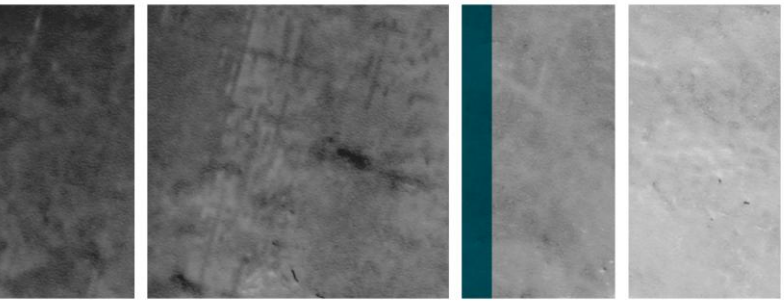


CyHV-1 (carp pox virus), CyHV-2 (goldfish haematopoietic necrosis virus) and CyHV-3; and, (iv) immunoassays for localisation of CyHV-3 in infected fish tissues and in cell cultures.

A crucial step in the project was to test the virulence of CyHV-3 for carp in Australia. *In vivo* infectivity trials were undertaken to determine if 4-10 cm carp in Australia are susceptible to infection by CyHV-3, and whether they develop disease and die. The susceptibility of older fish was also examined. Knowledge of the temporal pattern of excretion of virus from infected fish was necessary for an understanding of the epidemiology of the disease caused by CyHV-3 (koi herpevirus disease, KHVD).

The specificity of a potential biological control agent for the target species is, of course, critical. Therefore, a number of non-target species of fish were also tested in susceptibility trials. Furthermore, given that mortality in carp-goldfish hybrids challenged with CyHV-3 is lower than in pure carp, a system to allow differentiation of carp from goldfish and hybrids was considered essential.

In order to ensure that there were no cross-reactive cyprinid herpesviruses in the Murray-Darling Basin (M-DB) that might compromise the efficacy of CyHV-3 were it to be released into the M-DB, an extensive survey of carp from the M-DB was also conducted.



2. Methods: virus isolates & laboratory procedures

2.1 Introduction

In order to initiate a research program to investigate the use of CyHV-3 as a potential biocontrol virus for carp in Australia, a number of preliminary steps had to be undertaken. Importantly, given that CyHV-3 is exotic to Australia, all work in this program had to be conducted under the conditions of high biological security at the CSIRO-Australian Animal Health Laboratory (AAHL) in Geelong, Victoria.

Furthermore, appropriate strains of CyHV-3 had to be imported from overseas laboratories since this virus has never been reported (or suspected) in Australia. In order to isolate virus from infected fish, and to undertake any *in vitro* work with the virus, continuous cell lines that would support the growth of CyHV-3 also had to be imported.

Other laboratory techniques, critical for research and/or diagnostic use, also had to be introduced to AAHL. Many of these were simply reproduced from the literature, but some methods for research were developed at AAHL.

2.2 Methods and Results

2.2.1 Material imported into AAHL

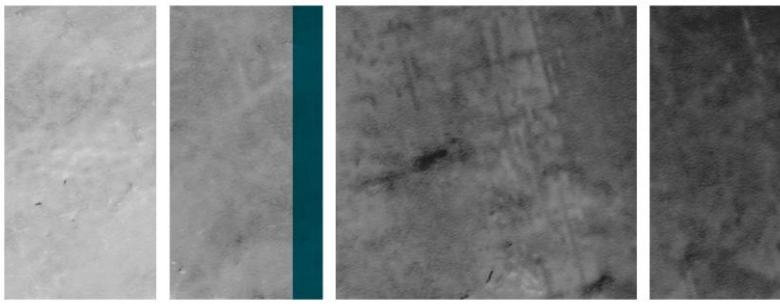
The Centre for Environment, Fisheries and Science (CEFAS) Weymouth Laboratory, UK (Contact: Keith Way) and The School of Veterinary Medicine, University of California, Davis, USA (Contact: Ron Hedrick), kindly provided both the common carp brain (CCB) and koi fin (KF-1) cell lines which are susceptible to infection by CyHV-3. These laboratories also provided reference strains of virus as cell culture supernatants (see Table 1).

Table 1: CyHV-3 isolates imported into AAHL

Virus isolate	Cell line used to grow the virus	Origin of isolate
G406	CCB	CEFAS, Weymouth, UK
G406	KF-1	CEFAS, Weymouth, UK
US 98-21	CCB	CEFAS, Weymouth, UK
US 98-21	KF-1	CEFAS, Weymouth, UK
USA F98-50	KF-1	Univ. California, Davis, USA

2.2.2 Purity and innocuity testing

Prior to any work being undertaken on imported material, purity and inocuity testing is required in order to exclude the presence of any adventitious fish viruses. Polymerase chain reactions (PCRs) specific for the known major viral pathogens of finfish were used to test for purity/inocuity. The imported cell lines (CCB and KF-1) were expanded using standard cell culture techniques (Nicholson, 1985). Total nucleic acid was extracted from an aliquot and



used as target for PCRs specific for a range of viruses. For DNA virus PCRs, the nucleic acid was used directly. For RNA virus PCRs, cDNA was obtained prior to performing the PCR (ie RT-PCR).

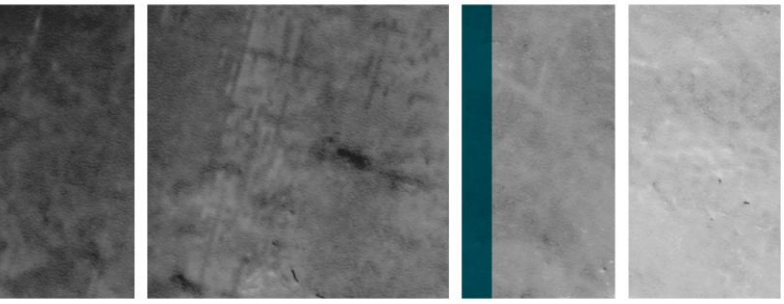
The PCR tests used were those obtained from the literature and recognised internationally as specific and sensitive for their respective target virus. The primers used for each PCR are shown in Table 2.

Using cell line nucleic acid as the target, all PCR tests yielded negative results demonstrating the absence of all viruses tested for, viz., CyHV-3, infectious pancreatic necrosis virus (IPNV), infectious salmon anaemia virus (ISAV), viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV) and spring viraemia of carp virus (SVCV). All control samples yielded expected results (Table 3).

Similarly, the KHV isolates were tested for purity using the PCR tests described above. All KHV isolates received from overseas laboratories were shown to be free from all other viruses tested for by PCR and they all yielded positive results using the KHV-specific PCR (Table 3).

Table 2: PCR Tests for Fish Viruses

Virus	PCR Primer set	Reference
CyHV-3	5' GACGACGCCGGAGACCTTGTG 3' 5' CACAAGTTCAGTCTGTTCCTCAAC 3'	Gilad et al., (2002)
IPNV	5' ACGAACCCTCAGGACAA 3' 5' CACAGGATCATCTTGGCATAGG 3'	Davies et al. (in prep.) Blake et al. (2001)
ISAV	5' GGCTATCTACCATGAACGAATC 3' 5' GCCAAGTGTAAGTAGCACTCC 3'	Mjaaland et al. (1997)
VHSV	5' GTCCCCAGGGATGATGNCC 3' 5' AGTCCCCAGGGATGATGNCC 3' Nested PCR set: 5' CACGAGTACCCGTTCTTCCC 3' 5' AGTCCCCAGGGATGATGNCC 3'	OIE (2011a)
IHNV	5' ATGATCACCACTCCGCTCATT 3' 5' CTCTGGACAAGCTCTCCAAGG3' Nested PCR set: 5' GATTGGAGATTTTATCAACA 3' 5' CTCTGGACAAGCTCTCCAAGG 3'	OIE (2011b)
SVCV	5' TCTTGGAGCCAAATAGCTCARRTC 3' 5' AGATGGTATGGACCCCAATACATHACNCAY 3' Nested PCR set: 5' TCTTGGAGCCAAATAGCTCARRTC 3' 5' CTGGGGTTTCCNCCTCAAAGYTGY 3'	OIE (2011c)



2.2.3 Optimisation of culture system

Following purity testing of the CyHV-3 isolates and the CCB and KF-1 cell lines, aliquots of the CyHV-3 cell culture supernatants were inoculated onto CCB and KF-1 cell cultures and grown to 100% cytopathic effect (CPE). Aliquots of these seed stocks have been stabilised in liquid nitrogen storage and in freezers at -80 ^{O}C. Working stocks, by growing aliquots in KF-1 cell cultures, have been created and aliquots of these working stocks have been stored frozen at -80 ^{O}C. These working stocks are used for all subsequent studies. Until further research shows otherwise, the KF-1 cell line has been accepted internationally as the cell line of choice for the isolation and growth of CyHV-3. Thus all subsequent work will be undertaken with this cell line.

Table 3: Purity testing of KF cell line and KHV isolates: Summary of Results

	PCR test for each specific virus					
Sample	KHV	SVCV	IPNV	IHNV	VHSV	ISAV
KF-1 cells	-	-	-	-	-	-
CCB cells	-	-	-	-	-	-
KHV isolates	+	-	-	-	-	-
SVCV		+				
IPNV			+			
IHNV				+		
VHSV					+	
ISAV						+

2.2.3.1 Host cell density

Host cell density to produce 80-95% confluency after overnight incubation at 25 ^{O}C in 24-well culture plates and 96-well microtitre plates has been determined (see Appendix 1 for details). For 96-well plates: a seeding rate of 4 million cells/11 mL (100 µL/well) is used. For 24-well plates: a seeding rate of 6 million cells/40 mL (250,000 cells/1.5 mL/well) is used.

2.2.3.2 Virus isolation

For virus isolation, standard procedures are used, as recommended in the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE, 2011). Using these techniques with KF-1 cell cultures incubated at 20 ^{O}C, virus was successfully re-isolated from the cell culture supernatants obtained from overseas laboratories (Figure 1).

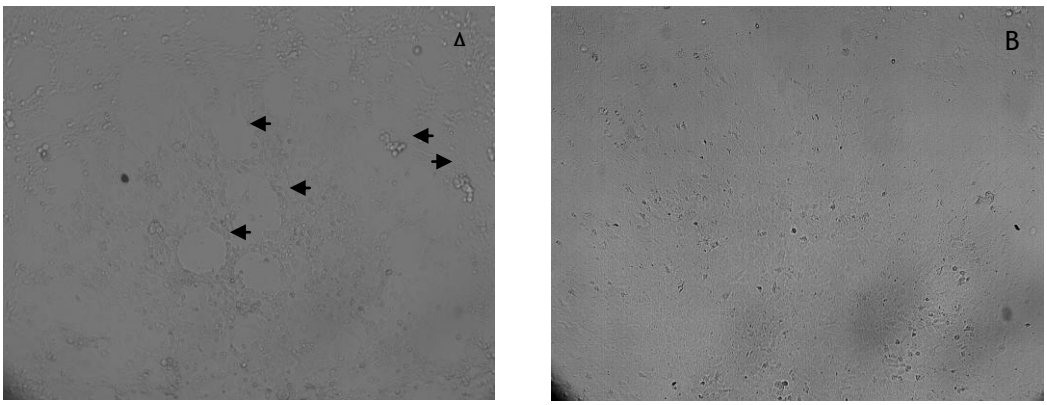


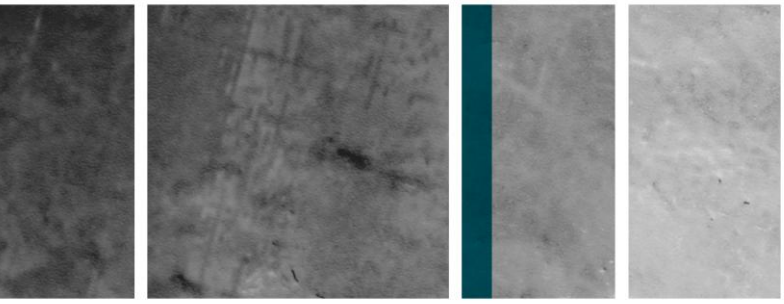
Figure 1: Photomicrographs of KF-1 cell cultures. The CyHV-3-infected culture (A), 20 days post-infection, showing cytopathic effect (CPE), rounded cells and plaques (arrowed). Note that the equivalent uninfected control cell culture (B) is fully confluent and does not display the typical CPE caused by CyHV-3.

2.3 Molecular detection methods

The PCR tests used were obtained from the literature and are recognised internationally as specific and sensitive for CyHV-3. The primers used for each PCR are shown in Table 4. In an effort to standardise PCR diagnostic tests for CyHV-3, the CEFAS Weymouth Laboratory, UK organised and implemented an international proficiency test program for CyHV-3 PCR testing. The AAHL Fish Diseases Laboratory (AFDL) has participated in each round of proficiency, or "ring", testing since 2006, and has been uniformly successful in identifying the unknown specimens. It is well-established that real-time (e.g., TaqMan) PCRs are more sensitive than conventional PCRs, and therefore the use of this test is likely to become standard for detection of low-level infections.

Table 4: PCR Tests for CyHV-3

PCR Primer set	Reference
5' GACGACGCCGGAGACCTTGTG 3' 5' CACAAGTTCAGTCTGTTCCTCAAC 3' Product size = 484 bp	Gilad et al. (2002)
Forward = 5' GGGTTACCTGTACGAG 3' Reverse = 5' CACCCAGTAGATTATGC 3' Product size = 409 bp	Bercovier et al. (2005)
Gray's PCR (using the CEFAS modified cycling conditions) Forward = 5' AGGACCGCTACAAGAGCCTC 3' Reverse = 5' GACACATGTTACAATGGTCGC 3' Product size = 151 bp	Gray et al. (2002)
Gray's modified PCR (official Japanese test for KHV) Forward = 5' GACACCACATCTGCAAGGAG 3' Reverse = 5' GACACATGTTACAATGGTCGC 3' Product size = 292 bp	Yuasa et al. (2005)



2.4 Immunocytochemical and immunohistochemical tests developed using commercial antiserum

2.4.1 Immunocytochemistry

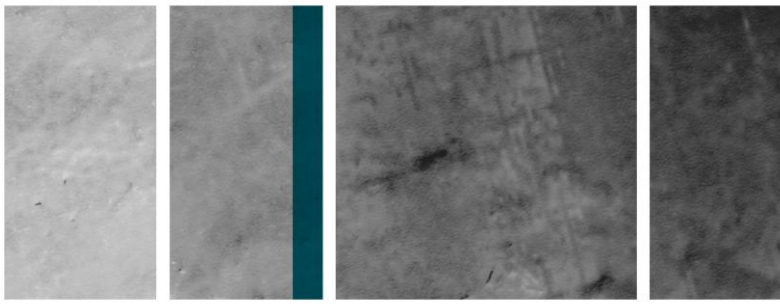
Localisation of CyHV-3 in cell cultures by immunocytochemical methodology has been established using a commercial monoclonal antibody (Aquatic Diagnostics Ltd, Stirling, UK). The technique has been added to a revised version of the Australian and New Zealand Standard Diagnostic Procedure (ANZSDP). ANZSDP was drafted in anticipation that one would be needed in Australia (see Appendix 1). However, during the course of this project, a diagnostic method was published in the OIE Manual of Diagnostic Tests for Aquatic Animals (2009). Policy in Australia states that if an OIE Manual Diagnostic Test exists that suits Australia's needs then an ANZSDP is not required. The draft ANZSDP for CyHV-3 is essentially the same as the OIE Manual Diagnostic Test. Thus the draft ANZSDP, while fully developed as part of this project, was not submitted to SCAHLS for review and publication.

2.4.2 Immunohistochemistry

Localisation of CyHV-3 in tissue sections of carp by immunohistochemical methodology has been established using a commercial monoclonal antibody (Aquatic Diagnostics Ltd, Stirling, UK). The technique has been added to a revised version of the ANZSDP.

2.5 Barcoding system introduced to confirm species of fish

The development of a Luminex-based system to differentiate carp from goldfish is reported in a later Section since the barcoding system was introduced in order to address the issue of the prevalence of hybrids.



3. Results 1: Susceptibility of Australian carp to CyHV-3

3.1 Introduction

Clearly, if a program were to be developed on the use of CyHV-3 as a potential biocontrol agent for carp in Australia, then the first question to be addressed is whether Australian strains of carp are susceptible to the virus. There have been many trials involving the virus and Australian carp, but this section describes the first trial that was conducted.

3.2 Methods

Due to a long-running drought in south-eastern Australia (and the consequent lack of breeding by carp), the young carp that were needed for susceptibility trials (and for later non-target species testing) were difficult to source throughout much of this project. However, periodically Keith Bell (K & C Global Fisheries Pty Ltd) and DPI Victoria were able to access wild fish for experimental purposes.

For this work, 107 juvenile carp (mean length +/-SD of 2.57 +/- 0.65 cm) were collected from the Goulburn River near Snobs Creek, Victoria. The fish were distributed into eleven groups of eight to ten fish. Each group was held in a 2-L tank containing fresh water at 23 oC. The treatment regime is shown in Table 5. In two of the groups, each fish was inoculated intraperitoneally (IP) with 10 $TCID_{50}$ of CyHV-3 per fish (in this case the KHV C07 isolate from Indonesia). Another three groups of fish were exposed by bath immersion to 30 $TCID_{50}$ per mL of CyHV-3 for 2 h, two groups to 6 $TCID_{50}$ per mL, and a final two groups to 0.6 $TCID_{50}$ per mL. One group of fish served as the injection control and another as the bath control. The control fish were treated identically to the experimental groups but received only L-15 medium without virus. Fish were fed a commercial feed at 1% bodyweight per day and were monitored twice daily for clinical signs of disease and mortality. Any showing extreme clinical signs of disease were immediately euthanized. Confirmation of infection was by one, or a combination, of the following: clinical signs of disease; CyHV-3-specific PCR; pathology.

3.3 Results and discussion

Initial infectivity trials demonstrated that carp sourced from Australian waters, and held at 23 oC, are susceptible to infection by CyHV-3 by either the IP route or by the more natural route of bath infection. Juvenile fish succumbed to disease and died within 1-2 weeks following exposure to the virus (Figure 2).

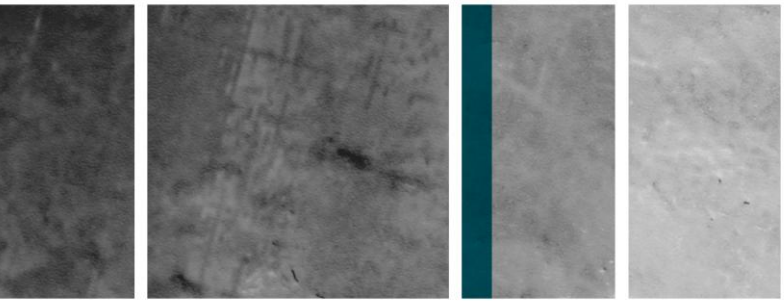


Table 5: Challenge trial for juvenile carp with CyHV-3

Tank[a]	Route of infection	Dose of virus	No. of fish in expt	Percent D/M[b]
1	Bath	Neg ctl	10	0
2	IP	Neg ctl	10	
3	Bath	0.6 $TCID_{50}$ per mL	10	10
4	Bath	0.6 $TCID_{50}$ per mL	10	
5	Bath	6 $TCID_{50}$ per mL	10	40
6	Bath	6 $TCID_{50}$ per mL	10	
7	Bath	30 $TCID_{50}$ per mL	8	96
8	Bath	30 $TCID_{50}$ per mL	10	
9	Bath	30 $TCID_{50}$ per mL	9	
10	IP	10 $TCID_{50}$	10	100
11	IP	10 $TCID_{50}$	10	

[a]: All experiments conducted at 21-22 °C. [b]D/M: Dead or Moribund (severe clinical signs requiring euthanasia).

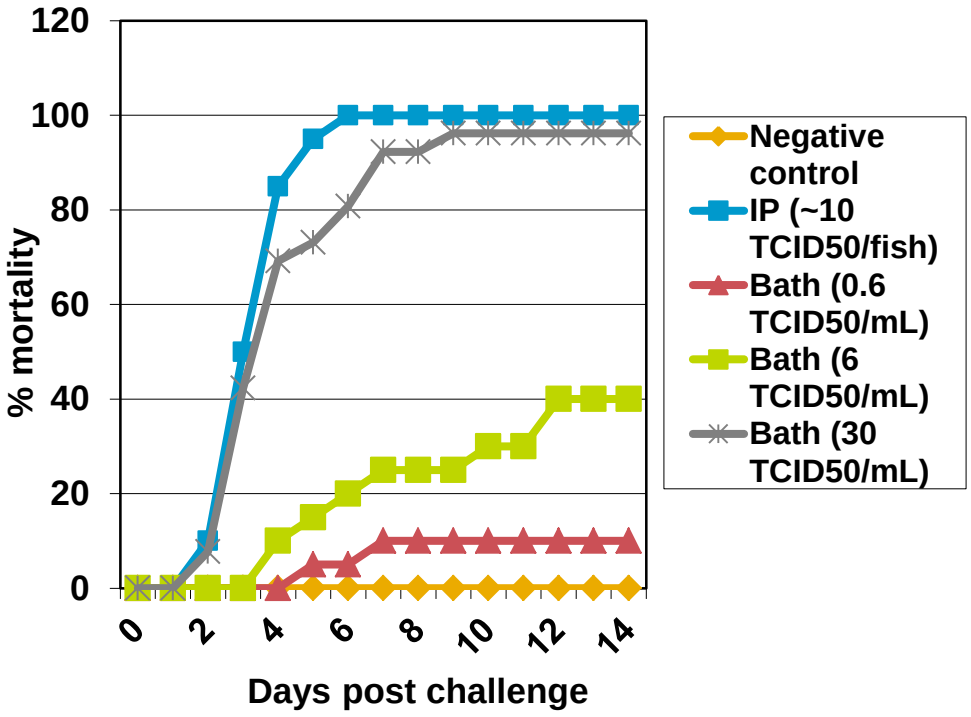
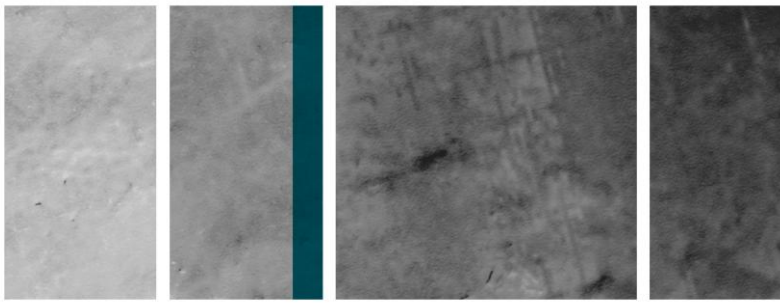


Figure 2: Cumulative mortality for carp (Cyprinus carpio) fry, sourced from Australian waters and infected with koi herpesvirus



4. Results 2: Susceptibility of carp of various ages to CyHV-3

4.1 Introduction

While the susceptibility of carp to CyHV-3 is certainly dependent on water temperature, the size of the fish is also a determinant of susceptibility. Young fish are said to be more susceptible to disease than adults. For example, Perelberg *et al* (2003) have stated that over 90% of 2.5 gm and 6 gm fish were killed following exposure to infected fish, whereas only 56% of 230 gm fish were killed. The aim of this current work was to gain some understanding of the susceptibility of different sized Australian carp to CyHV-3, using size of the fish as an indicator of age.

4.2 Methods

Wild-caught carp were utilized in susceptibility experiments on carp and non-target species. The fish were generally challenged with CyHV-3 either by direct intraperitoneal (IP) inoculation or by bath infection. On two occasions, fish were challenged by being placed in contact with infected fish. Following challenge, fish were monitored daily, and any showing extreme clinical signs of disease were immediately euthanized. Confirmation of infection was by one, or a combination, of the following: clinical signs of disease; CyHV-3-specific PCR; pathology.

4.3 Results and discussion

The results from a number of experiments are shown in Table 6. While no single experiment (using standard conditions of infection) was conducted to determine the age-susceptibility of Australian carp to CyHV-3, data from a number of experiments conducted over a number of years do provide some insights into the issue.

The data in Table 6 suggest a declining susceptibility to challenge with CyHV-3 as carp become larger (and older). The smallest (and presumably youngest) fish in Expt 1 were very susceptible, regardless of whether the virus was delivered IP or by bath infection. While the mean size of these fish was 2.6 cm, 3 of 20 that were inoculated IP, and 6 of 27 that were bath infected, were less than 2.0 cm in length (but none were under 1.0 cm long). Ito *et al* (2007) demonstrated that larvae bigger than 2 cm were, in general, about 100% susceptible, larvae between 1 and 2 cm were of mixed susceptibility (66% mortality), and larvae/fry less than 1 cm were generally insusceptible. These findings deserve to be tested with Australian carp, especially given that carp nursery sites are likely to be a major target for any future strategic release of CyHV-3.

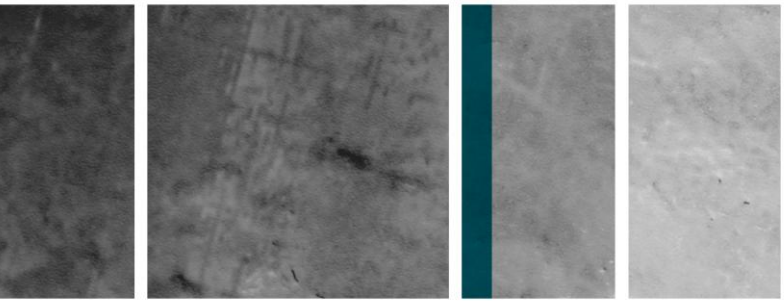


Table 6: The percent susceptibility to CyHV-3 of different-sized fish

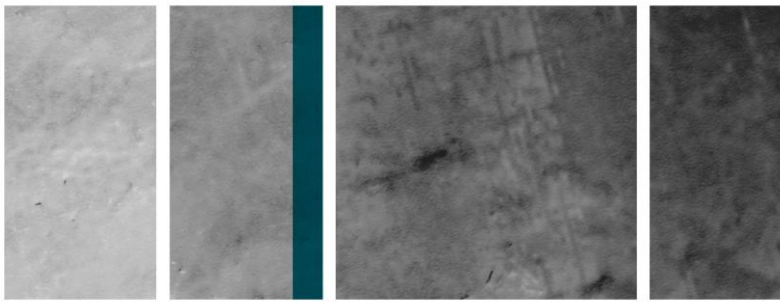
Expt[a]	Size (cm)[b]	Route of infection	Dose of virus	No. of fish in Expt	Percent D/M[c]
1	2.6 (0.7)	IP	10 $TCID_{50}$ per fish	20	100
		Bath	30 $TCID_{50}$ per mL	27	96
2	12.1 (1.0)	Bath	100 $TCID_{50}$ per mL	30	90
		In contact	NK[d]	20	75
3	18.5 (1.9)	IP	$10^{4.1}$ $TCID_{50}$ per fish	8	38[e]
4	30[f]	IP	ND[g]	6	33[h]
		In contact	NK	1	0[i]

[a]: All experiments conducted at 21-22 ºC. [b]: Mean +/- std deviation; [c]D/M: Dead or Moribund (severe clinical signs requiring euthanasia); [d]: Not known; [e]: In addition, three survivors had low Ct values for the KHV PCR; [f]: Estimated mean length; [g]: Not determined; [h]: In addition, four fish developed moderate clinical signs of disease, and were euthanized (2) or recovered (2); [i]: Developed mild clinical signs of disease, and was euthanized.

Although the numbers of larger carp that were tested in this study were small (due to the lack of facilities at AAHL for holding large carp), it appears that older carp are less susceptible to virus, reflected in the lower number of fish that were either found dead, or moribund with severe clinical signs of disease (eg, swimming erratically, or recumbent on the bottom of the tank). In addition, as carp become larger, and older, there was a tendency for them to develop mild clinical signs of disease (eg, patchy reddening of the skin), and, if not euthanized, to then recover. Perhaps many of these fish would die in the wild, but almost certainly some would survive, probably with a solid immunity against CyHV-3. Such fish, if females, could conceivably pass protective antibodies to their young through the yolk sac.

It is worth noting that in Expt 3, carp were held for 26 dpi, by which time mortality appeared to have finished. However, at the end of the experiment, 3 of the survivors were found to have PCR evidence for the presence of large amounts of virus in kidney and gills. It is possible that a few more of these fish may have died had the experiment been prolonged, but persistently-infected fish may also have been produced. Such fish may have an important role in the epidemiology of the natural disease (Uchii *et al*, 2011).

In summary, these data are consistent with the observation that larger (and older) carp are less susceptible to a lethal infection with CyHV-3.



5. Results 3: Sensitivity of juvenile Australian carp to CyHV-3

5.1 Introduction

The sensitivity of carp to infection with CyHV-3 will be an important factor in determining the strategy for the eventual release of the virus as a biological control agent in Australia. Gilad *et al* (2003) provided some limited results on the sensitivity of carp to infection with CyHV-3, but further data would be useful, particularly for Australian carp. The aim of the following work was to examine the sensitivity of juvenile carp to CyHV-3 via a natural route of infection.

5.2 Methods

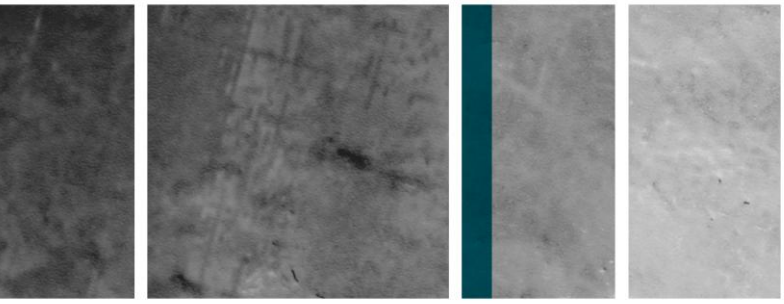
A trial was conducted on the sensitivity of juvenile carp to CyHV-3. The mean size (+/- the standard deviation) of the fish was 4.7 cm (+/- 0.7). Fish were exposed by bath infection for 2 hrs at 21 ^{o}C to 6 different concentrations of CyHV-3: 0.008, 0.08, 0.8, 8.0, 80 and 630 $TCID_{50}$/mL. As controls, five fish were also directly inoculated IP with 10-20 $TCID_{50}$ per fish. For each treatment, following exposure to virus, 10 fish were held individually in 1 L tanks, and a further 10 were held together in a single 1 L tank. Water temperature was maintained at 21 ^{o}C, and all fish were checked daily for the length of the trial (19 days). When mortalities occurred, fish were examined by a CyHV-3-specific PCR or by histopathology.

Water samples were also collected daily from each tank, and these were examined by the CyHV-3-specific PCR for the presence of virus. These results constitute part of the report for the Section on virus excretion (see 'Excretion of KHV from clinically affected carp').

5.3 Results and discussion

The results for the positive control IP-inoculated fish are shown in Figure 3. Note that only 3 of 5 (60%) fish were found dead or moribund in this trial. Two of the three dead fish were positive by PCR (the third was too autolyzed for further examination).

When fish were infected via a natural route of infection (Figure 4), mortalities were also relatively low. Thirty-three of the 38 dead or moribund fish were tested by PCR, and found to be positive for a specific CyHV-3 product in the gills, kidney or spleen. The exceptions were one moribund fish from the 0.8 $TCID_{50}$/mL dilution that was not examined, two dead fish from the 0.08 $TCID_{50}$/mL dilution that were both negative by PCR, and two moribund fish from the 630 $TCID_{50}$/mL dilution that were not examined.



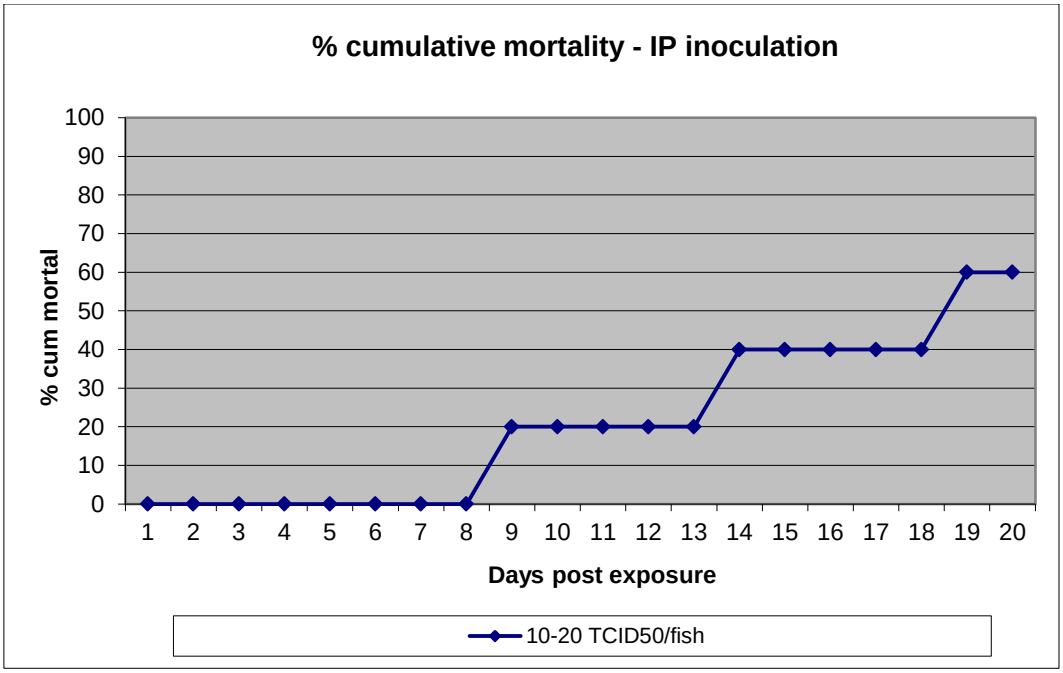


Figure 3: Percent cumulative mortality for carp inoculated IP with 10-20 TCID50/fish of CyHV-3

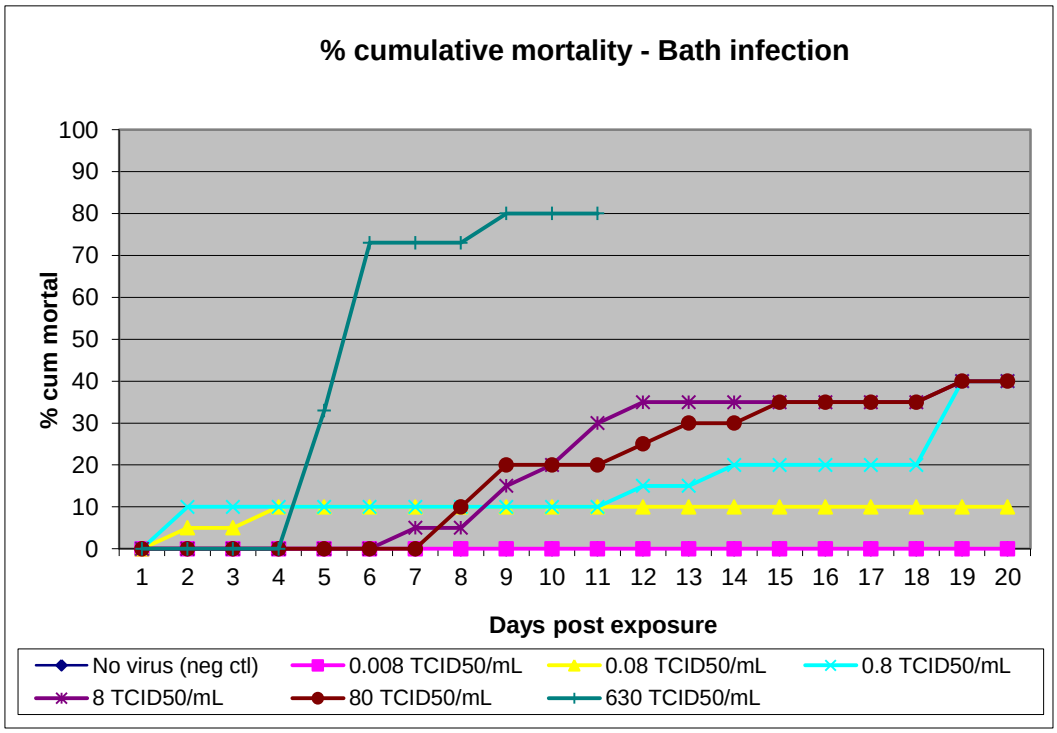
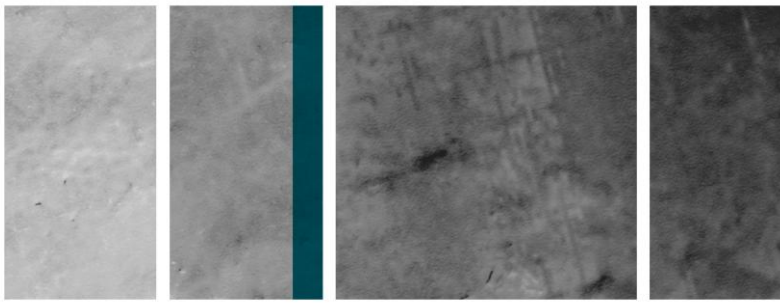


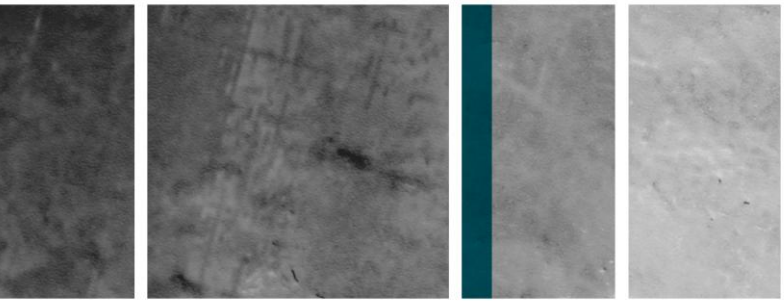
Figure 4: Percent cumulative mortality for carp infected with different doses of CyHV-3 via a natural route of infection



While the data in this work demonstrate that the sensitivity of juvenile carp to CyHV-3, via a natural route of infection, increases with increasing concentrations of virus, the unexpected finding was the relatively low mortality in the carp when challenged with 10-20 $TCID_{50}$/fish IP or with 630 $TCID_{50}$/mL in a bath infection. Results from an earlier, similar, experiment in juvenile fish (Figure 2) showed that mortalities of 100% and 96% could be expected for fish that were IP-inoculated or bath infected (30 $TCID_{50}$/mL), respectively.

The likely reason for the lower mortalities in the current trial is that the experimental fish were, in fact, carp-goldfish hybrids rather than pure carp. The fish had been collected from a Victorian billabong which, with hindsight, is a likely source of hybrids (Haynes *et al*, 2012). Twelve of the fish were bar-coded, using the mitochondrial cox-1 gene, and all appeared to be *Cyprinus carpio*. While they may, indeed, have been carp, it is more likely that they were hybrids with a carp mother (since the cox-1 gene is only inherited on the maternal side).

Because there is some doubt about the paternity of the fish used in this trial, the data generated in the current work do not really extend our knowledge about the sensitivity of juvenile carp to infection with CyHV-3. Data from an earlier Section (Figure 2) remain our best source of information on this issue. By assumption, the current data do, however, provide some guide about the sensitivity and susceptibility of carp hybrids to CyHV-3, and this may be helpful during any future epidemiological modelling of a biological control program for carp in Australia.



6. Results 4: Susceptibility of non-target species to CyHV-3

6.1 Introduction

Prior to the release of any biological control agent for carp, a critical consideration would be the effect that the agent has on non-target species. It is expected that the susceptibility to CyHV-3 of many species of fish (and possibly other species too) will need to be tested before this virus could even be considered for release in Australia. This section reports the results of the initial non-target species testing with CyHV-3.

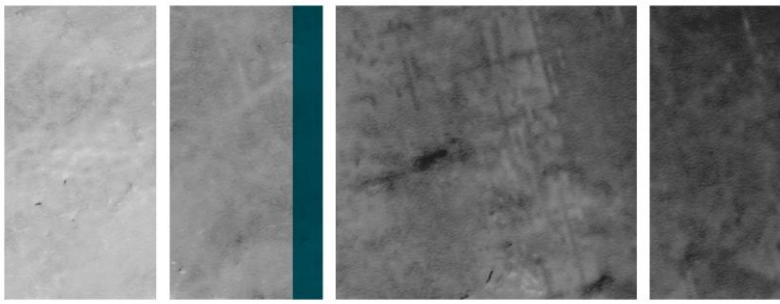
In these initial trials, Murray cod (*Maccullochella peelii peelii*), silver perch (*Bidyanus bidyanus*) and golden perch (*Macquaria ambigua*), all of which belong to the Order Perciformes, were tested, and later galaxiids (*Galaxias maculatus*, *O. Osmeriformes*) and rainbow trout (*Oncorhynchus mykiss*, *O. Salmoniformes*) were also selected for testing.

6.2 Methods

Juvenile carp (although out of season) became available, through the efforts of Keith Bell, in May/June, 2009, and Kylie Hall (Marine and Freshwater Fisheries Research Institute, Alexandra) obtained juvenile silver perch and Murray cod. This allowed the initial non-target species testing to begin. Batches of approximately 40 silver perch, Murray cod and carp were acclimatised to a water temperature of 21° to 23.5 °C, and then exposed to CyHV-3, either by intraperitoneal (IP) inoculation or by bath exposure. Negative controls were held at the same temperature. All dead and moribund fish were processed for detection of viral DNA (by polymerase chain reaction, PCR) or for histopathological examination.

A second non-target species testing program was conducted from mid-October to early December, 2009. Excess, unused juvenile carp and Murray cod from the first non-target trial were still available, and, through the efforts of Kylie Hall, Katie Doyle and Hui King Ho (Marine and Freshwater Fisheries Research Institute, Alexandra), we obtained juvenile golden perch from commercial breeders. Batches of golden perch and carp were acclimatised to a water temperature of 21 - 23.5 °C, and each species was then exposed to CyHV-3, either by IP inoculation (n=28 and 43, respectively), or bath infection (n=31 and 41, respectively). A single batch of Murray cod (n=31) was inoculated IP, a bath inoculation trial on Murray cod having already been completed. Negative control groups were slightly smaller, but were held at the same temperature. All dead and moribund fish were processed for detection of viral DNA (by PCR) or for histopathological examination.The titre of virus used in this trial was: $10^{5.3}$ $TCID_{50}$/mL (compared with $10^{5.5}$ in the first non-target spp trial).

The susceptibility of native galaxiids (*Galaxias maculatus*) and introduced rainbow trout (*Oncorhynchus mykiss*) to infection with CyHV-3 was determined using similar methods to those described above. *G maculatus*, about 3-5 cm in length, were collected from Lake Bullen Merri by Dr Paul Brown (DPI Victoria), and *O mykiss*, about 5-10 cm in length, were obtained from Snobs Creek Hatchery. Immature carp could not be found in south-eastern Australia, and therefore 6 mature carp (15-20 cm in length), obtained from NSW I&I (Narrandera, NSW), were used as positive controls. At AAHL, trout were acclimatised to a water temperature of 18 °C, while carp and galaxiids were acclimatised to 20-21 °C. All fish were then exposed to CyHV-3, either by intraperitoneal (IP) inoculation or by bath exposure. Negative controls were



held at the same temperature. All dead and moribund fish were processed for detection of viral DNA (by real-time PCR) or for histopathological examination. The experimental design is summarized in Table 7.

Table 7: Experimental design for testing the susceptibility of non-target species to CyHV-3

Room	Negative control[a]		Positive control[a]	Non-target species[a]	
	Bath	IP	IP	Bath	IP
1	Carp: Insuff. Trout: 18 Galaxiids: 22	Carp: 3 Trout: 23 Galaxiids: ND	-	-	-
2	-	-	Carp: 5	Trout: 31 Galaxiids: 41	Trout: 22 Galaxiids: ND

a: Number of fish in each treatment group. Fish held in 80 L tanks.
Insuff.: Too few carp for bath negative, and positive, control.
ND: Not done (galaxiids too small for IP inoculation).

6.3 Results

Figures 5A and B show the cumulative mortality (numbers of fish) for carp, following challenge with CyHV-3 by either (A) bath or (B) IP inoculation. The mortalities (64% bath, and 67% IP) are lower than those reported in a previous trial conducted at AAHL, but are still within the range reported with this virus by other workers overseas.

All carp that died, or were moribund (and were euthanized), were positive for CyHV-3 DNA by PCR in gills and kidney. Mean Ct values (+/- std deviation) for these fish were:

Bath - Gills: 29.1 +/- 4.2 Kidney: 31.1 +/- 3.9

IP - Gills: 25.8 +/- 2.9 Kidney: 25 +/- 2.5

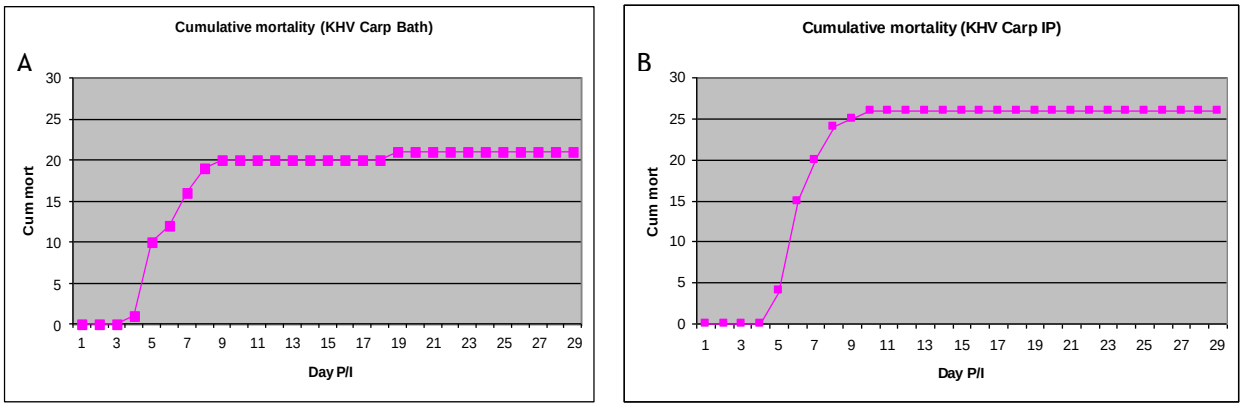
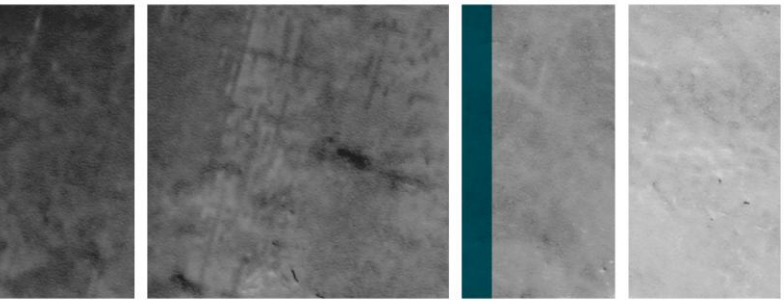


Figure 5: Cumulative mortality for carp following challenge with CyHV-3 by (A) bath, or (B) IP inoculation.

(Note that Ct values are measured on a scale of 0-40 where a Ct of 37, or greater, is considered negative, and the lower the Ct value, the more viral DNA is present). Note that in



those carp that were bath-inoculated with CyHV-3 (simulating a natural route of infection), the mean Ct value in the kidney for all dead/moribund fish up to 4 dpi was 32.3 +/- 4.3 whereas, for dead/moribund fish from 5 to 10 dpi, the mean Ct value was 28.9 +/-2.0. This finding is consistent with the fact that, following infection, fish undergo an initial viraemia with subsequent localization of the virus in the kidney. It then takes a further few days for the virus to replicate to high levels in the kidney.

Histologically, there was a paucity of lesions, but, in a number of fish, there were lesions in the gill-rakers. In one case, the mucosa lining the gill-raker was thickened and ragged. There was prominent epithelial hyperplasia in the affected mucosa, almost polypoid in places, and there were small multifocal areas of necrosis too. In affected areas, a proportion of cells (sometimes a very high proportion) contained intranuclear inclusion bodies with margination of nuclear chromatin. This latter change is very characteristic of herpesviruses. There were no lesions in the negative control carp (inoculated with, or bathed in, virus-free tissue culture medium).

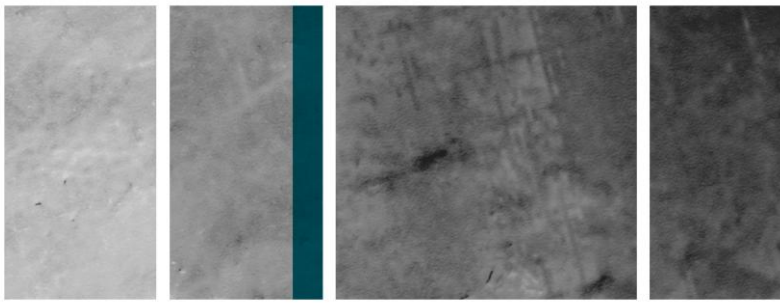
Because the carp that were used in this trial were wild-caught, it was considered important to confirm the species of these experimental fish, particularly those fish that survived the challenge with CyHV-3. Given that the fish were relatively small at the beginning of the experiment (approximately 4-6 cm long), was it possible that some carp-goldfish hybrids had been inadvertently included with the common carp? Hybrids, of course, would be expected to have a reduced susceptibility to CyHV-3. Subsequently, nucleic acid from 6 fish were examined by a "DNA barcoding" PCR - cytochrome C oxidase subunit 1 (Cox 1) using Fish F1 and Fish R1 primers and cycling conditions described by Ward *et al*. (2005). Specific PCR products were then sequenced to determine the species of each of these fish. Two fish that died from the CyHV-3 infection were shown to have a 99-100% match (over about 600 bases) with *Cyprinus carpio*. However, of 4 fish that survived challenge with CyHV-3, one was shown to match *Cyprinus carpio* (100%, approx. 600 bases), but three were shown to match *Carassius auratus* (99-100%, 600 bases). This is an important finding, not only in explaining our results, but also in thinking about the longer term aims of the project.

Figures 6 A and B show the cumulative mortality for a non-target species, silver perch, following challenge with CyHV-3 by either bath (A) or IP inoculation (B).

Mortalities in the silver perch (35% bath, 55% IP) were higher than expected. However, examination of all dead and moribund fish by PCR revealed that none of those that had been bath-inoculated were PCR-positive, and only 4 of 18 (22%) that had been IP-inoculated were positive for the presence of viral DNA. Furthermore, the mean (+/- std deviation) Ct values for these 4 fish were:

Gills: 35.6 +/- 0.8

Kidney: 39.2 +/- 1.2



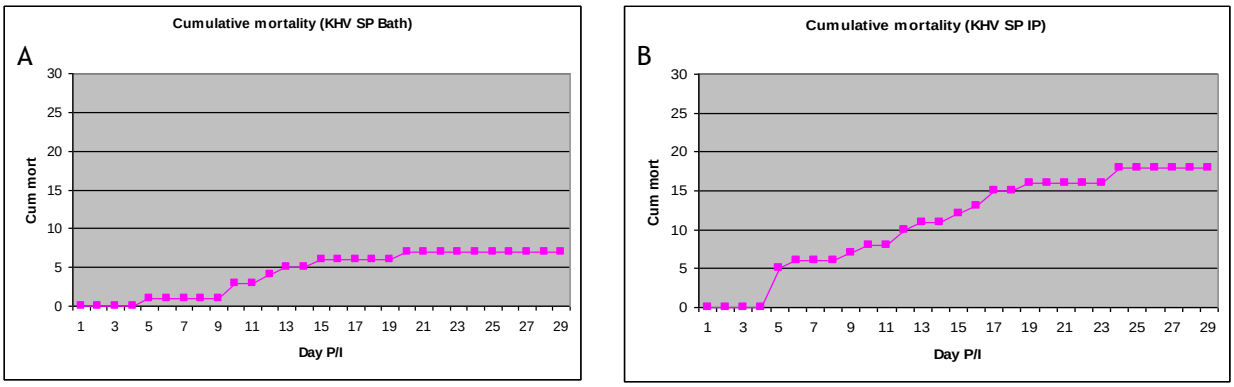
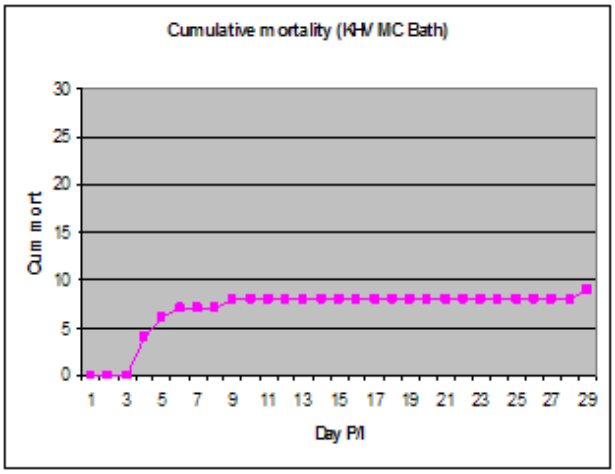


Figure 6: Cumulative mortality for silver perch following challenge with CyHV-3 by (A) bath, or (B) IP inoculation

It is notable that none of the silver perch that were exposed to CyHV-3 via a natural route of infection (bath inoculation) were positive by PCR. Of those that received an IP inoculation of virus, only 4 dead or moribund fish were PCR-positive, but all with relatively high Ct values that suggest that it may have been DNA from residual virus inoculum that was detected. Relatively low levels of viral DNA were also detected in the gill of one apparently normal fish when the experiment was terminated (Ct: 34.8).

There were few, if any, significant histological lesions observed in the silver perch. Mild infestations of *Epitheliocystis* sp have been observed in the gills of most silver perch, but it is unclear if these contributed to the mortality in these fish.

When the Murray cod, another non-target species, were transferred from their holding tank to the experimental tanks at the beginning of the experiment, there was a short problem with control of water temperatures in the new tanks, particularly in the tank where the cod were inoculated IP with CyHV-3. As a result, most of the latter fish became moribund within 2 days, and, by 3 days pi, the experiment had to be aborted (and the majority of fish were euthanized). PCR results showed that there was no evidence of CyHV-3 replication in any of the fish that were examined. Only one of four fish that were examined had any evidence of CyHV-3 DNA – a small amount of viral DNA, probably residual inoculum, in the kidney at 2 dpi.



In the Murray cod that were exposed to CyHV-3 by bath inoculation, the mortality curve is shown in Figure 7.

Mortality in the bath-inoculated fish was 24%. Histopathological examination revealed no significant lesions in these fish, and PCR results showed that viral DNA was found in only 2 of 15 dead or moribund fish that were examined. In both cases, low levels of CyHV-3 DNA (mean Ct value: 35.6 +/- 0.1) were found in the kidney of affected fish while gills of the same fish were negative for viral DNA.

Figure 7: Cumulative mortality for Murray cod following challenge with CyHV-3 by bath inoculation.

In the second trial, Figures 8A and B show the cumulative mortality for carp following challenge with CyHV-3 by either (A) bath infection or (B) IP inoculation. Data for negative control carp, challenged with tissue-culture medium in each case, are also shown. While the data demonstrate that the CyHV-3 inoculum was clearly virulent, mortality was slightly less than expected. Survivors and many of the affected fish were examined with a bar-coding PCR to determine if, as shown in the first non-target species trial, a proportion of the fish used in this trial were actually carp-goldfish hybrids (with an expected lower susceptibility to CyHV-3).

Figures 8C and D show the cumulative mortality for another non-target species, golden perch, following challenge with CyHV-3 by either (C) bath or (D) IP inoculation. In both cases, there was a reasonably high mortality over the course of the experiment (4 weeks), but, in each case, mortality in the negative controls was approximately similar to that in the CyHV-3-infected fish.

Figure 9 shows mortality in the Murray cod that were exposed to CyHV-3 by IP inoculation (bath inoculation having been undertaken in the first non-target species trial).

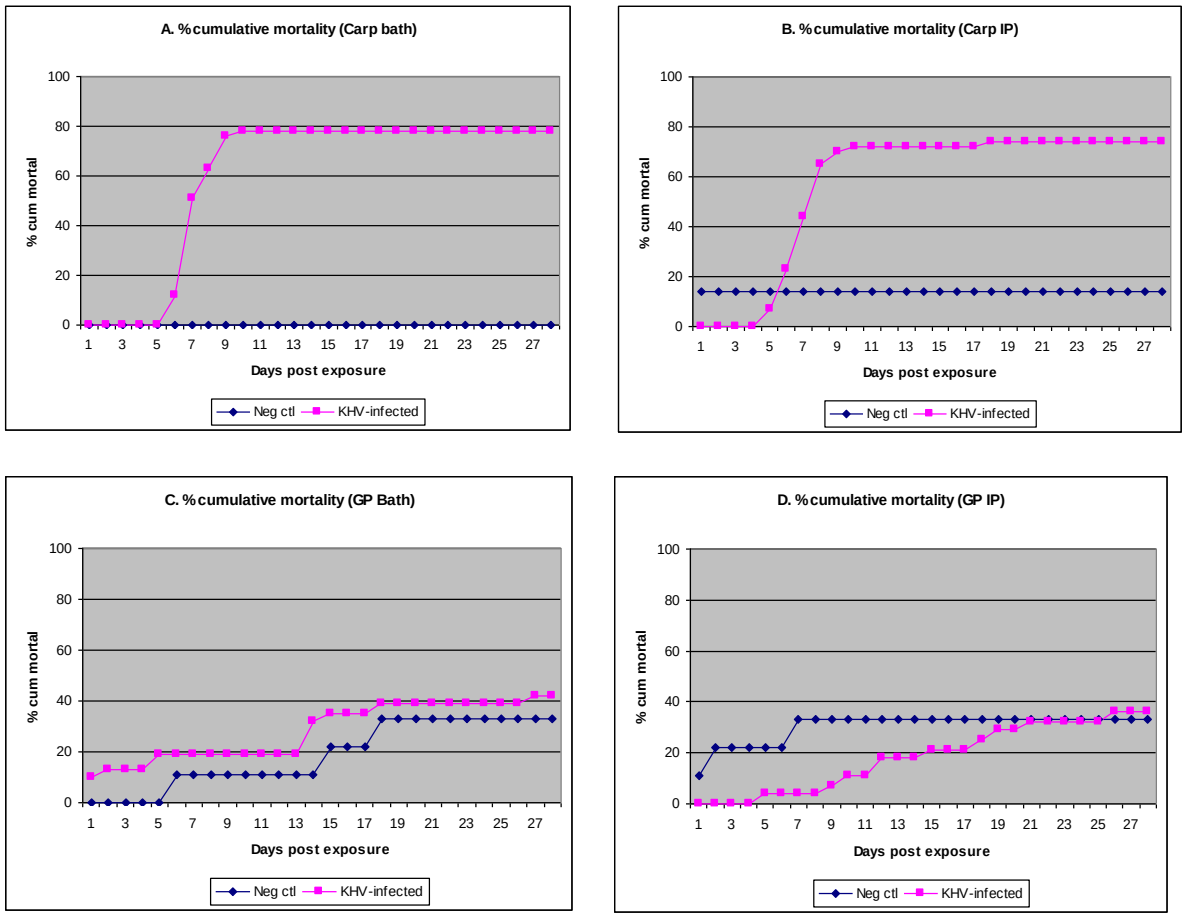
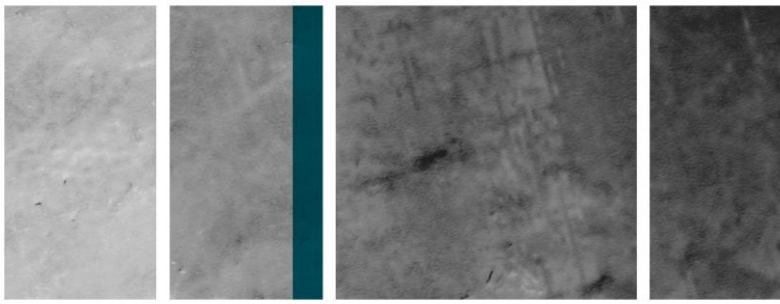


Figure 8: Cumulative mortality for carp (A, B) and golden perch (C, D) following challenge with CyHV-3 by bath (A, C), or IP inoculation (B, D).



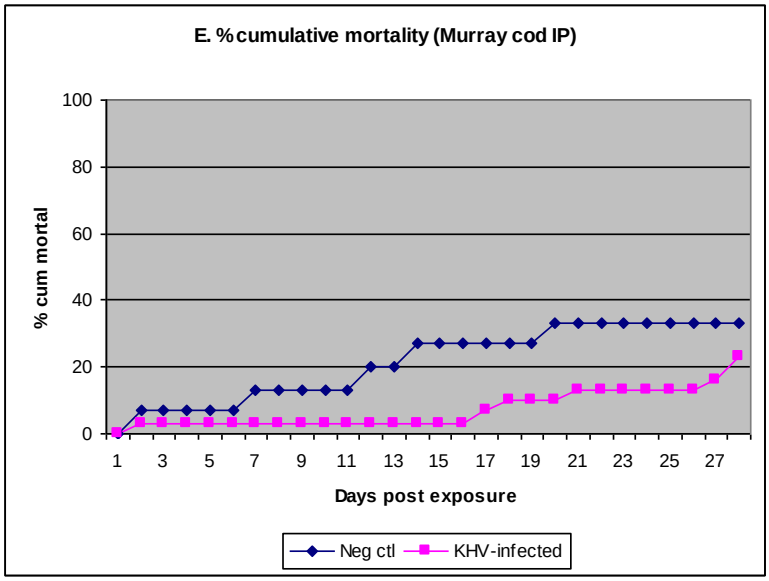


Figure 9: Cumulative mortality for Murray cod following challenge with CyHV-3 by IP inoculation.

As with the golden perch, mortality was moderately high, although worse in the negative controls than in the CyHV-3-infected fish.

While there was some evidence of an enteric metazoan parasite in some of the golden perch, there were no significant lesions that could be attributed to CyHV-3 in either the golden perch or the IP-inoculated Murray cod.

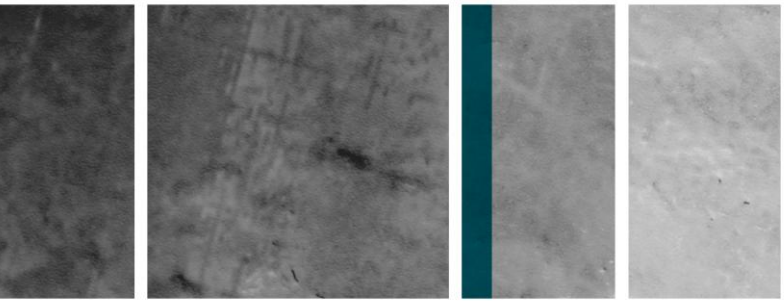
PCR revealed that no golden perch were PCR-positive (whether infected by bath or IP inoculation). Of the IP-inoculated Murray cod, only one fish was positive for the presence of viral DNA. Low levels of viral DNA were detected in the viscera (Ct: 34.9). The gills of this fish were PCR-negative, and it is likely that this weak PCR result was due to contamination from the work area. All carp that died, or were moribund (and were euthanized), were positive for CyHV-3 DNA by PCR in gills and kidney/spleen. Mean Ct values (+/- std deviation) for these fish were:

Bath - Gills: 25.1 +/- 1.8 Kidney/spleen: 25.3 +/- 2.1

IP - Gills: 23.8 +/- 2.4 Kidney/spleen: 21.6 +/- 1.6

For the galaxiid and salmonid susceptibility trials, it was found that, following titration of the CyHV-3 inoculum, IP-inoculated carp (each given a 100 uL dose) received $10^{4.1}$ $TCID_{50}$ of virus, while trout (50 uL dose) were challenged with $10^{3.4}$ $TCID_{50}$ (galaxiids were too small for IP inoculation). For bath-infected fish, both trout and galaxiids were immersed for 1.5 hrs in water containing CyHV-3 at a concentration of $10^{2.8}$ $TCID_{50}$/mL.

In Figure 10, the percent cumulative mortality for carp following challenge with CyHV-3 by IP inoculation is revealed. Although only two fish died (40%), one other also developed acute, red multifocal skin lesions that, over the course of 5-6 days, eventually resolved.



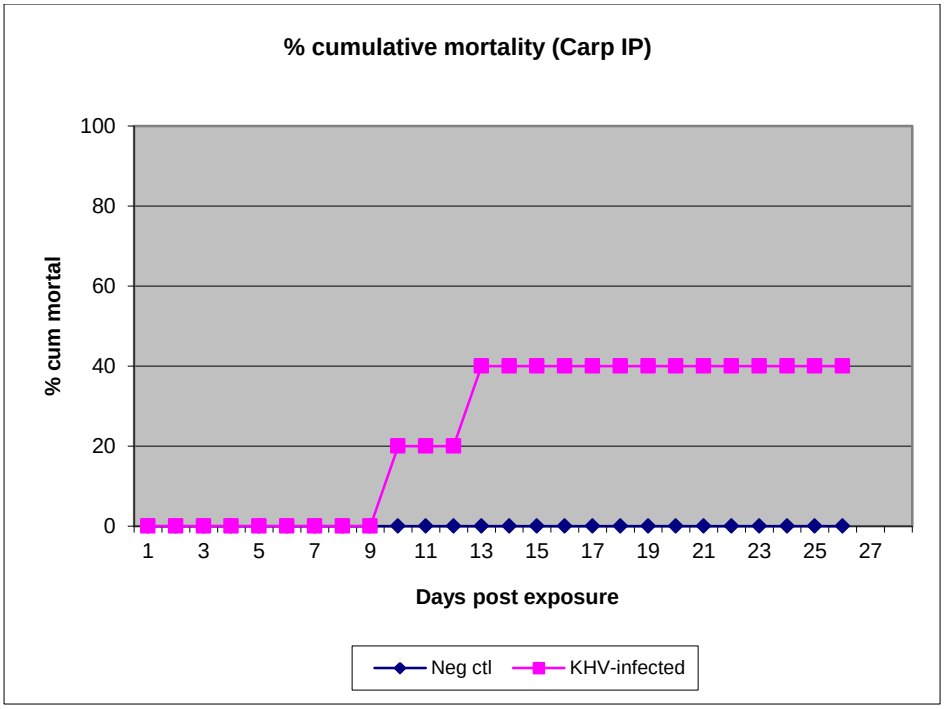


Figure 10: Percent cumulative mortality in positive control carp inoculated IP with CyHV-3

Both carp that were moribund (and were euthanized), were positive for CyHV-3 DNA by PCR. For the carp that was euthanized at 10 dpi, Ct values for gills, spleen and kidney were 16.5, 26.2 and 23.9, respectively. Values for the carp euthanized at 13 dpi were 21.9 and 23 for gills, and for kidney/spleen homogenate, respectively. Three apparently healthy carp were euthanized at the end of the experiment (26 dpi), and gills, and kidney/spleen homogenates, were positive by PCR in all three fish. Mean Ct values (+/- standard deviations) for these three fish were: gills: 30.3 (+/- 2.1); kidney/spleen homogenate: 35.0 (+/- 1.5). (Note that Ct values are measured on a scale of 0-40 where a Ct of 37, or greater, is considered negative, and the lower the Ct value, the more viral DNA is present).

Of three carp that were examined histopathologically, there was increased cellularity in the submucosa of the gill filaments of one fish, and apoptotic cells scattered throughout the mucosa of the gill raker. There were no significant lesions in other organs of this fish, nor in the other two fish.

The percent cumulative mortalities for rainbow trout exposed to CyHV-3 by IP-inoculation or bath infection are shown in Figure 11. Following IP inoculation, only 6 fish (27%) had died by 23 days post inoculation (dpi), three of these in the first 5 days of the experiment. There was a severe episode of mortality on the last two days of the experiment (24 and 25 dpi). By contrast, most mortalities in the bath-infected fish occurred by 4 dpi, with relatively few mortalities occurring thereafter.

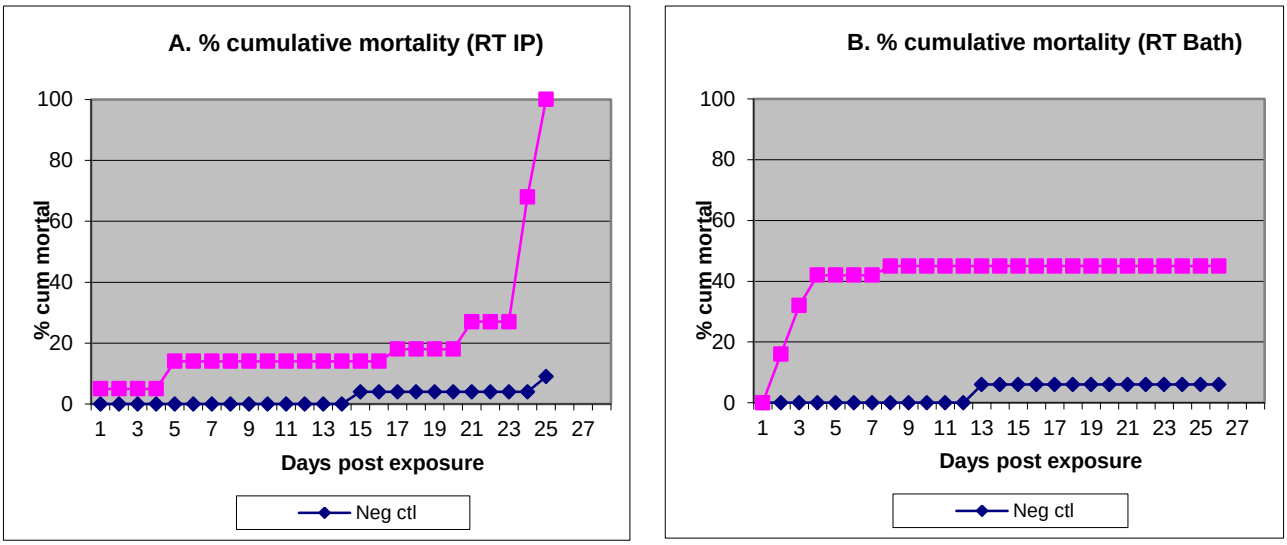


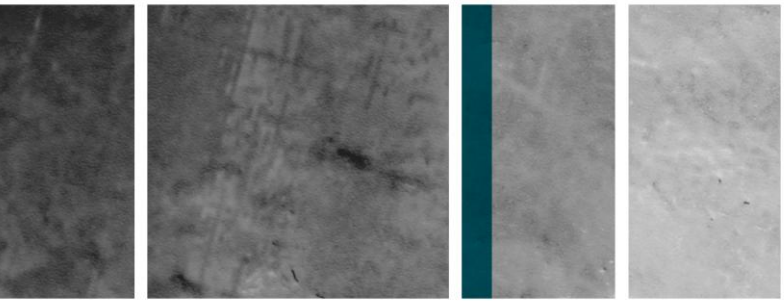
Figure 11: Percent cumulative mortalities in rainbow trout infected with CyHV-3. (A) IP inoculation; (B) bath infection

Examination of all dead and many moribund rainbow trout by PCR revealed that all of those that had been bath-infected were PCR-negative. Of those rainbow trout that had been IP-inoculated with CyHV-3, all, except one, were also PCR-negative. The one exception was a fish that was found dead at 1 dpi. The Ct value for a kidney/liver/spleen homogenate was 33.2, and is likely to have been due to residual inoculum in this fish.

Histopathological examination of rainbow trout revealed no significant differences between infected and uninfected trout (except for one CyHV-3 IP-inoculated fish with an acute peritonitis possibly due to the inoculation procedure). There were no significant lesions in one IP-inoculated moribund fish collected at 24 dpi (the majority of fish were found dead at 24 and 25 dpi, and therefore were unsuitable for histopathological examination).

In Figure 12, the percent cumulative mortalities for the galaxiids exposed to CyHV-3 by bath infection are shown. As seen in the mortality curves for the negative control fish, these were difficult fish to maintain in the tank-system that is available at AAHL. While the majority of mortality had occurred by 8-10 dpi, there was continued loss of fish throughout the entire experiment. For the bath-infected fish, most mortality occurred by 5 dpi, but, as with the negative controls, there were further sporadic deaths until the end of the experiment.

Examination of all dead and many moribund galaxiids by PCR revealed that all of those that had been bath-infected with CyHV-3 were PCR-negative.



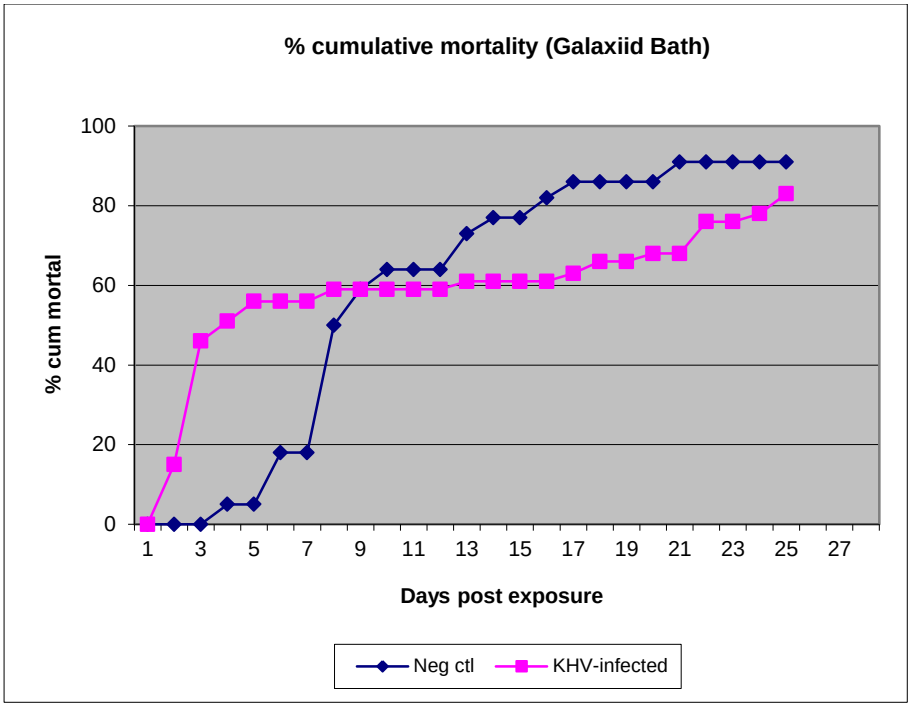


Figure 12: Percent cumulative mortalities in galaxiids bath-infected with CyHV-3

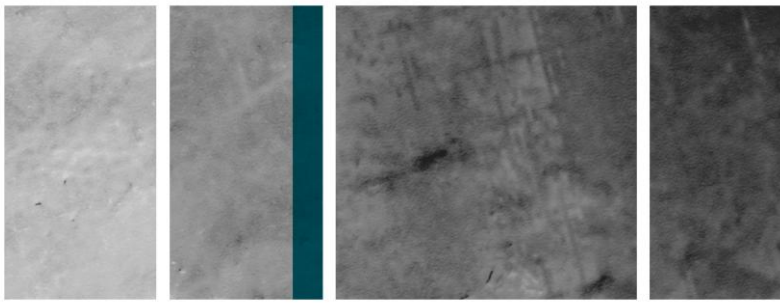
Histopathological examination of galaxiids revealed the presence of large acidophilic droplets in the cytoplasm of many hepatocytes in many negative control fish and bath-infected fish. The cause of this change is unknown (possibly dietary in origin). The only other significant change was seen in two fish exposed to CyHV-3, one sacrificed at 22 dpi and the other at 25 dpi. In the former, there was an acute branchitis characterized by oedema of gill filaments, foci of neutrophils, and fusion of many lamellae with formation of synechiae and subsequent cystic structures in the gill filaments, while the lesion in the latter was characterized by hyperplasia of the branchial epithelium and mucous secretory cells, fusion of lamella, and thickening of filaments.

6.4 Discussion

In summary, whereas CyHV-3 was clearly associated with the death of the experimental carp in all trials, there was little evidence that it affected the non-target species that were tested. In most of the latter, there was no evidence of viral DNA in dead (or surviving) fish, and, in those few fish where viral DNA was detected, it was present in such low levels that it encouraged the conclusion that it was simply residual inoculum (or contamination due to infected carp being processed in the same room).

Histopathological examination revealed lesions consistent with CyHV-3 infection in most, but not all, infected carp. By contrast, there were no lesions suggestive of a viral infection in any of the non-target species.

The inadvertent use of carp-goldfish hybrids in the first trial resulted in a lower than expected mortality in the positive control fish. More importantly, it raised the question of what is the proportion of carp-goldfish hybrids in the water systems of the Murray-Darling



Basin? If they form a substantial proportion of the carp population, then there could be a negative impact on any future use of CyHV-3 as a biological control agent. This issue is addressed in a later Section of this report.

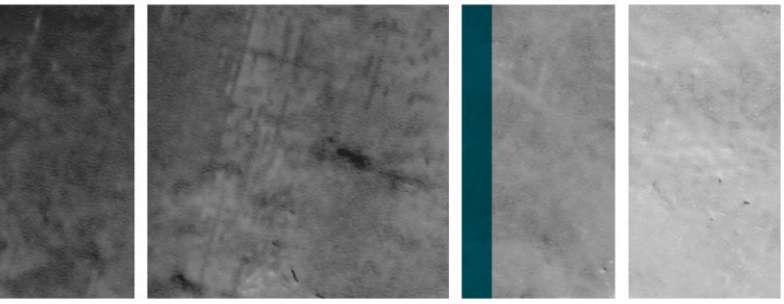
For the non-susceptibility trial involving galaxiids and rainbow trout, the abnormal clinical signs in three carp, plus the overall 40% mortality, are consistent with our previous findings in larger, more mature carp infected with CyHV-3 (where mortality is usually 30-40%). Furthermore, we rarely see histological lesions consistent with CyHV-3 infection except in 4-10 cm immature carp. PCR results in the carp were also consistent with infections in all 5 of the positive control carp (despite the fact that only two of the carp showed clinical signs of disease). Together, these findings confirm the virulence of the CyHV-3 inoculum used in this experiment.

For rainbow trout challenged with CyHV-3, mortality was either late (IP-inoculated fish) or early (bath-infected fish) (Figure 11). In both cases (with one exception), there was no PCR evidence for the presence of virus in either dead or live fish. The one exception was an IP-infected trout that was found dead at 1 dpi, far too early for the death to be attributed to CyHV-3. Most likely the positive PCR in this instance was due to residual inoculum administered to the fish the day before. Most importantly, there was no PCR evidence to link the marked mortality in the IP-inoculated fish at 24 and 25 dpi with CyHV-3.

Galaxiids proved difficult to maintain in the tank system used at AAHL as demonstrated by the constant mortalities in the negative control fish (Figure 12). Most mortalities in the CyHV-3 bath-infected galaxiids occurred by 5 dpi, too early to be attributed to CyHV-3. This conclusion was corroborated by PCR results which showed no evidence for the presence of viral DNA in any of the CyHV-3 bath-infected fish. Histological lesions were observed in the gills of two galaxiids that were euthanized at 22 and 25 dpi. These were non-specific lesions that could have been caused by a variety of pathogens. Although these two fish were not examined by PCR, it can be anticipated that, if the lesions were due to CyHV-3, then these two fish would have been excreting virus for many days (in one of the fish, the lesion was chronic-active, implying that it had been present for many days). In that case, it would be expected that at least some of the other fish collected at the same time would have been PCR-positive for CyHV-3. This was not the case; all fish examined from 22 to 25 dpi were PCR-negative.

To summarize the results from this section, whereas CyHV-3 was clearly associated with the death of the experimental carp, the combined clinical, histopathological and PCR evidence suggested that the two non-target species were not affected by CyHV-3. The one exception was a PCR-positive IP-inoculated rainbow trout that died at 1 dpi, too early for the death to be attributed to CyHV-3. It is likely that, in this case, the viral DNA that was detected was simply residual inoculum.

The range of non-target species that has now been tested includes five species of fish, four being native species and one an introduced species. Furthermore, these five species encompass three taxonomic Orders of teleost fish, the Perciformes, Osmeriformes and the Salmoniformes.



7. Results 5: Determine the presence of cross-reactive cyprinid herpesviruses in carp in the Murray-Darling Basin

7.1 Introduction

There are three known cyprinid herpesviruses – CyHV-1 (also known as carp pox), CyHV-2 (goldfish haematopoietic necrosis virus), and CyHV-3 (commonly known as KHV). Although CyHV-1 and -2 have not been isolated in Australia, CyHV-2 has been described in Australian goldfish (Stephens *et al*, 2004), and CyHV-1, although not reported formally, is generally recognised as being present (based on the presence of proliferative epidermal lesions that are occasionally seen in adult Australian carp).

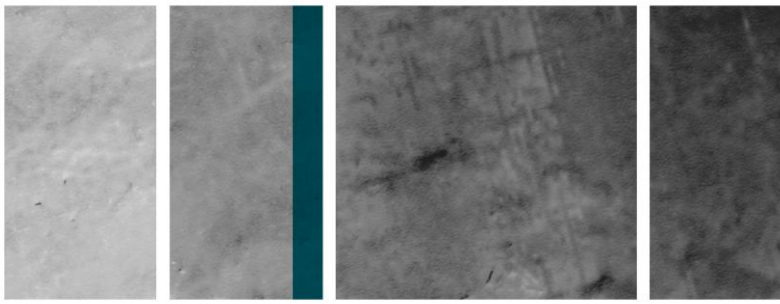
While carp appear to be refractory to infection with CyHV-2 (Hedrick *et al*, 2006), they are certainly susceptible to infection by CyHV-1 (Sano *et al*, 1985, 1991). It may cause an acute disease, where virus has been demonstrated by immunofluorescence studies in gills, liver, kidney, intestinal mucosa, oesophagus and LP, or, in older fish that survive acute infection, the virus may cause proliferative epidermal lesions (colloquially known as 'carp pox'). In the latter case, virus appears to be restricted to gills, cranial nerve ganglia, subcutaneous tissue and spinal nerves (reviewed in Fijan, 1999).

CyHV-1 is known to share common antigens with CyHV-3 (Adkison *et al*, 2005), and therefore it is possible that a prior, or concurrent, infection of carp with this virus might confer protection against CyHV-3. Past experience with rabbit haemorrhagic disease (RHD) virus as a biological control agent for rabbits in Australia demonstrated that undescribed, unexpected viruses may also be present in a target population, and that these may confer resistance on the host (Strive *et al*, 2009). For this reason, it was considered necessary to survey carp in the M-DB for cyprinid herpesviruses, known and unknown, that might be cross-reactive with CyHV-3. A nested generic cyprinid herpesvirus polymerase chain reaction (PCR) assay was designed for this purpose.

7.2 Methods

Carp were collected from throughout the M-DB by Dr Dean Gilligan and his team (NSW I&I). Following epidemiological advice from Dr Iain East (Department of Agriculture, Forestry and Fisheries), one hundred or more carp were collected from each of 8 locations, and 49 were collected from the remaining location. Locations, with specific sites, are shown in Table 8.

All fish were frozen, shipped to AAHL, and stored at -20 ^{o}C until processing. For most fish, after thawing, samples of kidney, liver and gills (approximately 10 mm^3 of each) were removed from each fish, pooled in a 5 mL tube, and homogenised. Where very small fish precluded the identification of individual organs, the head and tail of the fish were removed, and the remainder was homogenised. Some samples were very autolyzed, in which case only a gill sample was homogenised. Approximately 1 mL of each homogenate was added to a mixture of proteinase K and ATL buffer, and the mixture was incubated overnight at 56 ^{o}C, according to the manufacturer's directions (QIAamp DNA Mini kit). Total nucleic acid was extracted from the digested homogenate (MagMAX-96 Viral RNA isolation kit, Applied Biosystems), and the concentration of DNA determined (Nanodrop, Analytical Technologies) in 25 randomly-selected samples. A PCR for the gene of a mitochondrial enzyme, cytochrome c



oxidase subunit 1 (cox 1), was used to demonstrate the quality of the extracted DNA in 12 different samples.

All 849 carp DNA samples were tested for the presence of cyprinid herpesvirus-1, -2 or -3 using a nested, generic cyprinid herpesvirus PCR, developed at AAHL, that targeted a conserved region of the DNA polymerase gene of each virus. The primary PCR was run for 40 cycles, and the nested for 30 cycles, each with an annealing temperature of 55 ^{o}C.

Table 8: Sampling locations (with the specific sites) for the collection of carp from throughout the Murray-Darling Basin in NSW

Accession no.	Location	Site(s)	Number of fish tested from site	Mean size (mm) of collected fish[a]
12-0489	Darling River waterway	Menindee	100	145.2 (29.5)
12-0488	Darling River waterway	Bourke U/S	100	56 (19.7)
11-4662	Great Cumbung Swamp (Lower Lachlan wetlands)	Ulonga	34	83.4 (35.3)
		Geramy OP 1/2	16	ND
		Geramy OP 3	50	82.2 (19.9)
11-4664	Namoi wetlands		100	81.7 (16.7)
11-4667	Macquarie Marshes	Brewon	49	106.8 (40.5)
11-4670	Gwydir wetlands	Gingham watercourse	100	106.5 (22)
11-4672	Barmah Millewa Forest	Moira Lake	100	108.3 (30.6)
11-4674	Lake Cargelligo		100	74 (12)
12-0490	Murrumbidgee River	Murrell's Crossing	100	ND

[a] Value in parentheses is standard deviation

7.3 Results

The mean concentration of DNA (+/- std dev) in the 25 samples that were examined was 175.6 (+/-125.4) ng/uL with results ranging from 9.7 to 540.4 ng/uL. In 10 fish that were grossly autolyzed, the mean concentration was 124.5 (+/- 99.4) ng/uL. The cox-1 PCR product for 12 different fish was strong and specific (data not shown).

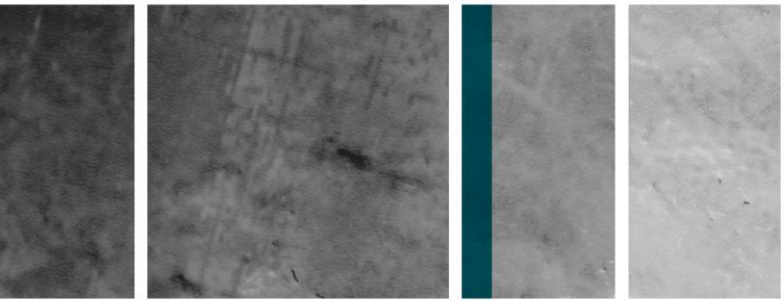


Figure 13: Specificity of a nested generic cyprinid herpesvirus PCR. Top row: primary PCR - product: 465 bp. Bottom row: nested PCR - product: 360 bp. Lane 1: CyHV-1; Lane 2: CyHV-2; Lane 3: CyHV-3.

Figure 13 illustrates the specificity of this generic cyprinid herpesvirus PCR, showing that it can detect all three known cyprinid herpesviruses. The double bands in the nested PCR (lanes 1 and 3) are likely to be due to carryover of primary PCR product.

In Figure 14, results from 24 of the 849 carp DNA samples tested with the nested PCR are illustrated. The annealing temperature for both the primary and nested PCRs was 55 ^{o}C, and, as seen in the Figure, this resulted in many non-specific products for some fish samples. This strategy (ie, low stringency annealing temperatures for primers) was chosen purposely in order to allow the best chance of detecting cyprinid herpesviruses that might only be partially related to CyHV-1, -2 or -3. Eleven PCR products, that approximated the expected 360 bp product, were purified from gels, and sequenced. Subsequent sequences were then subjected

to a BLAST search. None of the eleven sequences matched any known cyprinid herpesvirus sequence. The closest matches were with carp or zebrafish molecules, or with various species of bacteria belonging to the *Pseudomonas* genus.

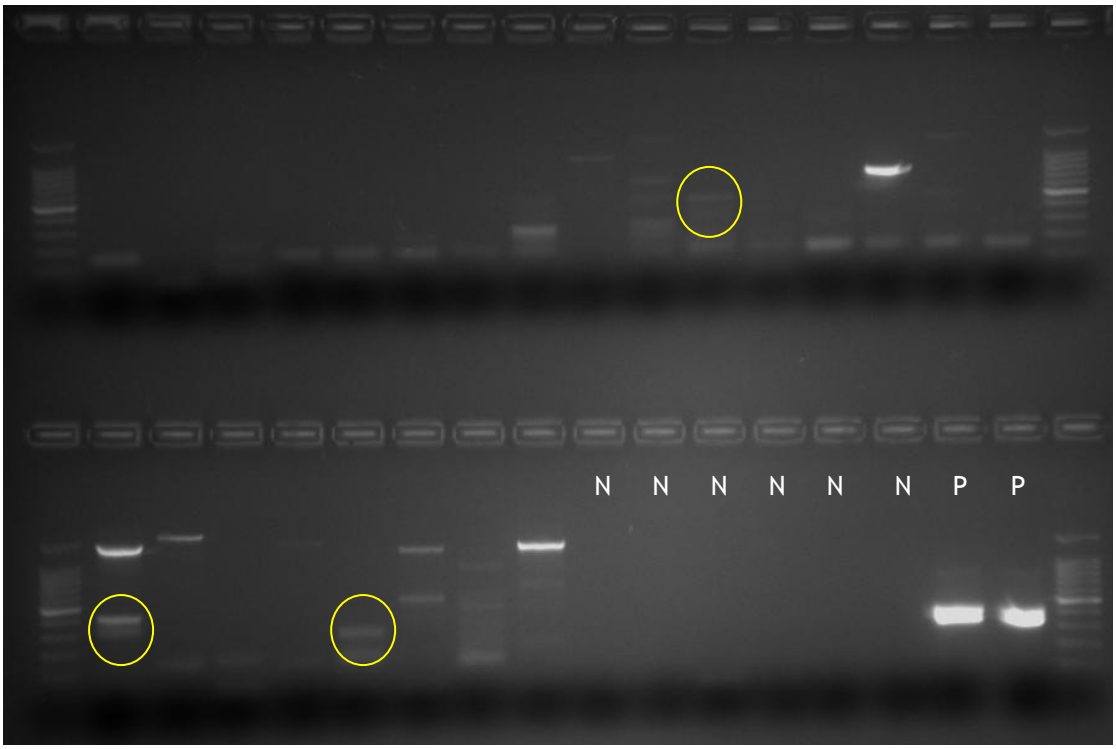
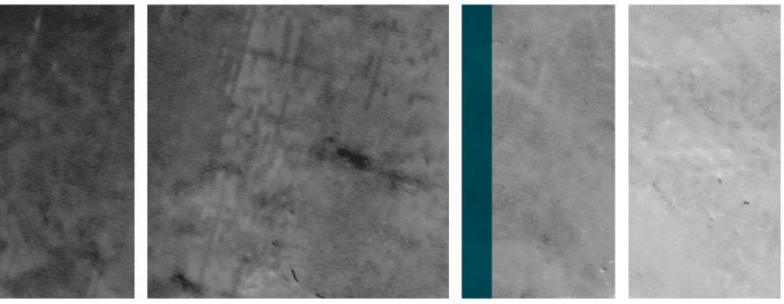


Figure 14: Results of the generic cyprinid herpesvirus PCR on 24 samples from Lake Cargelligo (11-4674). N: no template controls; P: positive controls (CyHV-3 DNA)

7.4 Discussion and conclusion

Of the known viruses in Australia, CyHV-1 would be the most likely to be potentially cross-reactive with CyHV-3 in carp. However, past experience with RHD virus demonstrated that it was important to extend the survey to allow the detection of potentially undescribed cyprinid herpesviruses. Because of the large number of samples that had to be tested, it was decided to use a generic cyprinid herpesvirus PCR, purposely designed for this study. There was perfect alignment of the primary and nested primers with sites in the DNA polymerase gene of CyHV-1, -2 and -3. Nevertheless, a low annealing temperature was used for both stages of the PCR in order to enhance the chances of detection of any other closely-related viruses.

Many PCR products were detected during examination of the 849 samples. However, although many of these products appeared to be about 360 bp (the size of a specific product), when purified and sequenced, none were shown to be most closely aligned to the DNA polymerase sequence of a cyprinid herpesvirus. Therefore, it was concluded that known, or previously undescribed, cyprinid herpesviruses were either absent from the carp populations that were surveyed, or their prevalence fell below the level of detection of this survey.



8. Results 6: A preliminary estimate of the prevalence of carp-goldfish hybrids from inland waters of Victoria

8.1 Introduction

Mortality in one batch of 4-6 cm carp that were used in the non-target species testing (see 6. Results) was less than expected. This raised the question of whether many (or all) of those fish may, in fact, have been carp (C)-goldfish (GF) hybrids. A preliminary examination of the mitochondrial cytochrome c oxidase subunit 1 (cox 1) gene sequence from a sample of these fish did, in fact, suggest that 2/2 fish that died following IP inoculation with CyHV-3 were *Cyprinus carpio* whereas 7/7 that survived were hybrids.

Given that carp-goldfish hybrids are recognized as being less susceptible to CyHV-3 (Bergmann *et al*, 2010a, b; Hedrick *et al*, 2006), it is clearly important to have some idea of the prevalence of hybrid carp in Australian inland waters. This small survey was established, firstly, to establish a method that would allow rapid differentiation of pure carp from hybrids, and, secondly, to obtain a preliminary estimate of the prevalence of carp-goldfish hybrids in a small sample of fish from a Victorian river.

The development of a Luminex-based system to differentiate carp from goldfish is reported here since the barcoding system was introduced in order to address the issue of the prevalence of hybrids.

8.2 Methods

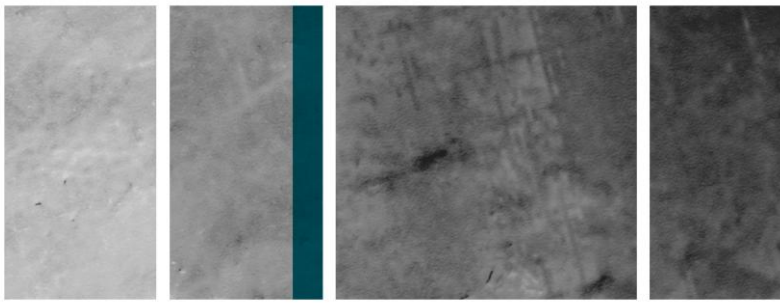
Dr Paul Brown (DPI Victoria) supplied 99 ethanol-fixed fin-clips collected from fish that phenotypically resembled carp. The origin of the fin-clips is shown in Table 9.

Total nucleic acid was extracted from the fin-clip samples (each about 10-20 mm^2) by digestion in ATL buffer (Qiagen) and Proteinase K at 56 ^oC overnight, followed by robotic extraction using a MagMAX-96 Viral RNA isolation kit (Applied Biosystems). In addition, total nucleic acid samples, previously extracted from the kidney of a carp that survived IP inoculation with CyHV-3 (# 116, a putative hybrid) and a carp that died following IP challenge (#168, a putative pure carp), were prepared as controls. A fin-clip from a goldfish (purchased from a local pet shop), also processed as for the carp samples, was used as a positive control.

Table 9: Sampling locations (with the specific sites) for the collection of fin-clips.

Waterway	Location	No. of fish	Mean size (mm) of collected fish[a]
Goulburn River	Lake Nagambie	27	467.8 (139.5)
Ovens River	Wangaratta	21	371.7 (195.5)
Goulburn River	Mooroopna	11	232.7 (128.4)
Ovens River	Unspecified	30	ND
Murray River	Upstream of Corowa	10	ND

[a] Value in parentheses is standard deviation ND: Measurements not recorded



Allele-specific primers (ASPs) for carp and for goldfish were designed. These were based on available sequences (Hardy *et al*, 2011) for the 18S (nuclear) and 12S (mitochondrial) small subunit ribosomal RNA genes for each of the two species of fish. Assays that differentiated between carp and goldfish 18S, and carp and goldfish 12S, were based on a Luminex platform, similar to that described for another application (Gleason and Burton, 2012).

In addition, two previously-described PCRs were also used (Yamaha *et al*, 2003). Each PCR amplifies repetitive DNA sequences specific for either carp or goldfish. The primers and conditions for the PCRs were exactly as described by Yamaha *et al* (2003). A number of the specific PCR products generated by these PCRs were extracted from gels (QIAquick Gel Extraction kit, # 28704), and sequenced using the manufacturer's protocol on a Prism 3130xl Genetic Analyzer (Applied Biosystems) using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).

8.3 Results

The results of tests on all samples are shown in Table 10.

Table 10: Results of the Luminex® assays (based on allele-specific primers for carp and goldfish 18S and 12S genes), and of the carp- and goldfish-specific PCRs.

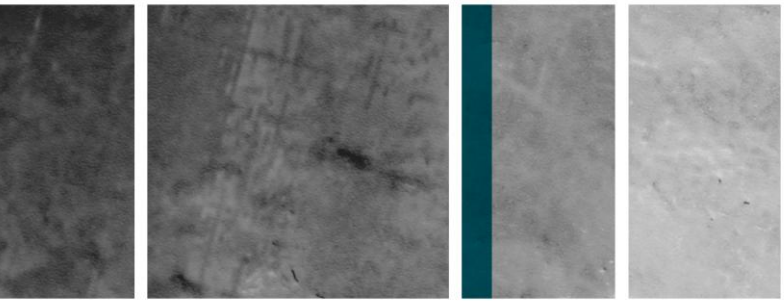
DNA samples	Luminex® assays with ASPs[a]				PCR assays[b]	
	Carp 18S (nuclear)	GF 18S (nuclear)	Carp 12S (mitoch)	GF 12S (mitoch)	Carp PCR	GF PCR
1-8[c]	**P**[g]	N[h]	**P**	N	**P**	N
9[c]	**P**	N	**P**	N	**P**	**P**
10-99[c]	**P**	N	**P**	N	**P**	N
116[d]	N	**P**	N	**P**	**P**	**P**
168[e]	**P**	N	**P**	N	**P**	N
GF fin-clip[f]	N	**P**	N	**P**	N	**P**

[a]: Allele-specific primers were designed for carp and goldfish (GF) nuclear (18S) and mitochondrial (12S) genes; [b]: Conventional carp- or goldfish-specific PCRs (Yamaha *et al*, 2003); [c]: DNA from the 99 fin-clips that were examined; [d]: DNA from a carp that survived IP inoculation with CyHV-3; [e]: DNA from a carp that died following IP challenge with CyHV-3; [f]: DNA from a fin-clip from a goldfish; [g]: Positive; [h]: Negative.

8.4 Discussion

Given that carp-goldfish hybrids are recognized as being less susceptible to CyHV-3 (Bergmann *et al*, 2010a, b; Hedrick *et al*, 2006), it is clearly important to have some idea of the prevalence of hybrid carp in Australian inland waters. This may be an important factor in determining the efficacy of CyHV-3 as a future biological control agent in Australia. What is the prevalence of these hybrids in Australian waters, and how are hybrids detected?

Initially, we used sequencing of the mitochondrial cytochrome c oxidase subunit 1 gene (cox 1; Ward *et al*, 2009; Ward *et al*, 2005) to detect hybrids. However, because mitochondria are only inherited in the egg, this method only determines the species of the mother. By contrast, Hardy *et al* (2011) proposed the use of nuclear (18S) and mitochondrial (12S)

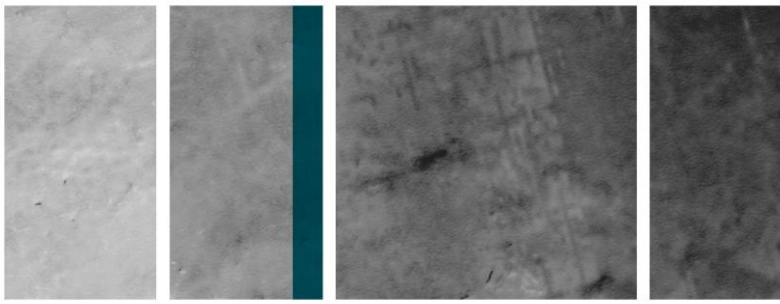


sequences to distinguish all freshwater fish species known to inhabit the M-DB. This method would also allow identification of hybrids. Using information developed by Hardy *et al* (2011), we developed a Luminex-based assay that relied on ASPs to differentiate carp, goldfish and hybrids. Theoretically, this method would allow the identification of hybrids without the necessity of sequencing 18S and 12S PCR products, a costly process for any survey involving large numbers of fish. We also used two PCRs, one specific for carp and the other for GF (Yamaha *et al*, 2003). These PCRs are based on highly repetitive sequences of DNA that are unique to each species. So, if both PCRs were positive for a particular fish (using fin-clips), the fish would be considered a hybrid.

The results in Table 10 demonstrate that, while the Luminex assay may be useful for identifying the species of individual fish in a large survey, the PCRs developed by Yamaha *et al* (2003) are more likely to allow the detection of hybrids. Luminex-based assays that rely on ASPs are already being used in the marine sciences to speciate fish eggs that cannot be easily identified morphologically (Gleason and Burton, 2012). Our work suggests that, if species-specific ASPs were designed, it could also be used to identify fish species without the need for sequencing of specific nuclear or mitochondrial PCR products. However, as seen with samples # 9 and # 116 (Table 10), the ASP approach failed to identify two fish that would be classified as hybrids based on the PCRs of Yamaha *et al* (2003). Based on the results for the 12S gene, fish # 9 had a carp mother, while # 116 had a goldfish mother. In each case, the results for the 18S gene were the same as those for the 12S gene, whereas, given that the specific PCR assays indicated that both these fish were hybrids, the 18S for # 9 and # 116 would be expected to have indicated a goldfish and carp origin, respectively.

One reason for the apparent failure of the ASP approach might be explained by gene conversion where a gene from one parent begins to change to the gene from the other parent, even before the next generation. This is less likely to have phenotypic effects in the case of a multi-copy gene (especially if the copies are in different loci), but it could make interpretation of the results of the ASP approach difficult.

In terms of the specific question that prompted this small survey of carp in Victorian rivers, it appears that one fish out of the 99 surveyed (1%) was a hybrid. This is very similar to the result obtained by Haynes *et al* (2012) who conducted a much more extensive survey of 1155 carp in the M-DB. Although the authors do not state explicitly the proportion of hybrids, it appears that approximately 3% of surveyed fish were hybrids. Furthermore, they also found that the majority of hybrids had a goldfish on the maternal side, whereas for sample # 9, the 12S result suggests that this fish had a carp on the maternal side. Regardless, the most important consequence of these two surveys is the suggestion that hybrids comprise a relatively small proportion of the carp population in most waterways of south-eastern Australia. It has been suggested that that proportion may be higher in special instances such as transient water courses (Haynes *et al*, 2012), and this may need to be taken into consideration in any future biological control program. The possibility of selecting a variant of CyHV-3 that is more virulent in hybrids should not be overlooked.



9. Results 7: Excretion of CyHV-3 from clinically affected carp

9.1 Introduction

In developing a biological control program for carp based on the use of CyHV-3, it is important to have a sound understanding of the epidemiology of the disease, and its effect on carp in Australia. Apart from understanding the infectivity, age-susceptibility and mortality in carp, the temporal pattern of virus excretion from infected fish is also important to understand. While Yuasa *et al* (2008) undertook some studies on virus excretion in infected carp, our aim was to establish an experimental system that would allow further study of CyHV-3 excretion in Australian juvenile carp).

9.2 Methods

A trial was conducted on the sensitivity of juvenile carp to CyHV-3 (see earlier Section). Fish were exposed by bath infection for 2 hrs at 21 ^{o}C to 5 different concentrations of CyHV-3: 0.008, 0.08, 0.8, 8.0, 80 and 630 $TCID_{50}$/mL. As controls, fish were also directly inoculated IP with 10-20 $TCID_{50}$ per fish. For each treatment, following exposure to virus, 10 fish were held individually in 1 L tanks, and a further 10 were held together in a single 1 L tank. Water temperature was maintained at 21 ^{o}C, and all fish were checked daily. When mortalities occurred, fish were examined by a CyHV-3-specific PCR or by histopathology.

One mL water samples were also collected daily from each tank, and these were stored at -20 ^{o}C until ready for processing. Following extraction of total nucleic acid from each sample, they were then examined by the CyHV-3-specific PCR for the presence of virus.

9.3 Results

The results in Figures 15 (ten fish per tank) and 16 (one fish per tank) demonstrate the temporal pattern of excretion of CyHV-3 from infected fish. Note that the amount of virus excreted is measured only in relative terms, low Ct values representing more virus than high Ct values.

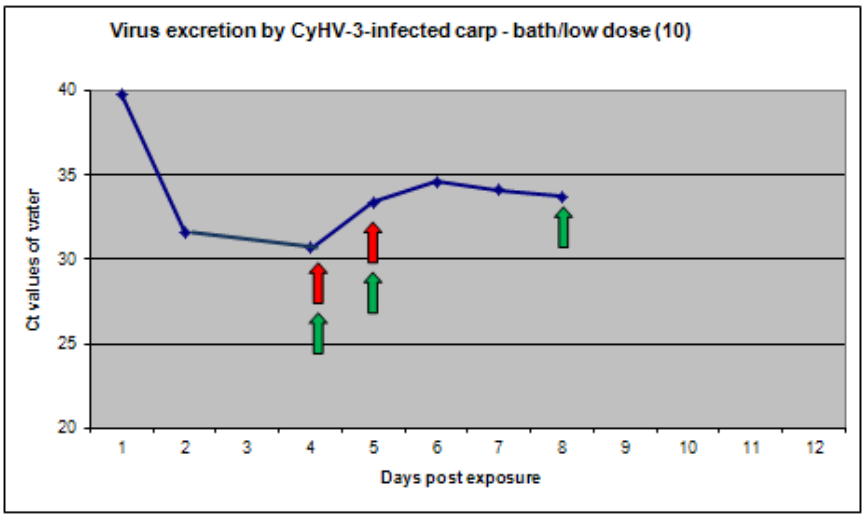


Figure 15: The temporal pattern of excretion of CyHV-3 from a group of fish. Ten juvenile carp were held in a 1L tank, and water samples were collected daily following infection of the fish. Red arrows indicate when dead fish were found in the tank; green arrows indicate the presence of moribund fish.

These data demonstrate that not only can excreted

virus be detected in the water when 10 infected fish share a 1 L tank (Figure 15), but also that virus can be detected in a tank of the same size when only one fish is present (Figure 16). Furthermore, it is apparent from Figures 15 and 16 that CyHV-3 is excreted from infected fish at least 2 days before a fish dies or becomes moribund.

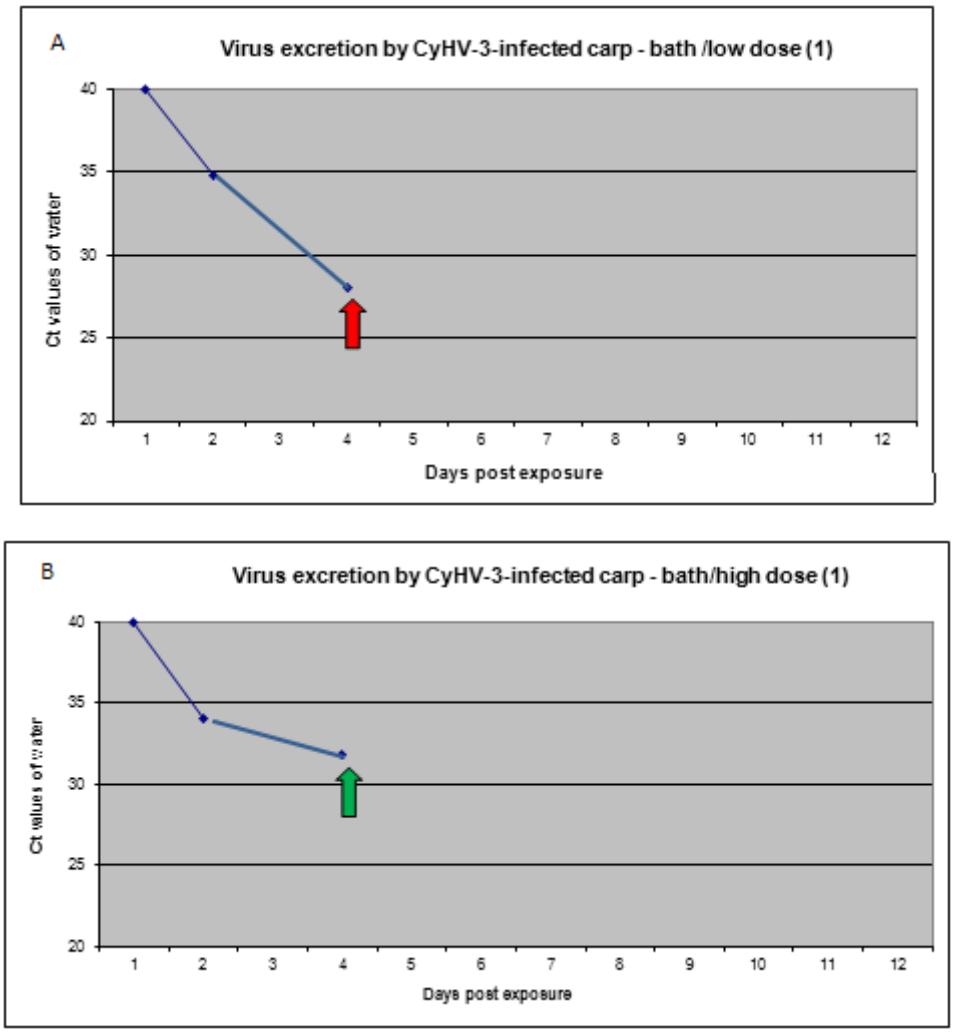
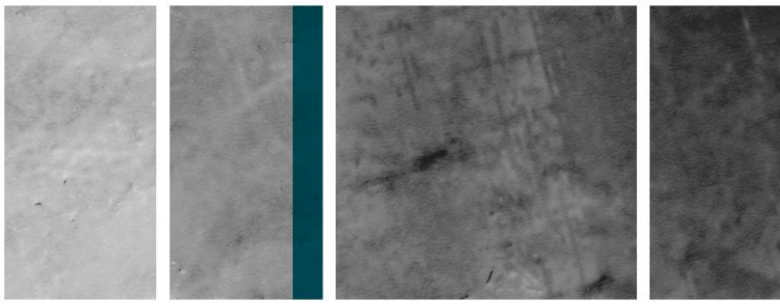


Figure 16: The temporal pattern of excretion of CyHV-3 in individual fish. One juvenile carp was held in a 1 L tank, and water samples were collected daily following infection of the fish. Red arrow (A) indicates when fish was found dead in the tank; green arrow (B) indicates the presence of a moribund fish.

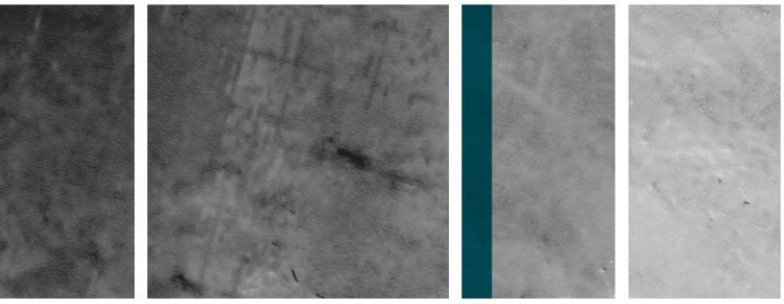
9.4 Discussion

This study demonstrated that an excellent model has now been developed to study viral excretion from juvenile CyHV-3-infected fish. The model allows virus excretion to be detected from a single fish. Although absolute quantification of excreted virus was not determined in this work, the use of quantitative PCR would allow this to be achieved. In addition, it would be possible to add 3 or 4 sentinel (fin-clipped) juvenile fish to a tank containing a single infected fish in order to demonstrate that the excreted virus was, indeed, virulent.



It is evident from the results that CyHV-3 is excreted for at least 2 days prior to clinical signs of disease, or death, occurring in an infected fish. This result is consistent with the findings of Yuasa *et al* (2008) who examined shedding of virus from infected common carp that were held at 16 º, 23 º or 28 ºC. Excreted virus was detected by co-habiting koi carp for 24 hr periods with the infected common carp. They demonstrated that virus was shed for extended periods (particularly at 16 ºC), and that virus excretion from infected common carp began before mortality began (eg, at 16 ºC, excretion began at 7 dpe, but mortality did not occur until 21 dpe). Estimates of the absolute amounts of virus excreted by infected fish would be very useful for developing epidemiological models of KHV in carp populations.

Finally, as noted in an earlier Section, it is likely that the fish used in this study were, in fact, hybrid carp. While it is useful to know that hybrids are capable of excreting virus, it would be more useful to have the equivalent data for known pure carp (in addition to absolute values for the amount of virus that is excreted).



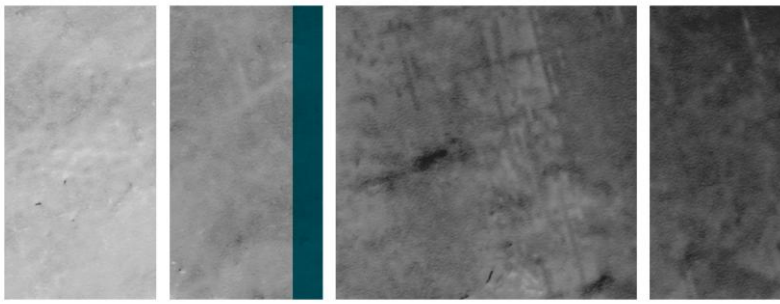
10. Overall conclusions

This Report represents the findings from Phases 1 and 2 of the Invasive Animals CRC-funded project "Koi herpesvirus: its potential as a biological control agent for carp in Australia.

The work was beset with unforeseen difficulties from the outset, the two most important being, firstly, the extended drought in south-eastern Australia that lasted for almost the duration of Phases 1 and 2. The main consequence of this was that for much of the time it was impossible to obtain the young susceptible carp that were essential for the work to progress. On occasion, it was thought that young carp had been found, but, on at least two of these occasions, these carp (which had been found in billabongs or irrigation channels) were subsequently shown to be carp-goldfish hybrids. There was a positive aspect to this finding, however, as mentioned later. The second major difficulty to impact on the project was the onset of a severe illness that affected one of the two Principal Co-investigators. This removed him from full-time work for 7 months, and then restricted him to part-time work for another 6 months. Unfortunately, this all happened at a time when young carp had once again become available! Nevertheless, despite these difficulties, all major Milestones/Objectives of Phases 1 and 2 have been met.

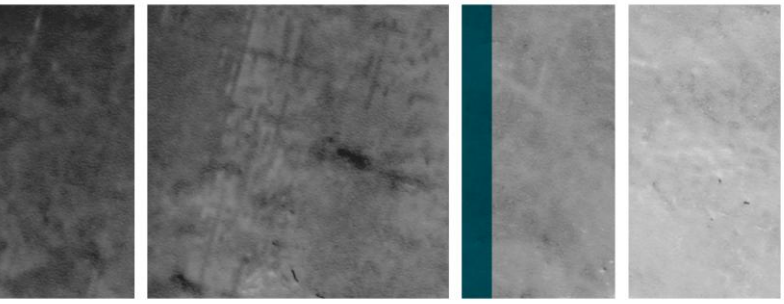
In summary, the following have been achieved:

- CyHV-3 reference strains and the KF-1 and CCB cell lines were imported into AAHL from CEFAS Weymouth Laboratory, UK and University of California, Davis, USA. An Indonesian strain of CyHV-3 was also introduced to the lab, and this was chosen for all subsequent experimental work.
- Assays for the detection of CyHV-3 infections of carp were introduced to AAHL. These included: (i) virus isolation and growth of CyHV-3 in carp cell lines; (ii) several PCRs used by international aquatic animal disease diagnostic laboratories; (iii) development of a generic cyprinid herpesvirus PCR with the potential to differentiate CyHV-1 (carp pox virus), CyHV-2 (goldfish haematopoietic necrosis virus) and CyHV-3 (KHV); and, (iv) immunoassays for localisation of CyHV-3 in infected fish tissues and in cell cultures.
- *In vivo* infectivity trials were completed, and demonstrated that 4-10 cm carp in Australia are not only susceptible to infection by CyHV-3, but that they develop disease and die in a matter of days following infection. Other studies revealed a much lower mortality in older fish.
- A number of non-target species (Murray cod, golden perch, silver perch, a galaxiid and rainbow trout) were also tested in susceptibility trials. There was no evidence of virus replication nor of histological lesions in the non-target species.
- An extensive survey of carp collected from eight sites across the Murray-Darling Basin (M-DB) showed no evidence for any cross-reactive cyprinid herpesviruses that might compromise the efficacy of CyHV-3 were it to be released into the M-DB.
- A new bar-coding system, based on Luminex technology, was introduced to allow differentiation of carp from goldfish and hybrids. Results were compared with a previously published PCR test. An examination of a small sample of carp (99) collected from Victorian waters appeared to detect one hybrid.



- The sensitivity of juvenile carp to infection with different doses of CyHV-3 was determined, although this work may have been compromised by the lack of availability of immature carp at the time.
- An experimental system was devised that enabled detection of excreted virus from CyHV-3-infected immature carp. By this means, a preliminary temporal pattern of excretion of virus was determined.
- An extensive written review of CyHV-3, and the disease caused by the virus, was prepared for use in any future attempts to gain approval for release of CyHV-3 into Australian waterways

Together, these findings provide the basis for developing an epidemiological modeling system for CyHV-3 in Australian waterways, and for opening broad-scale discussions on the possible use of CyHV-3 as a biological control agent for carp in Australia. Of course, further information is still required, particularly on the susceptibility of a wider range of non-target species.



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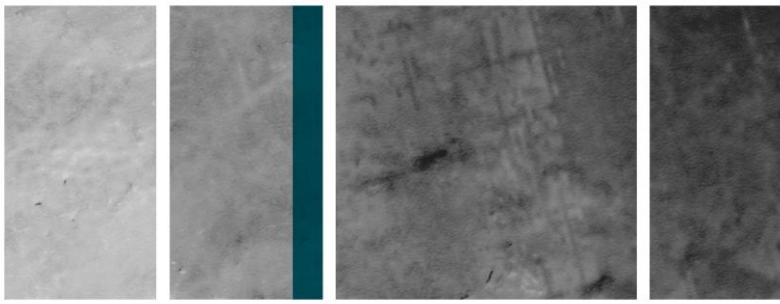

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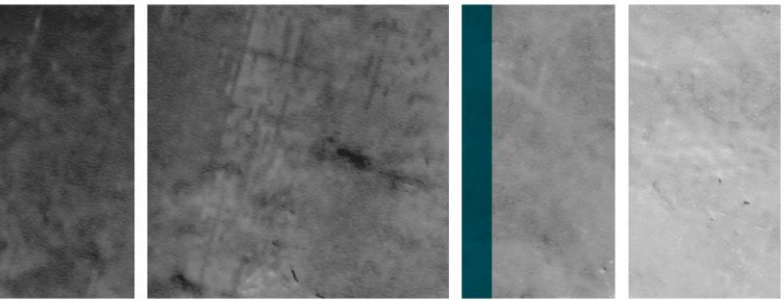


Appendix 1. Maintenance and use of Koi fin (KF-1) cell line (Koi, *Cyprinus carpio koi*)

General Information

KF-1 cell line (Hedrick *et al.*, 2000) was supplied by Prof. Ron Hedrick, University of California Davis, USA.

Incubation temperature at AAHL: 25^{o}C for growth and maintenance of stock cell cultures; 20^{o}C for maintenance of inoculated cell cultures during incubation of samples for the growth of cyprinid herpesvirus 3 (CyHV-3).

Materials

1. Growth Medium:

   Leibovitz L-15 medium

   2 mM Glutamine

   100 IU penicillin/100 μg streptomycin (Pen/Strep)/mL

   10% (v/v) foetal bovine serum (FBS)

2. 10X Trypsin-Versene (T/V; Invitrogen)

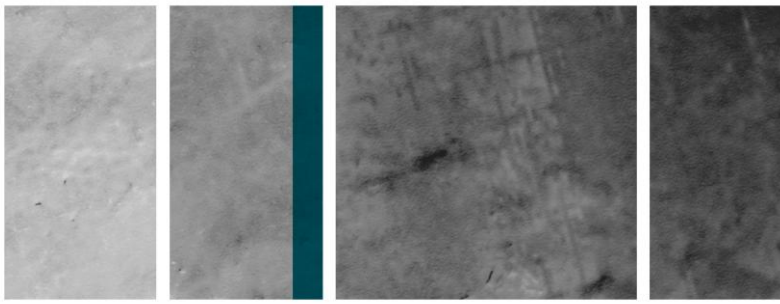
   Dilute one vial with 9 mL PBSA prior to use. Keep refrigerated (or on ice), as this solution will denature at room temperature.

3. Sterile phosphate buffered saline (PBSA; pH 7.4 without Ca^{2+}/Mg^{2+})
4. Tissue culture flasks or multi-well plates
5. Sterile pipettes
6. 50 mL sterile centrifuge tubes
7. Glass discard bottle

Methods

NOTE: All manipulations must be undertaken in a Class II safety cabinet and using aseptic techniques

1. Bring cell culture medium to room temperature (not too critical - cells will tolerate cold medium).
2. In safety cabinet, decant old medium from cell cultures into glass discard bottle.
3. Rinse cell monolayer with PBS-A and decant the fluid into glass discard bottle.
4. Add T/V solution [add solution (3 mL for 75 cm^2 flask or 5 mL for 150 cm^2 flask) to the flask on the opposite side to the monolayer]. Ensure the cell monolayer is completely covered then immediately remove excess. Rock the flask for a minute or two. Look for



the monolayer becoming translucent and patchy then tap the flask firmly against your hand to facilitate cell detachment.

5. Resuspend the detached cells in growth medium. To ensure neutralisation of the trypsin/versene, use three times the volume of T/V solution used previously. Transfer the suspension to a sterile centrifuge tube and centrifuge for 5 min. at 100-150 x *g*.
6. After centrifugation, carefully decant the supernatant medium-T/V solution mixture into the discard bottle leaving the cell pellet in the tube.
7. Resuspend the cell pellet in a known volume (for example 3 mL) of growth medium and perform a cell count using a haemocytometer.
8. Calculate the number of cells in the suspension and seed as per the table below.

Vessel	Total cells	Cells per well	Volume of medium
25 cm^2 flask	1.5 million	n/a	10-15 mL
75 cm^2 flask	4.5 million	n/a	20-30 mL
24-well plate	6 million in 40 mL	225,000	1.5 mL/well
96-well plate	4 million in 11 mL	36,000	100µL/well

Flasks seeded at these rates should produce a 100% confluent monolayer in 5-7 days at 25^{o}C). Cultures in multi-well plates should be approximately 80% confluent after overnight incubation.

9. Incubate cell cultures, in air, at 25^{o}C prior to inoculation and 20^{o}C after inoculation with test samples suspected of containing CyHV-3.

Cultures for virus isolation

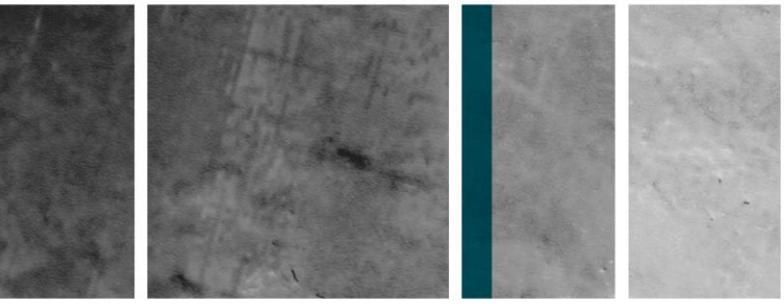
Twenty-four-well cluster plate cultures are used for virus isolation from fish tissues. Generally, plates are seeded with cells approximately 24 hours prior to use.

Procedure:

Plates are seeded at a cell density of approximately 6 million cells/plate (approximately 225,000 cells/1.5 mL/well).

NOTE: All manipulations must be undertaken in a safety cabinet and using aseptic techniques

1. Using a cell suspension prepared by the above method and, based on the cell count performed above, determine the volume of cells required. Note that approximately 40 mL of cell suspension is required to seed one 24-well plate.
2. Add the required volume of cell suspension to the required volume of growth medium to achieve a final cell density of 6 million cells/40 mL/plate.
3. Gently swirl to mix the suspension and using a sterile pipette dispense 1.5 mL of the cell suspension into each well.

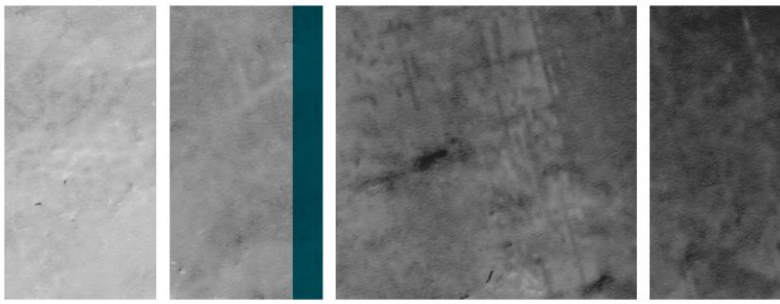


4. Replace plate lids and place in a sealed container e.g. plastic lunch box.
5. Place in cool temperature incubator at approximately 20^{O}C in air, until required.

The test is valid if the negative control cell cultures retain normal cellular morphology for the full period of incubation.

The test sample is negative if the inoculated cell cultures retain normal cellular morphology similar to the negative control cultures, i.e., do not demonstrate viral CPE.

If any of the cell cultures inoculated with test samples demonstrate CPE, further investigations are required such as: PCR (Appendix 2) or examination by electron microscopy.



Appendix 2. PCR-based tests

Reagents

Reagents stored at at -20°C

100% Ethanol AR grade

Primers (18 µM)

HotStar Taq Mastermix (QIAGEN Cat# 203445)

100 bp DNA ladder & loading dye (Promega Cat# G2101)

Reagents stored at 4OC

Buffer AVL

Reagents stored at room temperature

QIAamp viral RNA Extraction Kit (Cat # 52904)

QIAGEN Buffer AW1

QIAGEN Buffer AW2

QIAGEN Buffer AVE

Agarose (BIORAD Cat # 162-0134)

Ethidium bromide (BIORAD Cat #161-0430)

40x TAE Buffer (Promega Cat # V4281)

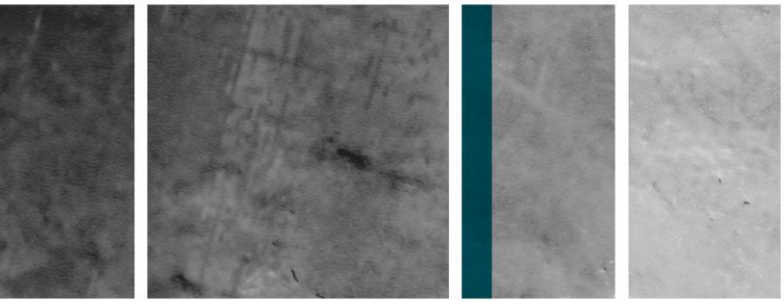
Equipment

Apart from the normal range of equipment required in the standard diagnostic laboratory (eg. refrigerators, freezers, vortex mixers, micropipettes, biological safety cabinets, centrifuges, balances, microwave oven, thermometers), specialised equipment required to undertake diagnostic PCR may include dry heat blocks, thermocycler, gel electrophoresis equipment, UV transilluminator, camera system and sequencer.

Quality control

Molecular diagnosis should be operated under an ISO 17025 accredited and audited quality assurance program. Thus, such a program would include initial evaluation of kits and validation of performance; ongoing internal evaluation through mandatory use of appropriate quality control samples where available; and performance monitoring through quality assessment or proficiency programs.

External quality control samples over the appropriate range of testing must be obtained or manufactured wherever possible. Wherever possible, quality control samples should be included in every assay run and the data presented so that run-to-run performance can be monitored. Positive, negative and reagent controls should be conducted as specified in the protocol. As a norm, formalin-fixed controls would be conducted with formalin-fixed test



samples, and appropriate unfixed controls would be conducted with fresh tissue or tissue-culture supernatant samples. Stocks of controls should be established. These controls should be evaluated prior to storage and used in a check-testing regimen and as controls for the conduct of disease investigations.

Procedure

Sample preparation

Due to the sensitivity of PCR tests, care at every step of sample preparation must be taken to ensure that cross-contamination of diagnostic samples does not occur. Thus all instruments and sample containers must be clean and uncontaminated, i.e., not pre-exposed to aquatic pathogens. Wherever possible it is recommended that disposable reagents and plastic-ware are used. At AAHL, samples would be handled and processed using sterile disposable single use containers, instruments and reagents to minimise the risks of contamination of the samples.

As a general principle, samples to be used in the PCR suite at AAHL for molecular diagnosis will be inactivated by an approved method prior to movement to the PCR suite.

Inactivation will be carried out by the following procedures by staff approved to work with the categories of agents

1. Cell-free samples, e.g., tissue culture supernatants at room temperature are added to an appropriate commercially prepared buffer (e.g., Qiagen AVL buffer) containing guanidinium isothiocyanate.
2. Tissue samples (including blood) and other specimens containing cells are homogenised at approximately 10% w/v in water, and then frozen and thawed. Samples are then microfuged (approximately 13000 x g for 20-30 sec), and the cell-free supernatant fluid collected. An appropriate volume of this is then mixed with an appropriate volume of a commercially prepared buffer (e.g., Qiagen AVL buffer).

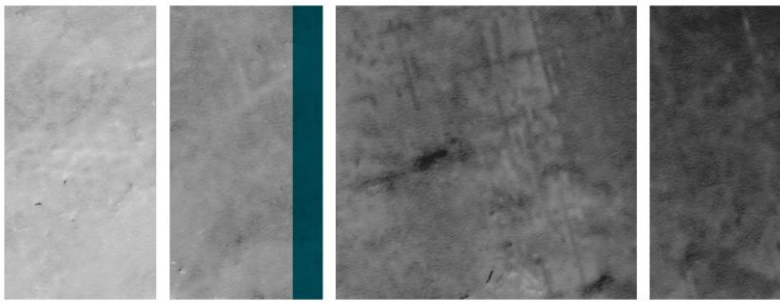
Nucleic acids are extracted from submitted samples in the Biological Safety Cabinet Class II in the PCR suite. All samples handled in the PCR suite will be less than 5mL and considered to be of low or intermediate titre (or inactivated if required as above).

Nucleic acid extraction

Nucleic acid (including CyHV-3 DNA) is obtained from cell-free samples using the QIAamp Viral RNA extraction kit (QIAGEN cat no. 52904). The manufacturer also recommends this kit for extraction of DNA viruses.

Conventional PCRs

Either of two conventional PCRs are routinely used in the PCR suite at AAHL: the Bercovier thymidine kinase PCR (Bercovier *et al.*, 2005), or the official Japanese PCR (Yuasa *et al.*, 2005). For either PCR, the PCR mixture for a single sample consists of the following reagents: 9.5 µL of water; 12.5 µL of HotStar Taq Master mix; 0.5 µL of the forward primer (18 µM); 0.5 µL of the reverse primer (18 µM); and 2 µL of DNA. For multiple samples, the volumes are multiplied appropriately. The mixture is incubated in an automatic thermal cycler (Perkin Elmer GeneAmp 2400) that is programmed appropriately for each PCR: 1. Bercovier TK PCR -



one cycle at 94^{o}C for 15 minutes; 35 cycles at 94^{o}C for 30 sec, 52^{o}C for 30 sec and 72^{o}C for 1 minute; and, finally, one cycle at 72^{o}C for 7 minutes. 2. Official Japanese PCR – one cycle at 94^{o}C for 15 minutes; 40 cycles at 94^{o}C for 30 sec, 63^{o}C for 30 sec and 72^{o}C for 30 sec; and, finally, one cycle at 72^{o}C for 7 minutes. Amplified DNA is detected by agarose gel electrophoresis.

The Bercovier TK primers are:

Forward = 5' GGGTTACCTGTACGAG 3'

Reverse = 5' CACCCAGTAGATTATGC 3'

Product size = 409 bp

The Japanese primers are:

Forward = 5' GACACCACATCTGCAAGGAG 3'

Reverse = 5' GACACATGTTACAATGGTCGC

Product size = 292 bp

Interpretation

At the completion of either PCR, specific PCR fragments of the correct size are identified by agarose gel electrophoresis:

- The negative control sample must have no evidence of specific amplified products.
- A positive control sample must yield a specific CyHV-3 fragment (either 409 bp, or 292 bp) (Figure 17).
- Amplified fragments of the correct size are then eluted from the gel and used to determine the DNA sequence (by using the PCR primers as sequencing primers). Note that, an apparently specific PCR product only provides a tentative diagnosis. *The PCR product must be sequenced to make a definitive diagnosis*.
- Sequence identity and genotype are determined by a Blast search of the Genbank database.

An assay is valid only when all controls yield the expected results.

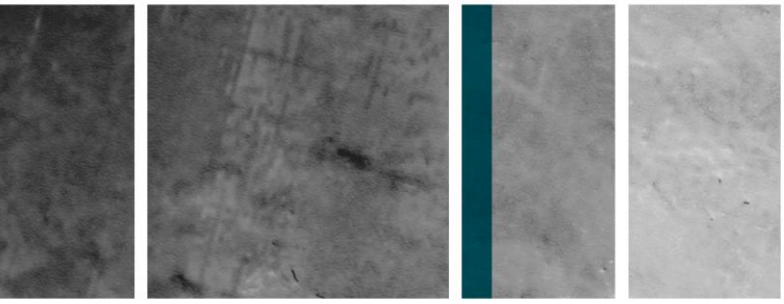
Real-time Taqman PCR

The Taqman PCR is based on that developed by Gilad *et al*. (2004) using the following primers and probe:

KHV-86f (forward): 5' GACGCCGGAGACCTTGTG 3'

KHV-163r (reverse): 5' CGGGTTCTTATTTTTGTCCTTGTT 3'

KHV-109p (probe): 5' FAM-CTTCCTCTGCTCGGCGAGCACG-TAMRA 3'



The PCR mixture for a single sample (one well only) consists of the following reagents: 6.75 uL of water; 12.5 uL of TaqMan 2x Universal PCR master mix (Applied Biosystems, Cat # 4304437); 1.25 uL of primer 1; 1.25 uL of primer 2; 1.25 uL of the TaqMan probe; and 2 uL of extracted DNA. For multiple samples (and if more than one well per sample is required), the volumes are multiplied appropriately.

Interpretation

After completion of the assay, results should be read using the following guide:

- Save and analyse data
- Determine if the assay is valid (Figure 18) by:
    - Visualizing a characteristic amplification curve in positive control wells, FAM dye layer
    - Determine the absence of contamination by observing no characteristic amplification curves in the NTC wells for the FAM dye layer.

If the above criteria are met and the assay is deemed valid, the test sample results may be read; if not the assay must be repeated in consultation with the technical manager.

Read Test sample:

- Determine the absence or presence of characteristic amplification curves for the test sample wells.
- Read the CT value for the test sample.

If the test is deemed valid, the results for the test sample wells may be interpreted using the following criteria:

- Positive test results are defined as the presence of specific amplicons expressed as a characteristic amplification curve similar to the positive control(s) and having a cycle threshold (CT) value less than 37.
- Negative test results are defined as the absence of specific amplicons expressed by a characteristic amplification curve similar to the No Template control (NTC) and having a cycle threshold (CT) value greater than 40.
- Indeterminate test results are defined as having a characteristic amplification curve similar to the positive control but a cycle threshold (CT) value between 37 and 40. This necessitates repeating the assay with at least 6 test sample wells.

References

Bercovier H, Fishman Y, Nahary R, Sinai S, Zlotkin A, Eyngor M, Gilad O, Eldar A & Hedrick RP. 2005. Cloning of the koi herpesvirus (KHV) gene encoding thymidine kinase and its use for a highly sensitive PCR based diagnosis. *BMC Microbiol.*, 5: 1-9.

Gilad O, Yun S, Zagmutt-Vergara FJ, Leutenegger CM, Bercovier H, and Hedrick RP. 2004. Concentrations of a koi herpesvirus (KHV) in tissues of experimentally infected

Cyprinus carpio koi as assessed by real time TaqMan PCR. *Dis. Aquat. Org.* 60: 179-187.

Hedrick RP, Gilad O, Yun S, Spangenberg JV, Marty GD, Nordhausen RW, Kebus MJ, Bercovier H & Eldar A. 2000. A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. *J. Aquat. Anim. Health*, 12: 44-57.

Yuasa K, Sano M, Kurita J, Ito T & Iida T. 2005. Improvement of a PCR method with the Sph I-5 primer set for the detection of koi herpesvirus (KHV). *Fish Pathology* 40:37-39

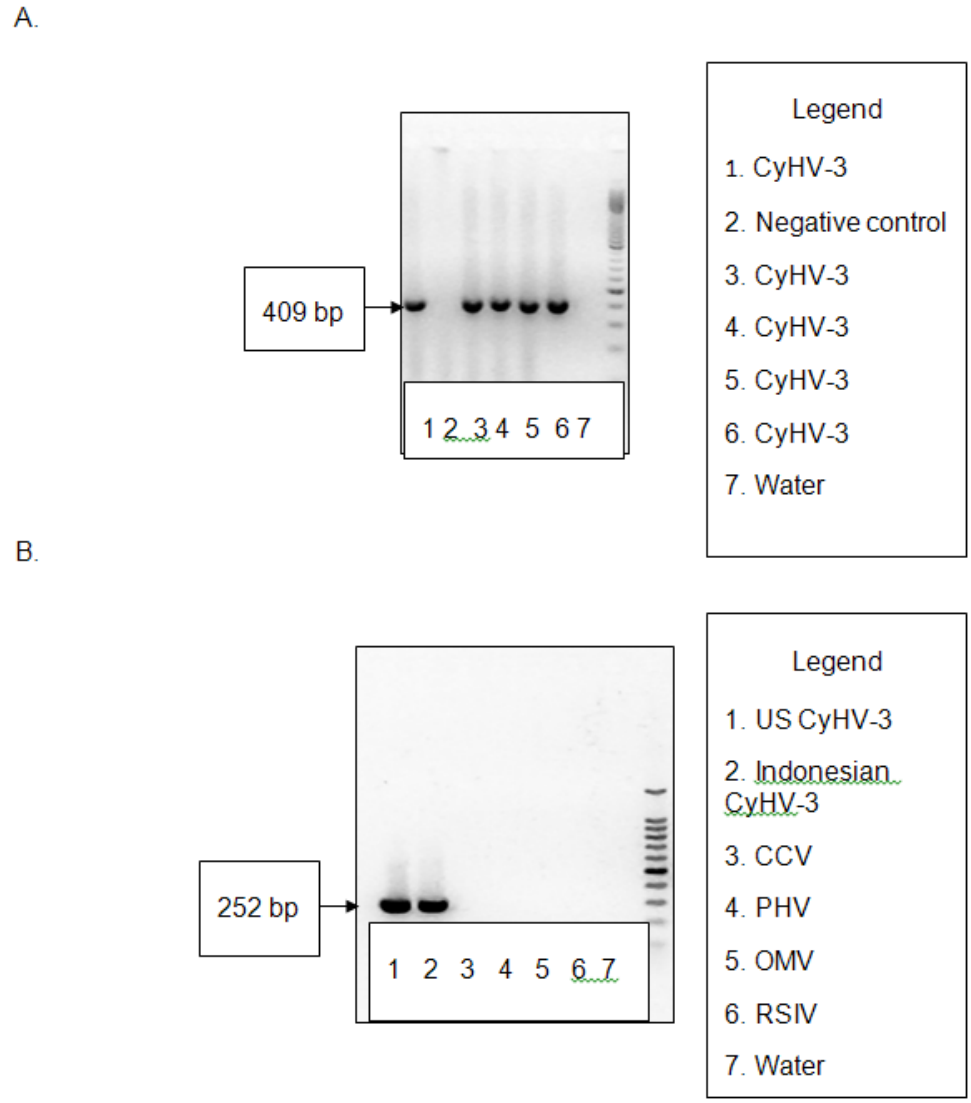
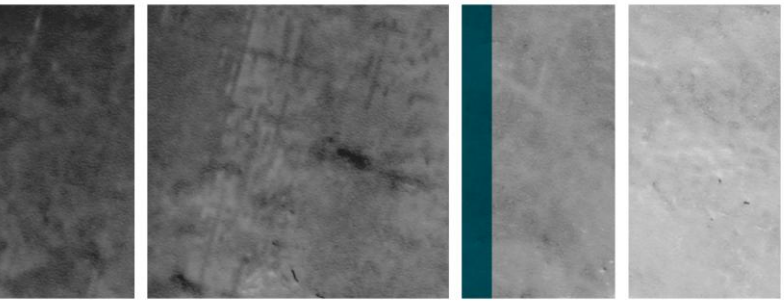


Figure 17: Specificity of (A) the Bercovier, and (B) the official Japanese, CyHV-3 PCRs. In (A), the samples are from the 2006 CyHV-3 International Proficiency test. In (B), samples are: 1. a US isolate of CyHV-3; 2. an Indonesian isolate of CyHV-3; 3. Channel catfish virus (IcHV); 4. Pilchard herpesvirus; 5. *Oncorhynchus masou* virus; 6. Red sea bream iridovirus.



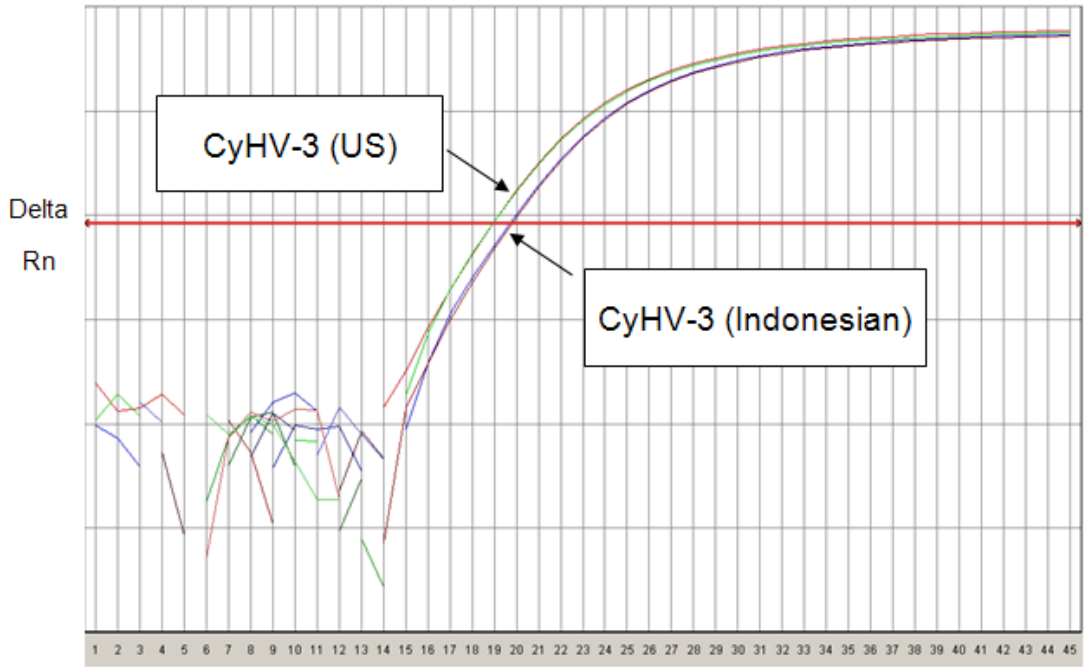
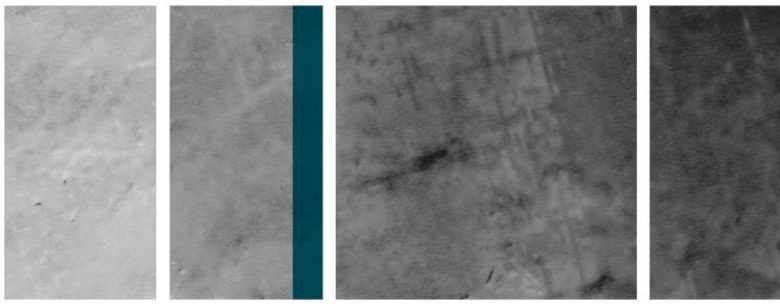


Figure 18: CyHV-3 TaqMan PCR



Appendix 3. Immunocytochemistry

Equipment

Humid chamber, for example, a plastic container with an air-tight lid

Plate shaker

Refrigerator

Inverted light microscope fitted with 4X and 10X objectives

Pipettes capable of dispensing 25µL to 500µL

Wash bottles or plate washer

Reagents

Phosphate-buffered saline without Ca^{2+} and Mg^{2+} ions, pH 7.4 (PBSA)

16% (v/v) formalin in PBSA

PBST 0.05% (v/v) Tween 20 in PBSA (Tween-20: Sigma-Aldrich P1379)

1% (w/v) skim milk powder solution in PBSA

0.1% (w/v) skim milk powder solution in PBSA

Mouse monoclonal antibody raised against CyHV-3 (Aquatic Diagnostics Ltd, Stirling UK)

Normal mouse IgG (Amersham Biosciences)

Biotinylated sheep anti-mouse Ig (Amersham Biosciences RPN1001)

Streptavidin-horseradish peroxidase conjugate (Amersham Biosciences RPN1231V)

9-ethylcarbazol-3-amine (AEC) Sigma-Aldrich 20mg tablets (A6926)

N, N-dimethylformamide (DMF), Sigma-Aldrich (D8654)

Acetate buffer 0.05 M, pH 5.0

Hydrogen peroxide H_2O_2 Sigma-Aldrich (H1009)

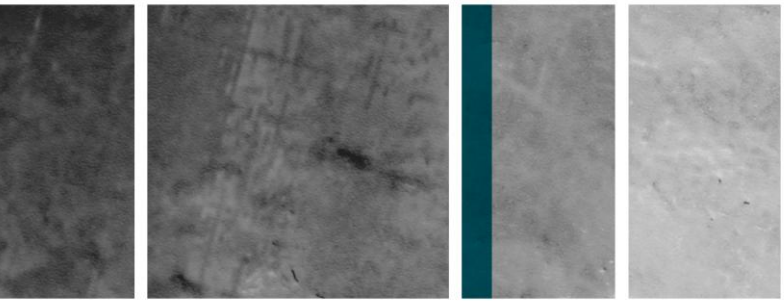
Deionised water

Mayer's haematoxylin (Lillie's modification) DAKO (S3309)

Scott's tap water (Drury RAB, Wallington EA. Carleton's Histological Technique 5th Ed. 1980)

Procedure

1. Fix cells by adding formalin solution to existing medium in a volume that gives a final concentration of 4% (v/v) formalin. Incubate in a humidified chamber in the refrigerator for at least one hour (but for no more than one day) to fix the cells. If the immunocytochemical test cannot be performed within one day remove the formalin solution, wash twice with PBSA. Add PBSA and return the plates to the refrigerator until the test can be performed.



2. Dilute anti-CyHV-3 antibody (1/2000) and normal mouse IgG (1/100) to working strength in 1% skim milk.
3. Dilute biotinylated sheep anti-mouse antibody (1/300) and streptavidin-horseradish peroxidase conjugate (1/300) to working strength in 0.1% skim milk.
4. Remove supernatant fluid from the wells of the cell culture, and wash the wells twice with 0.05% PBST.
5. Add 50µL (96-well plate) or 200µL (24-well plate) of primary antibody to each well. Incubate at room temperature for 1 h on a plate shaker set at low speed.
6. Remove the anti-CyHV-3 and normal mouse IgG solutions, and wash the wells 3 times with PBST. Add the biotinylated sheep anti-mouse antibody solution to all wells. Incubate at room temperature for 30 min on a plate shaker set at low speed.
7. Remove the biotinylated sheep anti-mouse antibody, and wash the wells 3 times with PBST. Add streptavidin-horseradish peroxidase conjugate to each well. Incubate at room temperature for 30 min on a plate shaker set at low speed.
8. Prepare the substrate just before use. Add one AEC tablet to 2.5mL of dimethlyformamide. Once dissolved add this solution to 47.5 mL of 0.05 M acetate buffer, then add 50µL of 30% [v/v] hydrogen peroxide to activate the solution.
9. Remove the streptavidin-horseradish peroxidase conjugate from the wells, and wash the wells 3 times with PBST.
10. Add 50µL (96-well plate) or 200µL (24-well plate) of active substrate solution and incubate at room temperature for 20 min. Use of a plate shaker is optional.
11. Discard the substrate and wash the cells with deionised water to stop the reaction.
12. Counterstain with Mayer's haematoxylin 50µL (96-well plate) or 200µL (24-well plate). Incubate at room temperature for 60-90 sec, rinse twice with water.
13. Add Scott's tap water for 90-120 sec to develop background blue colour.
14. Rinse with tap water and allow to air dry.
15. Examine processed wells by inverted light microscopy

Interpretation

Positive reaction: grainy, focal, brick-red staining of cells indicates the presence of CyHV-3 identified by the diagnostic antibody (Figure 19A).

Negative reaction: no red staining apparent. All cells should be stained pale blue due to the counterstain (Figure 19B).

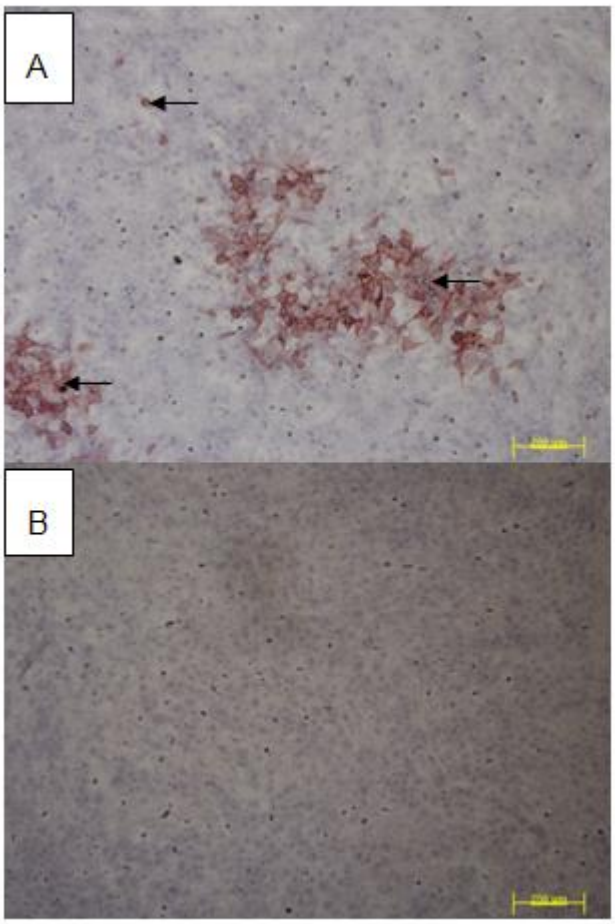
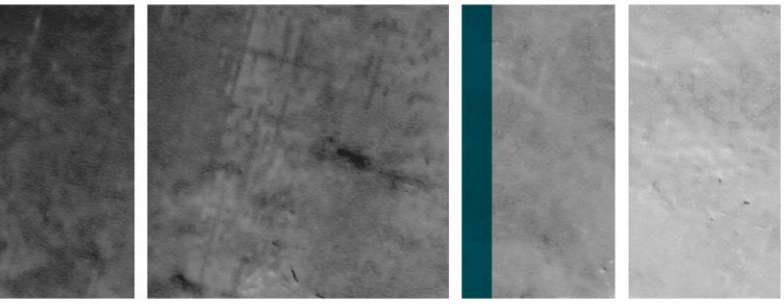


Figure 19: Photomicrographs demonstrating localisation of CyHV-3 in cultures of KF-1 cell line. CyHV-3-infected cell culture (A) and uninfected cell culture (B) treated with a commercial mouse anti-CyHV-3 monoclonal antibody. Red-brick staining (examples are arrowed) demonstrates presence of CyHV-3 in the infected culture.



Appendix 4. Immunohistochemistry

Equipment

60°C incubator/oven

Light microscope

Coverplate™ chambers (Thermo Fisher Scientific Inc. Waltman, MA, USA).

Reagents

Anti-cyprinid herpesvirus (CyHV-3) monoclonal antibody (Aquatic Diagnostic Ltd. Product No P14, Stirling, UK)

Vial contents: Each vial contains 200 µg of lyophilised protein prepared from bovine-free culture medium and contains no animal-derived stabilisers. This is sufficient for between 100-200 tests depending on the area of tissue to be screened in IHC.

The product should be reconstituted as follows:

1. Add 1 ml of Tris buffered saline (TBS) plus 1% skim milk to the vial and store as aliquots in 20°C.
2. Dilute 1/10 in TBS before use.

Storage: Store at -20°C prior to reconstitution. For prolonged storage, the MAb solution should be stored at -20°C. Repeated freeze/thawing of the product should be avoided.

Tris-buffered saline (TBS):

Trisma base 2.42g

NaCl 29.24g

Dissolve in approximately 900 ml distilled water, adjust pH to 7.2 using HCl and make up to 1 litre

TBS plus 1% skim milk

Skim Milk 0.1 g

10ml TBS

0.3% Hydrogen peroxide

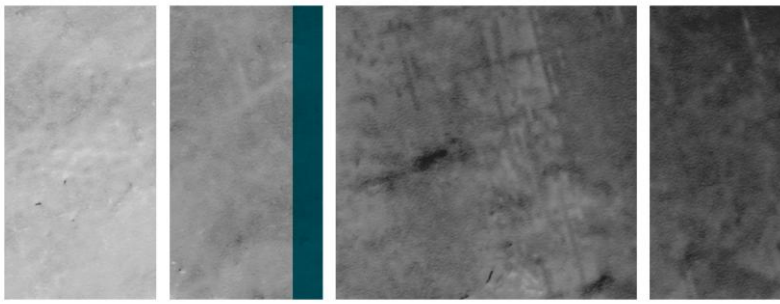
(Peroxidase Block, EnVision$^+$ Systems, DakoCytomation Inc., California, USA, cat no K4005).

Anti-mouse secondary antibody:

Anti-mouse immunoglobulins in Tris-HCl conjugated with peroxidise labelled polymer (Labelled polymer-HRP anti-mouse, EnVision$^+$ Systems, DakoCytomation Inc., California, USA, cat no K4005).

3-amino-9-ethylcarbazole:

(AEC$^+$ substrate chromogen, EnVision$^+$ Systems, DakoCytomation Inc., California, USA, cat no K4005).



Faramount aqueous mounting medium (DakoCytomation Inc., California, USA, cat no S30250).

Xylene

Ethanol

Haematoxylin

Scott's tap water

Procedure

1. Prepare paraffin-embedded tissue sections
2. Dewax at 60°C for 5 minutes.
3. Rehydrate sections in:
    a. xylene 4x 30sec
    b. 100% 4x 30sec
    c. 70% ethanol 1x 1min,
    d. Rinse in distilled water to remove the ethanol.
4. Place slides in Coverplate™ chambers (Thermo Fisher Scientific Inc. Waltman, MA, USA).
5. Wash the slides with 2mL TBS for 5 minutes, by filling the Coverplate™ well with 2mL TBS and incubating for 5 minutes. Following application of the buffer into the well, allow the buffer to slowly wash down the tissue section, leaving the last 80uL in the chamber for 5 minutes.
6. Block endogenous peroxidase activity by incubating the slides for 10 min at room temperature (≈22°C) with 10 drops of 0.03% H_2O_2 (Peroxidase Block, EnVision$^+$ Systems, DakoCytomation Inc., California, USA, cat no K4005).
7. Wash the slides with 2mL TBS for 5 minutes as in Step 5.
8. Place 100µL of reconstituted anti-CyHV-3 MAb (1:3 v/v with TBS+1% skim milk) onto the Coverplate™ well, and incubate overnight at 4°C.
9. Wash the slides with 2mL TBS for 5 minutes as Step in 5.
10. Application of enzyme (horseradish peroxidise, HRP) conjugated to secondary antibody: Incubate the slide for 45 minutes with 200µL of Dual Link Systems-HRP (EnVision$^+$ Systems, DakoCytomation Inc., California USA).
11. Wash the slides for 5 minutes.
12. Take out the slides from Coverplate™ chambers.
13. Encircle the tissue sections with Dako Pen
14. To visualise the reaction, incubate the slides for 30 min* with 3-amino-9-ethylcarbazole (AEC$^+$ chromogen, EnVision$^+$ Systems, DakoCytomation Inc., California, USA, cat no K4005). *If necessary, check after 10 min, if the signal is low, then add fresh AEC and incubate for another 10 min.

15. Stop the reaction by immersing the slides in distilled water.
16. Counterstain sections with haematoxylin for 3 min, soak in tap water, and wash with Scott's solution for 1 minute.
17. Rinse in tap water for 10 min.
18. Mount the slides with Faramount aqueous mounting medium (DakoCytomation Inc., California, USA, cat no S30250).
19. Wait 15 minutes to allow the mounting medium to dry.
20. Examine tissue under a light microscope – cells infected with the virus appear brick red in colour.

Interpretation

Negative reaction: no red staining apparent. All cells should be stained pale blue due to the counterstain (Figure 20A).

Positive reaction: grainy, focal, brick-red staining of cells indicates the presence of CyHV-3 identified by the diagnostic antibody (Figure 20B).

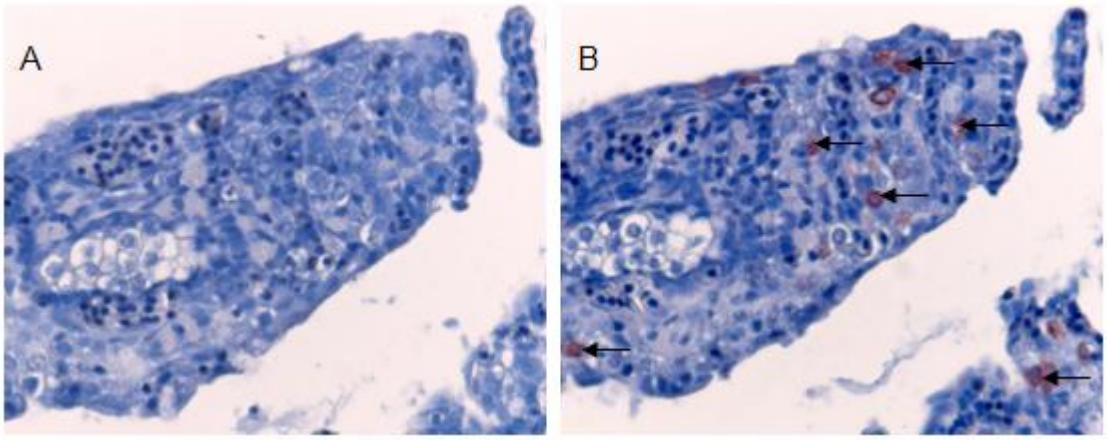


Figure 20: Photomicrographs demonstrating CyHV-3-specific immunohistochemical staining of gills from a CyHV-3-infected carp. A: Section treated with normal mouse serum (no staining); B: Virus-specific staining of section treated with anti-CyHV-3 MAb (arrows).

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