RESEARCH **12**

2019-176

WHAT ARE THE CARP VIRUS BIOCONTROL RISKS AND HOW CAN THEY BE MANAGED?

NATIONAL CARP CONTROL PLAN

Determination of the susceptibility of Silver Perch, Murray Cod and Rainbow Trout to infection with CyHV-3

This suite of documents contains those listed below.

NCCP TECHNICAL PAPERS

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

NCCP RESEARCH (peer reviewed)

Will carp virus biocontrol be effective?

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

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

9. 2017-055 and 2017-056: Water-quality risk assessment of carp biocontrol for Australian waterways
10. 2016-183: Cyprinid herpesvirus 3 and its relevance to humans
11. 2017-127: Defining best practice for viral susceptibility testing of non-target species to Cyprinid herpesvirus 3
12. 2019-176: Determination of the susceptibility of Silver Perch, Murray Cod and Rainbow Trout to infection with CyHV-3
13. 2016-152 and 2018-189: The socio-economic impact assessment and stakeholder engagement
    Appendix 1: Getting the National Carp Control Plan right: Ensuring the plan addresses community and stakeholder needs, interests and concerns
    Appendix 2: Findings of community attitude surveys
    Appendix 3: Socio-economic impact assessment — commercial carp fishers
    Appendix 4: Socio-economic impact assessment — tourism sector
    Appendix 5: Stakeholder interviews
    Appendix 6: Socio-economic impact assessment — native fish breeders and growers
    Appendix 7: Socio-economic impact assessment — recreational fishing sector
    Appendix 8: Socio-economic impact assessment — koi hobbyists and businesses
    Appendix 9: Engaging with the NCCP: Summary of a stakeholder workshop
14. 2017-237: Risks, costs and water industry response
15. 2017-054: Social, economic and ecological risk assessment for use of Cyprinid herpesvirus 3 (CyHV-3) for carp biocontrol in Australia
    Volume 1: Review of the literature, outbreak scenarios, exposure pathways and case studies
    Volume 2: Assessment of risks to Matters of National Environmental Significance
    Volume 3: Assessment of social risks
16. 2016-158: Development of strategies to optimise release and clean-up strategies
17. 2016-180: Assessment of options for utilisation of virus-infected carp
18. 2017-104: The likely medium- to long-term ecological outcomes of major carp population reductions
19. 2016-132: Expected benefits and costs associated with carp control in the Murray-Darling Basin

NCCP PLANNING INVESTIGATIONS

1. 2018-112: Carp questionnaire survey and community mapping tool
2. 2018-190: Biosecurity strategy for the koi (*Cyprinus carpio*) industry
3. 2017-222: Engineering options for the NCCP
4. NCCP Lachlan case study (in house) (refer to Technical Paper 9)
5. 2018-209: Various NCCP operations case studies for the Murray and Murrumbidgee river systems (refer to Technical Paper 8)

FRDC
FISHERIES RESEARCH & DEVELOPMENT CORPORATION

NCCP: Determination of the susceptibility of silver perch, Murray cod and rainbow trout to infection with CyHV-3

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Aquatic Animal Health Subprogram: Determining the susceptibility of Australian *Penaeus monodon* and *P. merguiensis* to newly identified enzootic (YHV7) and exotic (YHV9 and YHV10) Yellow head virus (YHV) genotypes

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Contents

Tables iii

Figures iv

Acknowledgments v

Abbreviations vi

Executive Summary vii

Introduction 1

Objectives 2

Assessment of the susceptibility of silver perch and Murray cod to infection with CyHV-3. 3

1.1 Methods 3

1.1.1 Preparation of CyHV-3 3
1.1.2 Experimental animals 3
1.1.3 Exposure to CyHV-3, monitoring and sampling of fish. 5
1.1.4 Nucleic acid extraction 6
1.1.5 Molecular tests 7
1.1.6 Histopathology 7

1.2. Results 8

1.2.1 Testing of the inocula 8
1.2.2 Morbidity in negative control and CyHV-3 exposed finfish 8
1.2.3 Molecular testing of negative control and CyHV-3-exposed finfish 17
1.2.4 Histological examination of carp, silver perch and Murray cod 24

Discussion 26

Conclusions 27

Implications 28

Recommendations 29

Extension and Adoption 30

References 31

Appendices 32

Appendix 1. List of Project staff 32
Appendix 2. Details of mortalities observed in silver perch and Murray cod exposed to CyHV-3 33
Appendix 3. Details of CyHV-3 Gilad qPCR positive and negative detections in CyHV-3 exposed carp. 35
Appendix 4. Development of the CyHV-3 Terminase RT-qPCR for the detection of CyHV-3 mRNA 37

Tables

Table 1. Details of fish deliveries 4
Table 2. Water quality parameters measured and acceptance ranges 4
Table 3. Distribution of fish across rooms 4
Table 4. Grading of clinical signs to reflect the appearance of mild, moderate and severely affected animals and intervention required 6
Table 5. Samples collected for molecular testing and histopathology 6
Table 6. Gilad CyHV-3 qPCR results for negative control carp 17
Table 7. Gilad CyHV-3 qPCR results for negative control Murray cod 17
Table 8. Gilad CyHV-3 qPCR results for negative control silver perch 18
Table 9. Summary molecular test results for carp, Murray cod and silver perch exposed to CyHV-3 18
Table 10. Gilad CyHV-3 qPCR and AFDL CyHV-3 Terminase RT-qPCR results for CyHV-3-exposed carp. Results are mean C_T values of samples tested in duplicate. 20
Table 11. Gilad CyHV-3 qPCR and AFDL CyHV-3 Terminase RT-qPCR results for CyHV-3-exposed Murray cod. Results are mean C_T values of samples tested in duplicate. 22
Table 12. Gilad CyHV-3 qPCR and AFDL CyHV-3 Terminase RT-qPCR results for CyHV-3-exposed silver perch. Results are mean C_T values of samples tested in duplicate. 23
Table 13. Summary of observations and test results combined for each species (CyHV-3 exposed and negative control fish) 27

Figures

Figure 1. Cumulative morbidity in negative control carp across the four replicate tanks 8
Figure 2a. Cumulative morbidity in carp after exposure to CyHV-3. 9
Figure 2b. Daily morbidity in carp after exposure to CyHV-3 10
Figure 3. Mild clinical signs in carp after exposure to CyHV-3 (arrow = examples of patches of reddened skin). Day 4 post exposure 10
Figure 4a. Moderate clinical signs in carp after exposure to CyHV-3. Day 4 post exposure 11
Figure 4b. Moderate clinical signs in carp after exposure to CyHV-3. Day 4 post exposure. 11
Figure 5a. Cumulative morbidity in negative control silver perch across the four replicate tanks. 12
Figure 5b. Daily morbidity in negative control silver perch across the four replicate tanks. 13
Figure 6a. Cumulative morbidity in silver perch after exposure to CyHV-3. 13
Figure 6b. Daily morbidity in silver perch after exposure to CyHV-3. 14
Figure 7a. Cumulative morbidity in negative control Murray cod across the four replicate tanks. 15
Figure 7b. Daily morbidity in negative control Murray cod across the four replicate tanks. 15
Figure 8a. Cumulative morbidity in Murray cod after exposure to CyHV-3. 16
Figure 8b. Daily morbidity in Murray cod after exposure to CyHV-3. 16
Figure 9. Detection of CyHV-3 DNA in different organs of CyHV-3-exposed carp across all tanks 19
Figure 10. Detection of CyHV-3 DNA (Gilad qPCR) and CyHV-3 mRNA (Terminase RT-PCR) of CyHV-3-exposed carp across all tanks 19
Figure 11. Detection of CyHV-3 DNA in different organs of CyHV-3-exposed Murray cod across all tanks 21
Figure 12. Detection of CyHV-3 DNA in different organs of CyHV-3-exposed silver perch across all tanks 21

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Abbreviations

ACDP	Australian Centre for Disease Preparedness
C_T	Threshold cycle
CyHV-3	*Cyprinid herpesvirus 3* (also known as koi herpesvirus; CyHV-3)
DNA	Deoxyribonucleic acid
EtOH	Ethanol
FBS	Foetal bovine serum
KF-1	Koi fin cell line
KHVD	Koi herpesvirus disease
L-15	Tissue culture medium
mRNA	messenger RNA
NCCP	National Carp Control Program
nPCR	Nested polymerase chain reaction
NEC	Negative extraction control
NTC	No template control
NTS	Non-target species
PBSA	Phosphate-buffered saline without Ca^{++} and Mg^{++} ions (pH 7.4)
PCR	Polymerase chain reaction
qPCR	Real-time (quantitative) polymerase chain reaction
RNA	Ribonucleic acid
RT-qPCR	Veal-time reverse-transcriptase (quantitative) polymerase chain reaction
$TCID_{50}$	Median tissue culture infective dose
TCSN	Tissue culture supernatant
v/v	Volume per volume

Executive Summary

To assist in the making of an informed decision regarding the potential for release of CyHV-3 as a biological control agent for carp, the National Carp Control Program (NCCP) requested additional information regarding the susceptibility of several non-carp species to infection with CyHV-3. This report describes the exposure of carp, silver perch and Murray cod to CyHV-3 and the outcomes for the three species.

Background

Despite evidence of non-susceptibility of non-target species (NTS) to CyHV-3 infection presented by McColl et al. (2016), it was felt by some stakeholders that while the study provided much compelling data, given the importance of understanding non-target species susceptibility in Australia, it would be important to consider best practice in evaluating this. In response to this stakeholder feedback, the NCCP commissioned a review of best practice in non-target species susceptibility (Pyecroft, 2018). While the review acknowledged that the work undertaken by McColl et al. (2016) used the most advanced techniques available at the time, it has been recommended that some species which had experienced unexpected mortalities in the former study be reassessed using the most recent methodological recommendations.

Aims/objectives

The objectives of the project was to determine the susceptibility of silver perch, Murray cod and rainbow trout to infection with cyprinid herpesvirus 3 (CyHV-3).

Methodology

Susceptibility to infection was determined after exposing carp, silver perch and Murray cod to CyHV-3 by intraperitoneal injection followed by immersion in water containing CyHV-3 and observation and sampling for 28 days. Equal numbers of fish were exposed to cell culture media as negative, non-exposed control fish.

All fish were monitored twice daily for signs of disease, with an additional 11pm monitoring point added when disease was observed. Samples were collected from dead and humanely killed fish for analyses including PCR testing, to determine the presence of the viral DNA and mRNA, and histology to observe the presence of any lesions indicative of KHVD.

Results/key findings

1. Based on development of clinical signs and detection of CyHV-3 DNA and CyHV-3 mRNA, carp were susceptible to infection with CyHV-3.

2. Based on no observation of clinical signs, low level detection of CyHV-3 DNA and no detection of CyHV-3 mRNA, silver perch and Murray cod were not susceptible to infection with CyHV-3.

3. Rainbow trout were not included in the trials due to acute unexpected mortalities on two occasions (87% in 2020 and 63% in 2021), within 48 hours of arrival into the ACDP Aquatic Facility. The cause of these acute mortalities was due to chlorine toxicity caused by chlorine and/or chloramine in the municipal source of the freshwater used to hold the fish. Free chlorine is removed by the aeration system in place while chloramine requires neutralization (i.e. sodium thiosulphate) or removal (i.e. catalytic activated carbon). The presence of chlorine at levels toxic to rainbow trout was confirmed by testing undertaken by ACDP and external consultants.

Implications for relevant stakeholders

The results presented in this report are based on experimental infections in an artificial environment. However, the results indicate that if CyHV-3 were released into the environment, there would be no major infection and disease event caused by CyHV-3 in silver perch or Murray cod.

Recommendations

It is important to confirm the expected mortality in carp after exposure to CyHV-3 using death as an endpoint. The morbidity observed in this project is not a useful indicator of mortality as it is unknown how many of the fish that were humanely killed for animal welfare reasons, when moderate clinical signs were observed, would have recovered from the disease.

Keywords
Cyprinid herpes virus 3, CyHV-3, carp, silver perch, Murray cod, susceptibility, bioassay, real-time PCR, qPCR, mRNA.

Introduction

The National Carp Control Plan (NCCP), an initiative of the Australian government, was developed to determine the feasibility of using CyHV-3 as biological control agent for common carp in Australia. The NCCP commenced in 2016 with completion planned for December 2019.

While the origins and evolutionary history of CyHV-3 remain unclear, the virus has clearly been active across an extensive geographical region for at least the last 20 years. During this time, captive and wild populations of common carp infected with CyHV-3 have cohabitated with a diverse range of species from various taxonomic groups, including other cyprinids. Despite this, naturally occurring CyHV-3 disease has only been recorded from common carp and its ornamental variety, koi, indicating host specificity. Hybrids of common carp × goldfish (*Carassius auratus*) and common carp × crucian carp (*Carassius carassius*) have also been shown to be susceptible to infection by CyHV-3.

Despite evidence of non-susceptibility of non-target species (NTS) to CyHV-3 infection presented by McColl et al. (2016), it was felt by some stakeholders that while the study provided much compelling data, given the importance of understanding non-target species susceptibility in Australia, it would be important to consider best practice in evaluating this. In response to this stakeholder feedback, the NCCP commissioned a review of best practice in non-target species susceptibility (Pyecroft, 2018). While the review acknowledged that the work undertaken by McColl et al. (2016) used the most advanced techniques available at the time, it has been recommended that some species which had experienced unexpected mortalities in the former study be reassessed using the most recent methodological recommendations (e.g. detection of KHV mRNA by more sensitive real-time PCR testing) .

Unexpected mortalities of greatest concern among non-target species exposed to CyHV-3 and in negative control animals included the following: after exposure to CyHV-3 by intraperitoneal inoculation and bath exposure, mortalities of 46% and 27% respectively were observed in silver perch (*Bidyanus bidyanus*) and 100% and 45% respectively were observed in rainbow trout (*Oncorhynchus mykiss*). After intraperitoneal inoculation and bath exposure of Murray cod (*Maccullochella peelii*) with CyHV-3, mortalities of 23% and 23% respectively were reported. Negative control mortality in Murray cod after intraperitoneal inoculation and bath exposure to uninfected cell culture media was 33% and 35% respectively. The authors stated that CyHV-3 was not the cause of the mortalities on the basis that PCR tests showed no viral replication in fish cells examined but did not provide further supporting evidence which might explain why the fish had perished. Furthermore, false-positive molecular test results in NTS exposed to CyHV-3 were only briefly explained.

The NCCP Scientific Advisory Group has determined that susceptibility of silver perch, Murray cod and rainbow trout remains a significant knowledge gap and additional experimentation is required to generate the knowledge regarding the susceptibility of these species. This project has been commissioned by the NCCP as a critical component of a larger body of work to determine the feasibility of using CyHV-3 as a biological control for introduced feral common carp (*Cyprinus carpio*). In order to make an informed decision regarding the consequences of release to native and commercially important finfish species, the NCCP requires scientifically valid data regarding the susceptibility of non-carp species to infection with CyHV-3 building on the most recent knowledge of best practice in non-target species susceptibility testing.

Objectives

1. To determine the susceptibility of rainbow trout (*Oncorhynchus mykiss*) to infection with CyHV-3,
2. To determine the susceptibility of silver perch (*Bidyanus bidyanus*) to infection with CyHV-3.
3. To determine the susceptibility of Murray cod (*Maccullochella peelii*) to infection with CyHV-3.

Assessment of the susceptibility of silver perch and Murray cod to infection with CyHV-3.

The specific aims of the experiment were:

1. To determine the susceptibility of rainbow trout to infection with CyHV-3.
2. To determine the susceptibility of silver perch to infection with CyHV-3.
3. To determine the susceptibility of Murray cod to infection with CyHV-3.
4. To confirm the susceptibility of carp to infection with CyHV-3
5. To compare any clinical signs, histopathology and molecular test results observed in silver perch and/or Murray cod to those obtained after concurrent infection of carp, a species know to be susceptible to infection with CyHV-3.

1.1 Methods

1.1.1 Preparation of CyHV-3

An Indonesian isolate of CyHV-3 was used in this study. The isolate, CyHV-3 C07 (Sunarto et al, 2011), was originally isolated from common carp suffering mass mortalities in West Java, Indonesia, in 2007 and was transferred to ACDP (formerly AAHL) in the same year. This isolate has been used in several *in vivo* experiments at ACDP, with the most recent being the study preceding this study, Essential studies on cyprinid herpesvirus 3 (CyHV-3) prior to release of the virus in Australian waters: Seasonality studies (FRDC 2017-135).

Cultures of the koi fin cell line (KF-1) were used to propagate the virus. KF-1 cell cultures, in 150 cm^2 tissue culture flasks (Corning), were grown in Leibovitz's L15 medium (L-15) supplemented with 10% (v/v) foetal bovine serum (FBS). Cultures were incubated at 25°C in an atmosphere of 100% air. When cell cultures were at approximately 95-100% confluence the culture medium was decanted, and cells inoculated with CyHV-3 (2 mL virus/flask: passage 2 in cell culture). After incubation for 2 hours at 25°C to allow virus adsorption, culture medium was added to the flask without removal of the inoculum. The virus-inoculated cell cultures were incubated at 25°C, and observed daily, using inverted light microscopy, for the development of cytopathic effect (CPE). When suitable CPE had developed, the tissue culture supernatant (TCSN) from all flasks was decanted, pooled and clarified by low-speed centrifugation (3,800 x *g* for 15 minutes at 15°C). The clarified TCSN (CyHV-3 TCSN, passage 3 in cell culture) was stored at -80°C, until used as inoculum in this study.

Prior to the start of the *in vivo* experiment, an aliquot of the CyHV-3 TCSN was removed from -80°C storage and viral titre quantified by titration, in cell culture, to determine the 50% tissue culture infective dose ($TCID_{50}$/mL; Reed & Muench, 1938). Briefly, 10-fold dilution series (10^0 – 10^{-7}) of TCSN were prepared in culture medium (L-15 + 10% FBS) and inoculated onto KF-1 cell cultures in 96-well tissue culture plates (Thermo). The inoculated titration plates were incubated at 25°C and at 10 days post-infection the titration plates were observed, by inverted light microscopy, to determine the viral titre.

1.1.2 Experimental animals

Carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*), silver perch (*Bidyanus bidyanus*) and Murray cod (*Maccullochella peelii*) were received at ACDP the week of 10 to 14 June (Table 1). On arrival to ACDP, for each species, temperature, general hardness, carbonate hardness, nitrate, nitrite, ammonia and dissolved oxygen were measured in the bags the fish were transported in and the receiving tank water at

ACDP, to confirm levels were within those acceptable for each species (Table 2). Bags were floated on the tank water until temperatures had equilibrated. This generally took less than 60 minutes as tank water temperatures had been pre-set based on advice from the fish suppliers. When temperatures had equilibrated, fish were transferred to individual tanks in each room according to the experimental design described in Table 3.

Table 1. Details of fish deliveries

Species	Delivery Date	Number	Size (cm)	Source
Carp	10/05/2021	162	8-20	Wild (various)
Rainbow Trout	11/05/221	153	12-14	Farmed
Silver perch	12/05/2021	157	4-5	Farmed
Murray cod	14/05/2021	160	8-9	Farmed

Table 2. Water quality parameters measured and acceptance ranges

Parameter	Murray cod	Silver perch	Rainbow trout	Carp
Temperature (°C)	22-28	10-30	10-22	17-30
General hardness (mg/L)	No data	20-300	No data	No data
Carbonate hardness (mg/L)	No data	>50	>50	>50
pH	6.8 – 7.0	6.0-9.5	6.0-9.0	6.0-9.0
Nitrite NO_2 (ppm)	<0.1	<0.1	<0.2	<0.6
Nitrate NO_3 (ppm)	No data	<0.1	<0.2	<1.0
Ammonia NH_3 (mg/L)	<0.5	<0.1	<0.1	<0.1
Dissolved oxygen (mg/L)	>5.0	>3.0	>7.0	>3

Each tank containing 80 L of freshwater was fitted with a canister filter, an additional airstone and tubing to provide somewhere for the fish to hide. Water quality parameters, described in Table 2, were monitored daily and water quality maintained by daily 30% water exchange when fish were fed commercial fish feed at 8am. Additional monitoring was also undertaken at 3pm.

Table 3. Distribution of fish across rooms

Room	Species	Tanks and fish/tank	Total	Treatment
C3	Carp	4 tanks with 16 fish/tank	64	Inject and bath expose
C4	Silver perch	4 tanks with 16 fish/tank	64	Inject and bath expose
C5	Murray cod	4 tanks with 16 fish/tank	64	Inject and bath expose
C6	Rainbow trout	4 tanks with 16 fish/tank	64	Inject and bath expose
C7	Carp Silver perch	4 tanks with 16 fish/tank 4 tanks with 16 fish/tank	64 64	Negative controls
C8	Murray cod Rainbow trout	4 tanks with 16 fish/tank 4 tanks with 16 fish/tank	64 64	Negative controls

Due to unexpected mortalities within 24 hours of arrival into ACDP, rainbow trout were not available for exposed to CyHV-3.

1.1.3 Exposure to CyHV-3, monitoring and sampling of fish.

For exposure to CyHV-3, carp and Murray cod received an intraperitoneal injection of 50 µL cell cultured CyHV-3 at a concentration of 10^3 $TCID_{50}$/mL. Due to their smaller size, silver perch were intraperitoneally injected with 20-30 µL of inoculum. After injection fish were placed into 20 to 30 L of aerated tank water containing CyHV-3 at a final concentration of 10^2 $TCID_{50}$/mL. Fish were bath exposed for 60 minutes then 16 fish were transferred back into each 80 L tank. Prior to infection with CyHV-3 rooms were set to BSL 3E with a 3-minute shower required on exit and all sample tubes and containers were exposed to Virkon for 10 minutes prior to removal from these rooms.

Fish were monitored twice daily and dead fish sampled. Once disease was observed to have commenced, based on the appearance of mild clinical signs, an additional 11pm monitoring point was added. Depending on the grading of the clinical signs observed, interventions of monitoring for 24 hours or immediate humane killing were implemented (Table 4) and fish sampled. One fish from each tank for each species and each treatment (CyHV-3 exposed and negative controls) was also sampled every four days. Samples taken are described in Table 5. Moribund fish, fish reaching the humane endpoint and fish for planned sampling were humanely killed by addition of 500 µL of Aqui-S anaesthetic to 9 L of water. When fish were heavily sedated, they were removed, killed by cervical dislocation, rinsed in fresh water and sampled. Samples collected are described in Table 5. Samples were collected into both 80% (v/v/) ethanol and RNALater to ensure optimal sample collection for testing DNA (CyHV-3) and RNA (CyHV-3 mRNA). The molecular testing plan was; samples from all fish species were to be tested by the CyHV-3 Gilad qPCR to detect viral DNA. If any fish exhibited clinical signs, then all samples would also be tested by the CyHV-3 Terminase RT-qPCR to detect viral mRNA. Any samples from fish not exhibiting clinical signs, but testing positive for CyHV-3 viral DNA, were also to be tested for CyHV-3 mRNA. As RNALater is typically used for RNA targets (viral RNA and mRNA) testing samples collected into both ethanol and RNAlater for CyHV-3 DNA would give us the opportunity to compare the stability of samples collected for DNA targets in ethanol and RNALater. Non-disposable instruments (i.e. scissors and forceps) were soaked in 1% bleach and rinsed in freshwater between each fish. Fish were exposed to CyHV-3 on 18 May 2021 and the 28-day trial was terminated on 15 June 2021.

Table 4. Grading of clinical signs to reflect the appearance of mild, moderate and severely affected animals and intervention required

Grading	Clinical sign	Intervention
Mild	• Excess mucus production (foam on the water surface). • Localised areas of pale skin due to increased skin mucus • Darkened skin lesions and focal reddening of the skin • Fin and tail rot	Additional monitoring point at 11pm commences
Moderate	• Skin discolouration with raised scales • Small, round skin lesions • Erosion/ulceration of the skin • Sunken eyes. • Fish swim lethargically at the water surface.	Monitoring and humanely killed with 24 hours. As fish will not be identified individually, any appearance of moderate lesions will result in humane killing of the fish at the next 8am monitoring point.
Severe	• Fish gasping constantly at the surface • Nervous signs (shaking, twitching, uncoordinated movements, erratic swimming). • Discoloured, swollen (and necrotic) gill filaments with excess mucus • Dorso-ventral recumbency on aquarium bottom, i.e., lying or resting in the normal upright position. • Lateral recumbency	Immediate human-killing

Table 5. Samples collected for molecular testing and histopathology

Fish Being Sampled	Samples for molecular testing		10% Buffered Neutral Formalin for histology
	80% (v/v) Ethanol CyHV-3 qPCR	RNA Later CyHV-3 mRNA RT-qPCR	
Dead	Gill and kidney Skin lesion (if present)	Gill and kidney Skin lesion (if present)	No (samples too autolysed)
CyHV-3 clinical signs	Gill and kidney Skin lesion (if present)	Gill and kidney Skin lesion (if present)	Gill and kidney
Moribund (non-specific)	Gill and kidney Skin lesion (if present)	Gill and kidney Skin lesion (if present)	Gill and kidney
Sampling Day	Gill and kidney Skin lesion (if present)	Gill and kidney Skin lesion (if present)	No (if no evidence of disease)

1.1.4 Nucleic acid extraction

Tissues were homogenised in 2 ml Lysing Matrix D tubes, containing 600 µl PBSA buffer, using FastPrep-24 or FastPrep-5G bead-beaters (MP Biomedicals). The homogenates were clarified by centrifugation at 10 000 × *g* for 5 min and total nucleic acid extracted from 50 µl of the supernatant using the MagMAX-96™ Viral RNA Isolation Kit on a MagMAX™ Express-96 Deep Well Magnetic Particle Processor (Life Technologies). Nucleic acid was eluted in 50 µl elution buffer and stored at -80°C prior to testing.

1.1.5 Molecular tests

Nucleic acids extracted from samples collected into ethanol and RNAlater were tested using the following assays:

1. CyHV-3 qPCR. This assay described by Gilad et al. (2004) detects CyHV-3 genomic DNA. This assay used the following reaction and cycling conditions; each 25 µl reaction mix contained 2 µl extracted nucleic acid template, 12.5 µl TaqMan Universal PCR Master Mix (Life Technologies), a final concentration of 900 nM for each primer, 250 nM for the CyHV-3-specific probe, 250 nM for the artificial probe and molecular-grade water. The artificial probe was added as an additional risk-mitigation measure for samples tested using the CyHV-3 qPCR to ensure samples testing positive for CyHV-3 were not due to contamination with the positive control plasmids (Moody et al., 2020). The CyHV-3 qPCR assays are performed in a 7500 Fast Real-Time PCR System or QuantStudio 5 Real-Time PCR System (Life Technologies) and analysed with the 7500 software or QuantStudio design and analysis software, respectively. PCR amplifications were programmed as follows: 1 cycle of 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A threshold of 0.1 was used to determine CT value. Nucleic acids were tested in duplicate and two plasmid positive controls, with different expected CT values, a negative extraction control (NEC) and a no template control (NTC) were included on each plate.

2. CyHV-3 Terminase mRNA RT-qPCR. This was in-house assay developed by AFDL for this project in a real-time format to increase the sensitivity of detection of CyHV-3 mRNA. mRNA is transcribed from viral DNA during replication and is a critical component of viral replication therefore detection of CyHV-3 mRNA would indicate an active CyHV-3 infection. This assay used the following reaction and cycling conditions; each 25 µl reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl 2× RT-PCR buffer, 1 µl 25× RT-PCR enzyme mix (AgPath-ID™ One-Step RT-PCR, Life Technologies), a final concentration of 900 nM for each primer, 250 nM for the probe and molecular grade water. The RT-qPCR assays are performed in a 7500 Fast Real-Time PCR System or QuantStudio 5 Real-Time PCR System (Life Technologies) and analysed with the 7500 software or QuantStudio design and analysis software respectively. PCR amplifications are programmed as follows: 30 min reverse transcription at 48°C followed by an initial 10 min denaturation at 95°C followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A threshold of 0.1 is used to determine C_T value. Samples were tested in duplicate and a positive control, a negative extraction control (NEC) and a no template control (NTC) were included for each run.

3. 18S rRNA qPCR (Applied Biosystems) to ensure nucleic acids had been extracted and were free of PCR inhibitors. This assay used the same reactions concentrations and cycling conditions as the CyHV-3 qPCR, with samples tested in singlicate. A negative extraction control (NEC) and a no template control (NTC) were included for each run.

1.1.6 Histopathology

Tissues for histology were processed for paraffin-embedding, sectioning and H&E staining using standard procedures. Sections were examined using a compound light microscope by an expert pathologist who was not provided with the treatment (exposed to CyHV-3, not exposed to CyHV-3 applied to the fish)

1.2. Results

1.2.1 Testing of the inocula

The titre of the inoculum was $10^{4.3}$ $TCID_{50}$/mL with a corresponding CyHV-3 qPCR mean C_T value of 17.8.

1.2.2 Morbidity in negative control and CyHV-3 exposed finfish

1.2.2.1 Carp

Due to observation of a possible *Lernea* spp. infestation, all CyHV-3-exposed and negative control carp were treated on Day 9 post exposure with Coopers Strike (250 g/L diflubenzuron), at a dose rate of 0.066 milligram of diflubenzuron per litre of tank water. The diflubenzuron treatment was repeated for all CyHV-3-exposed and negative control carp on Day 18 post exposure.

No clinical signs of any disease, including koi herpesvirus disease (CyHV-3D) were observed in negative control carp, none of these carp required humane killing and at the termination of the experiment on Day 28, cumulative morbidity was 0% (Figure 1). For the duration of the experiment the mean water temperature was 21.13°C (range of 20.1°C to 22.3°C).

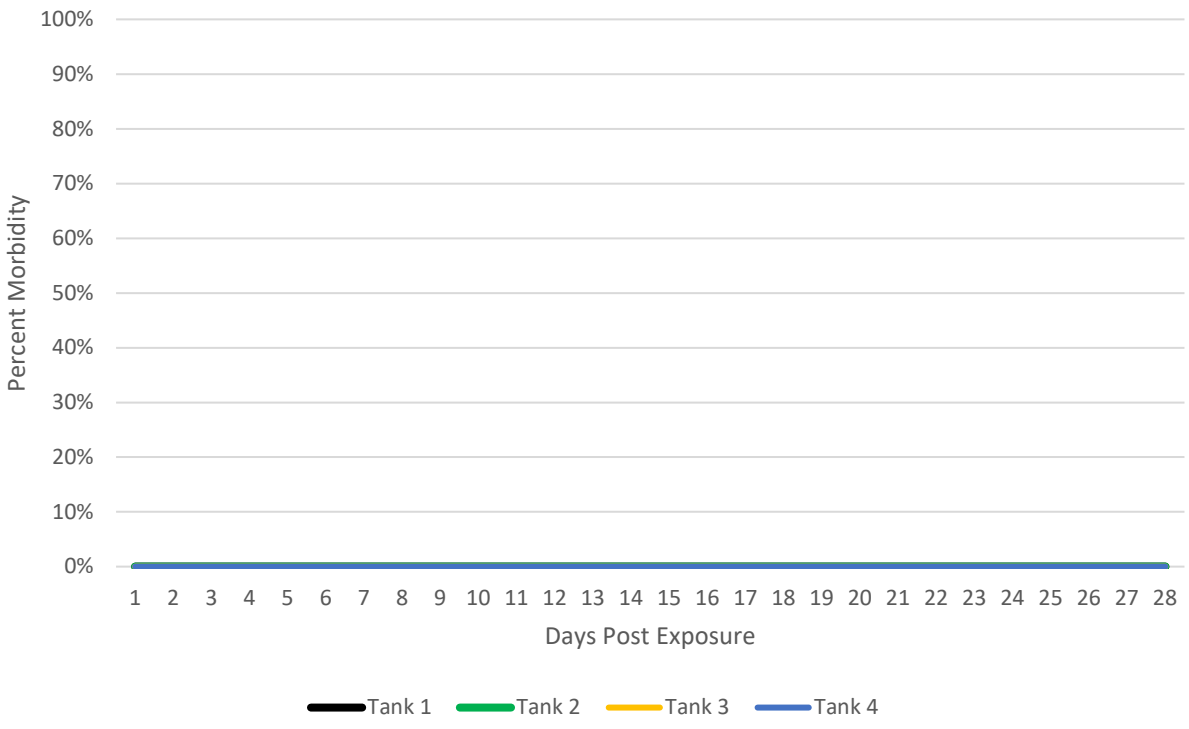

Figure 1. Cumulative morbidity in negative control carp across the four replicate tanks

In carp exposed to CyHV-3, mild clinical signs of disease (Table 4, Figure 3) were observed on Day 2 post exposure and additional 11pm monitoring was implemented. Signs of disease progressed in all four replicate tanks with moderate clinical signs (Figure 4a and 4b) observed from Day 4 to Day 12 post exposure. It is worth noting that it is the same fish in both Figure 4a and 4b, with the images taken a few seconds apart. The darker colour and appearance of discoloured, roughened skin was a lot more apparent when the fish was deeper in the water column. From Day 13 post exposure, moderate clinical signs were no longer observed, the disease appeared to be declining and additional monitoring at 11pm was no

longer required. From Day 18 to the end of the trial on Day 28, no mild clinical signs of CyHV-3D were observed. Across the four tanks 36 carp were humanely killed for welfare reasons due to the presence of moderate clinical signs consistent with CyHV-3D, with cumulative morbidity of 50%, 62.5%, 50% and 62.5% across the four replicate tanks. For the duration of the experiment the mean water temperature was 22.00°C (range of 21.1°C to 22.9°C).

Morbidity between the two treatments was statistically significantly different ($p<0.0001$, Kaplan-Meier survival analysis, MedCalc)

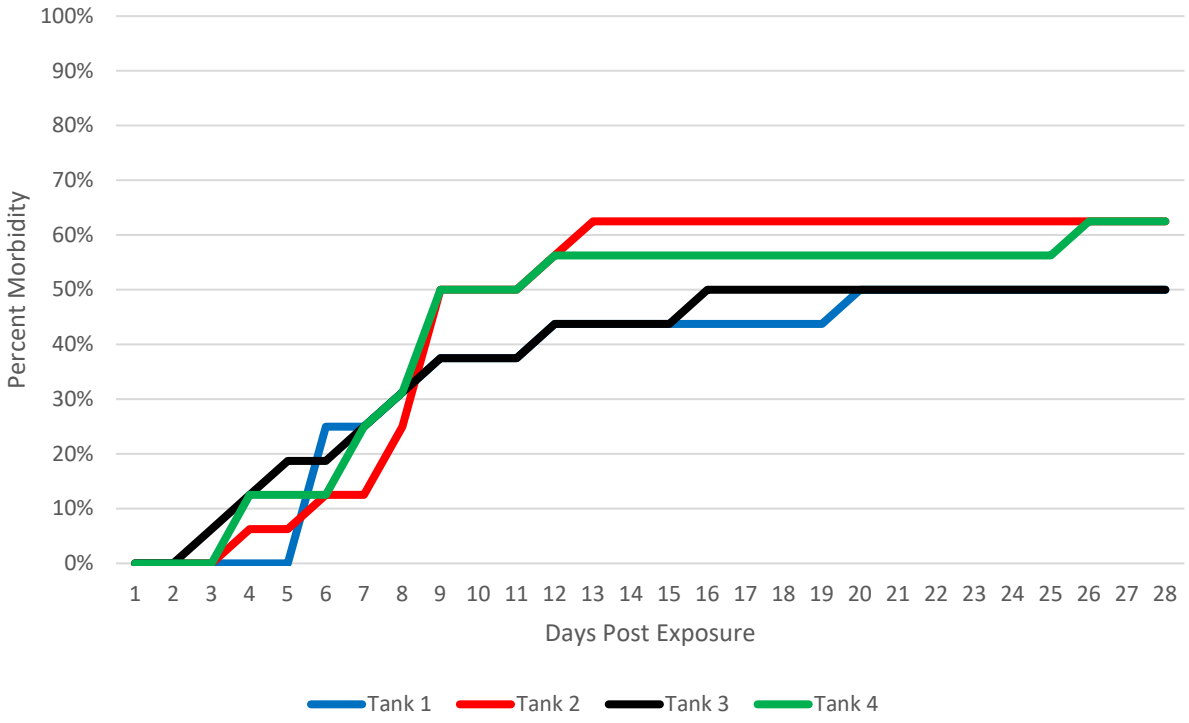

Figure 2a. Cumulative morbidity in carp after exposure to CyHV-3.

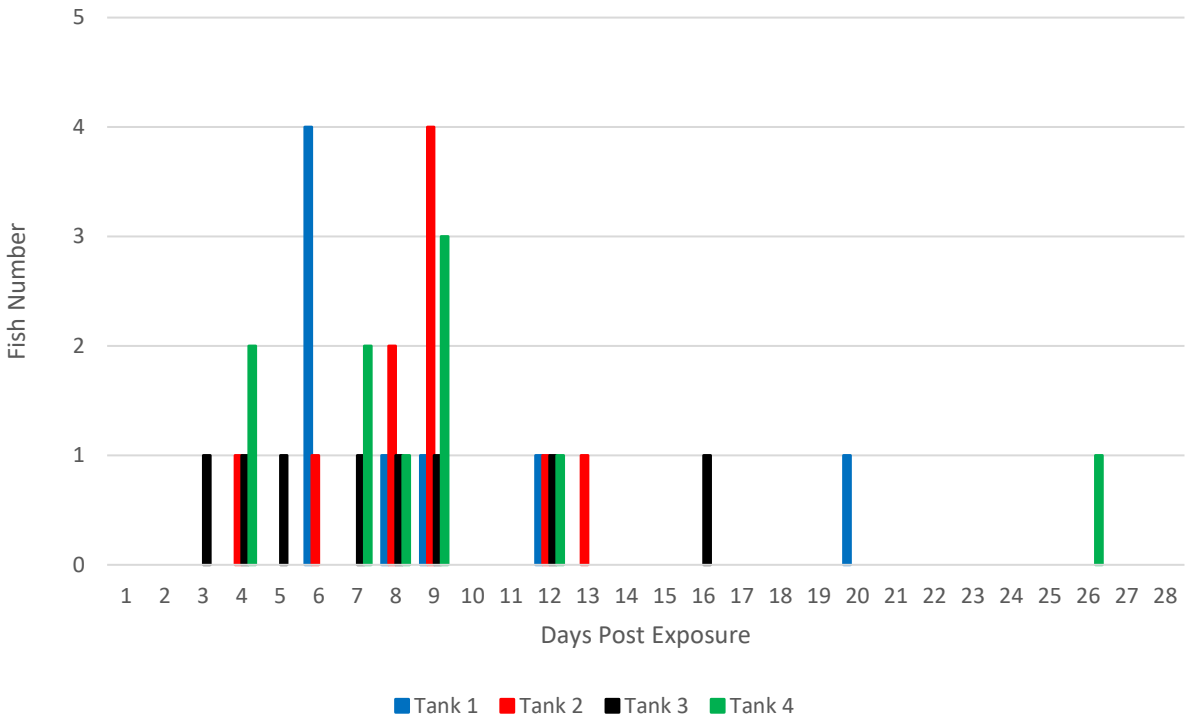

Figure 2b. Daily morbidity in carp after exposure to CyHV-3.

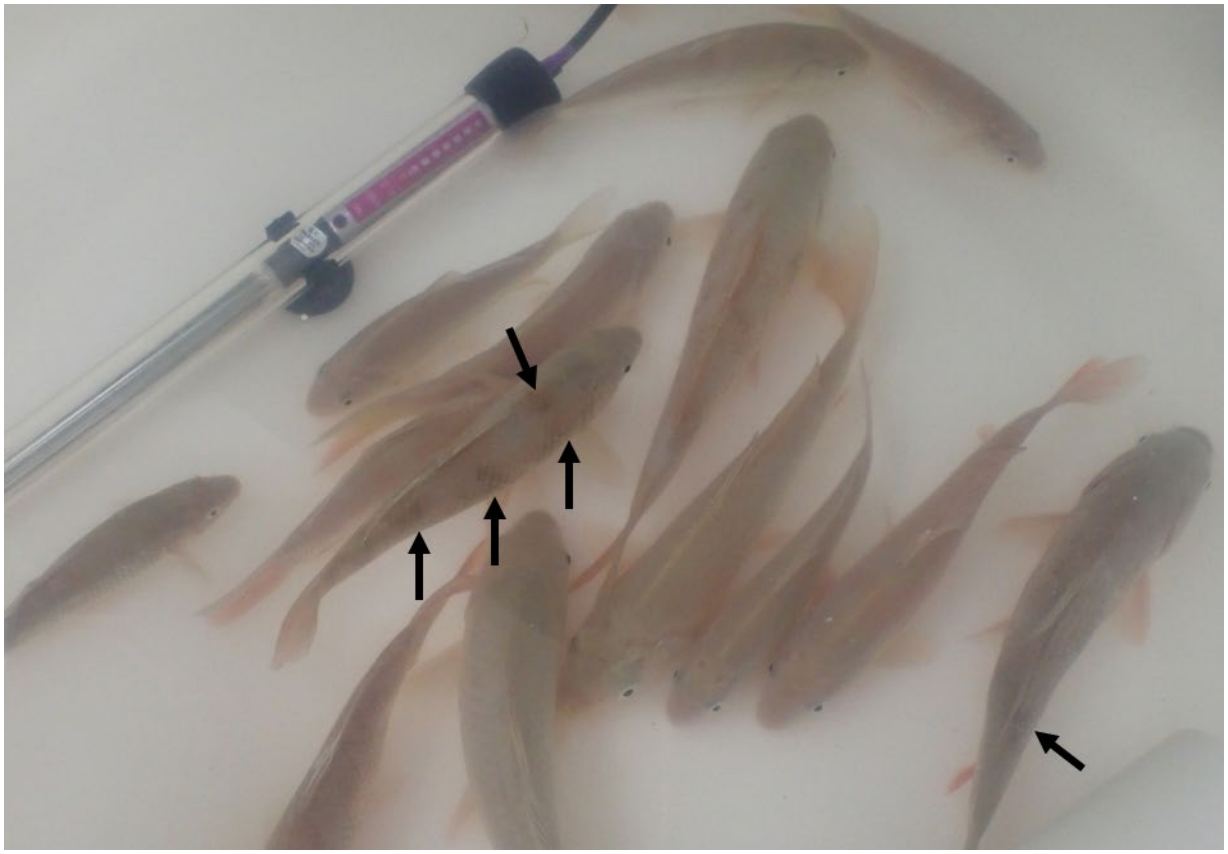

Figure 3. Mild clinical signs in carp after exposure to CyHV-3 (arrow = examples of patches of reddened skin). Day 4 post exposure.

Figure 4a. Moderate clinical signs in carp after exposure to CyHV-3. Day 4 post exposure.

Figure 4b. Moderate clinical signs in carp after exposure to CyHV-3. Day 4 post exposure.

1.2.2.2 Silver perch

No clinical signs of any disease, including koi herpesvirus disease (CyHV-3D) were observed in negative control silver perch. Cumulative morbidity (Figure 5a) ranging from 6% to 26% across the four tanks was observed due to seven fish found dead (Figure 5b) from natural attrition during the 28-day trial in the negative control room. There were no consistent clinical signs indicative of a specific disease observed in

any of these silver perch. For the duration of the experiment the mean water temperature was 21.66°C (range of 20.8°C to 22.3°C).

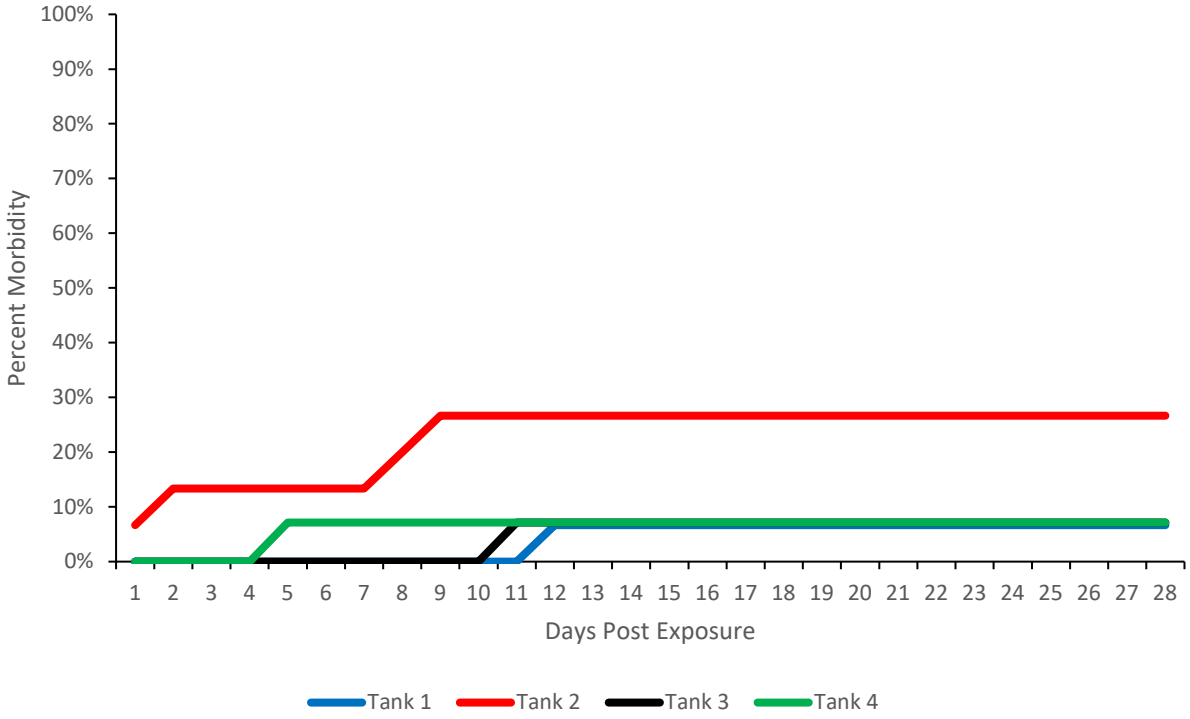

Figure 5a. Cumulative morbidity in negative control silver perch across the four replicate tanks.

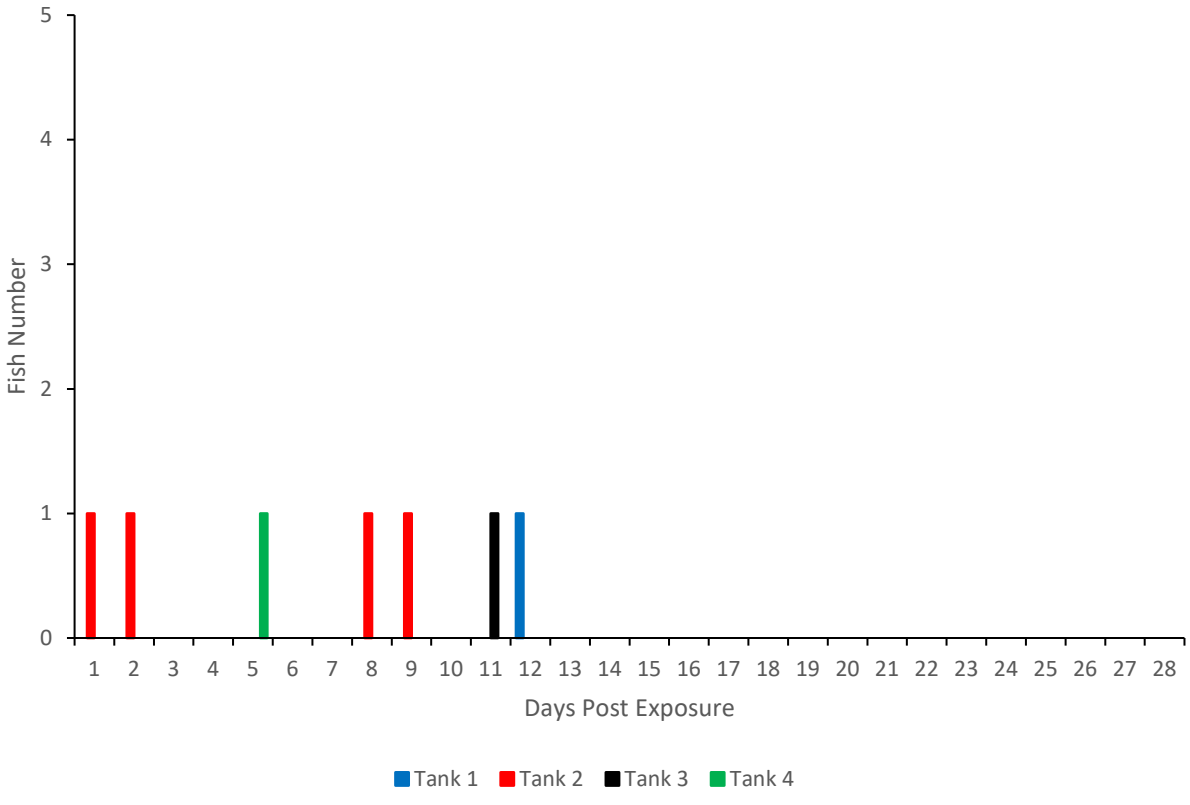

Figure 5b. Daily morbidity in negative control silver perch across the four replicate tanks.

No clinical signs of any disease, including koi herpesvirus disease (CyHV-3D) were observed in silver perch exposed to CyHV-3. Cumulative morbidity (Figure 6a) ranging from 14% to 25% across the four tanks was observed due to 11 dead fish, and two moribund fish that were humanely killed due to welfare reasons, during the 28-day trial (Figure 6b). There were no consistent clinical signs indicative of a specific disease observed in any of these silver perch. For the duration of the experiment the mean water temperature was 21.55°C (range of 20.4°C to 23.6°C).

Morbidity between the two treatments (CyHV-3 exposed and negative control silver perch) was not statistically significantly different (p=0.2663, Kaplan-Meier survival analysis, MedCalc)

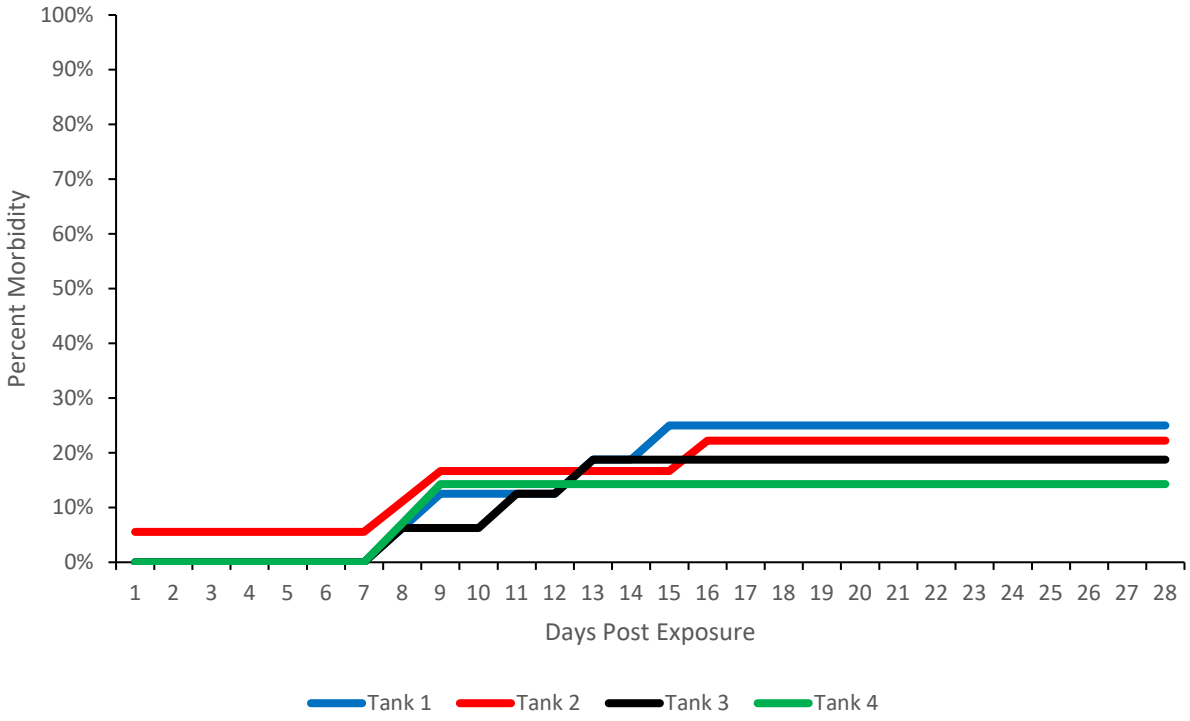

Figure 6a. Cumulative morbidity in silver perch after exposure to CyHV-3.

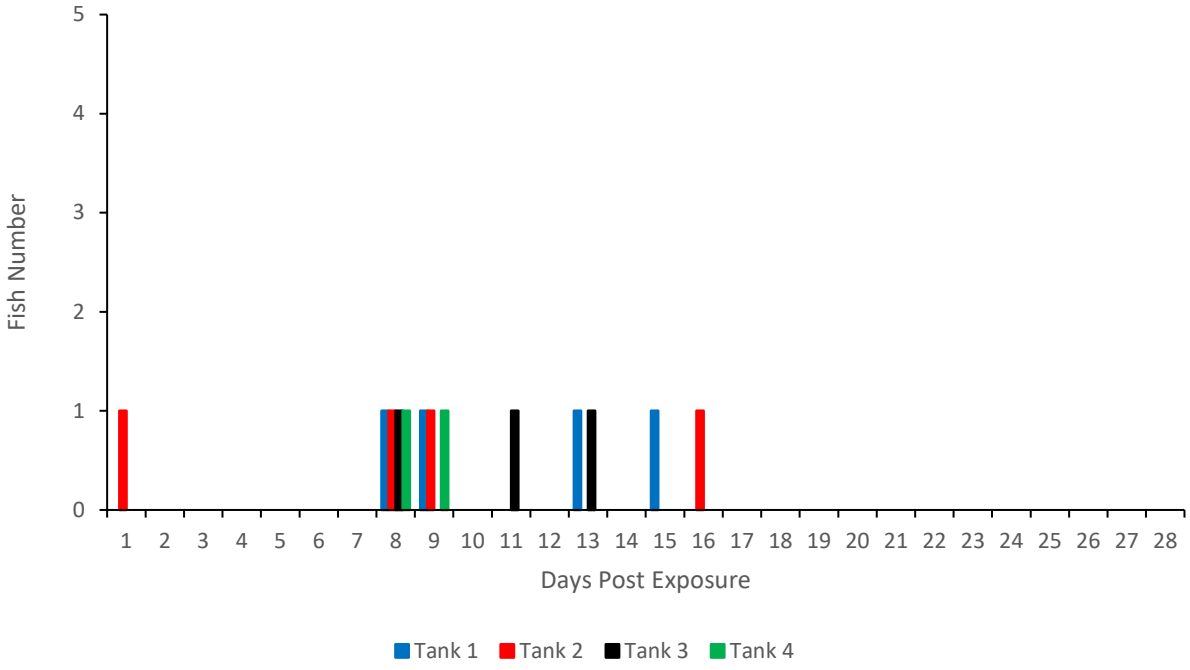

Figure 6b. Daily morbidity in silver perch after exposure to CyHV-3.

1.2.2.3 Murray cod

No clinical signs indicative of koi herpesvirus disease (CyHV-3) was observed in negative control Murray cod. Cumulative morbidity (Figure 7a) ranging from 0% to 25% across the four tanks was observed due to four dead fish and three moribund fish, that were humanely killed due to welfare reasons (Figure 7b), during the 28-day trial in the negative control room. There were no consistent clinical signs indicative of a specific disease observed in any of these Murray cod. For the duration of the experiment the mean water temperature was 22.37°C (range of 21.17°C to 24.0°C).

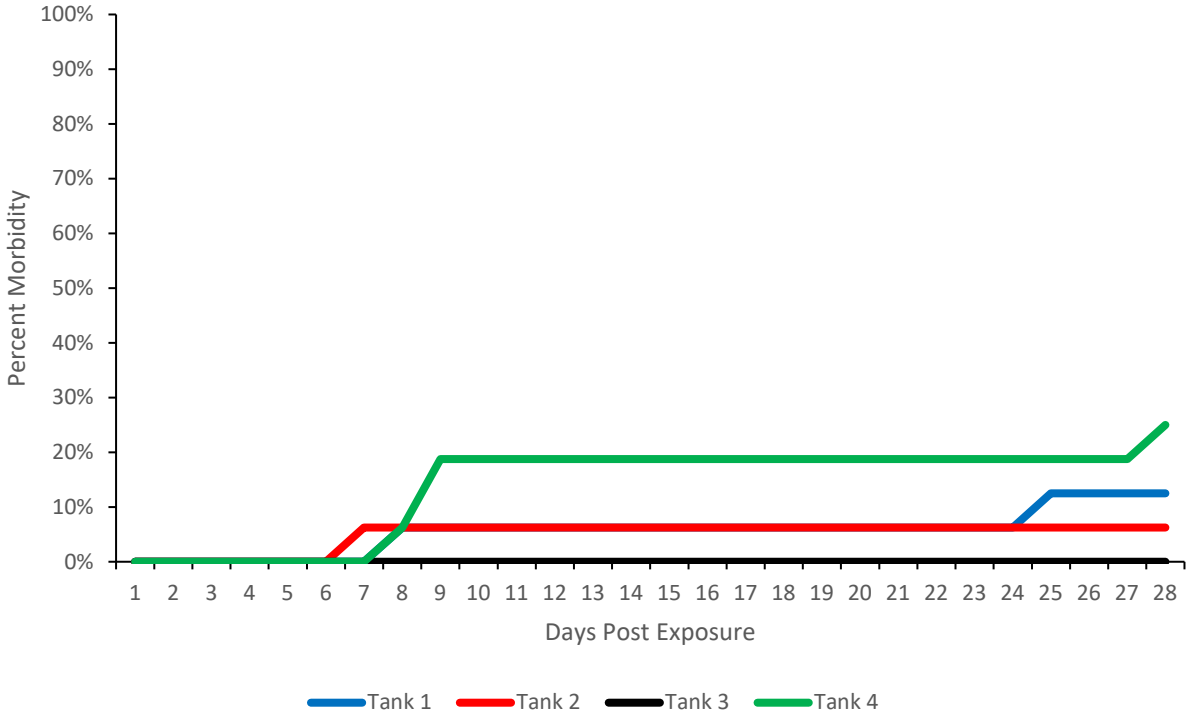

Figure 7a. Cumulative morbidity in negative control Murray cod across the four replicate tanks.

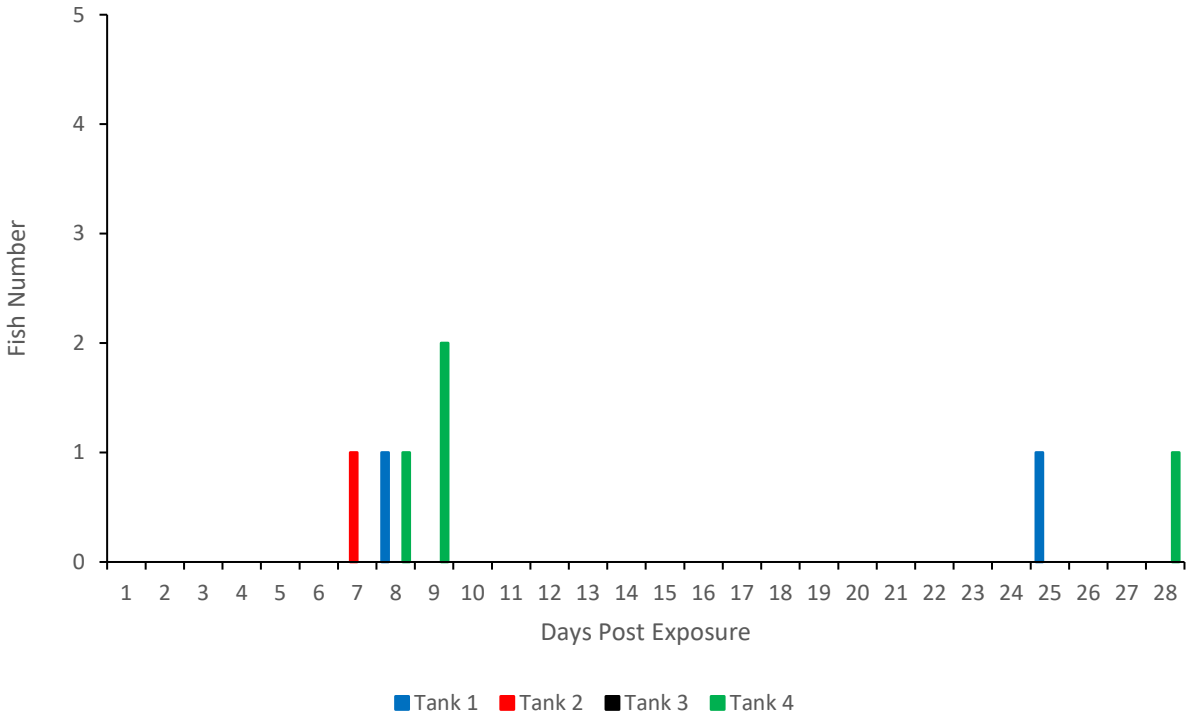

Figure 7b. Daily morbidity in negative control Murray cod across the four replicate tanks.

No clinical signs indicative of koi herpesvirus disease (CyHV-3D) was observed in Murray cod exposed to CyHV-3. Cumulative morbidity (Figure 8a) ranging from 6% to 18% across the four tanks was observed due to five dead fish and two moribund fish that were humanely killed due to welfare reasons during the 28-day trial (Figure 8b). For the duration of the experiment the mean water temperature was 21.67°C (range of 20.5°C to 22.0°C).

Morbidity between the two treatments (CyHV-3 exposed and negative control Murray cod) was not statistically significantly different (p=0.9685, Kaplan-Meier survival analysis, MedCalc)

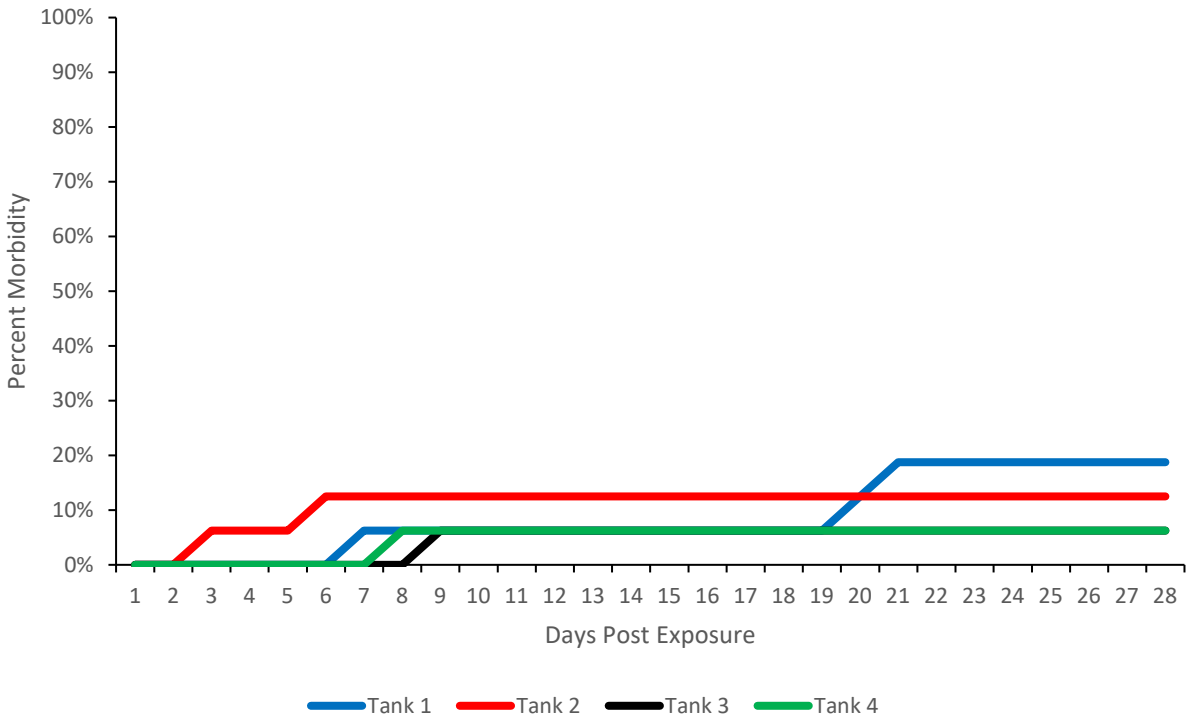

Figure 8a. Cumulative morbidity in Murray cod after exposure to CyHV-3.

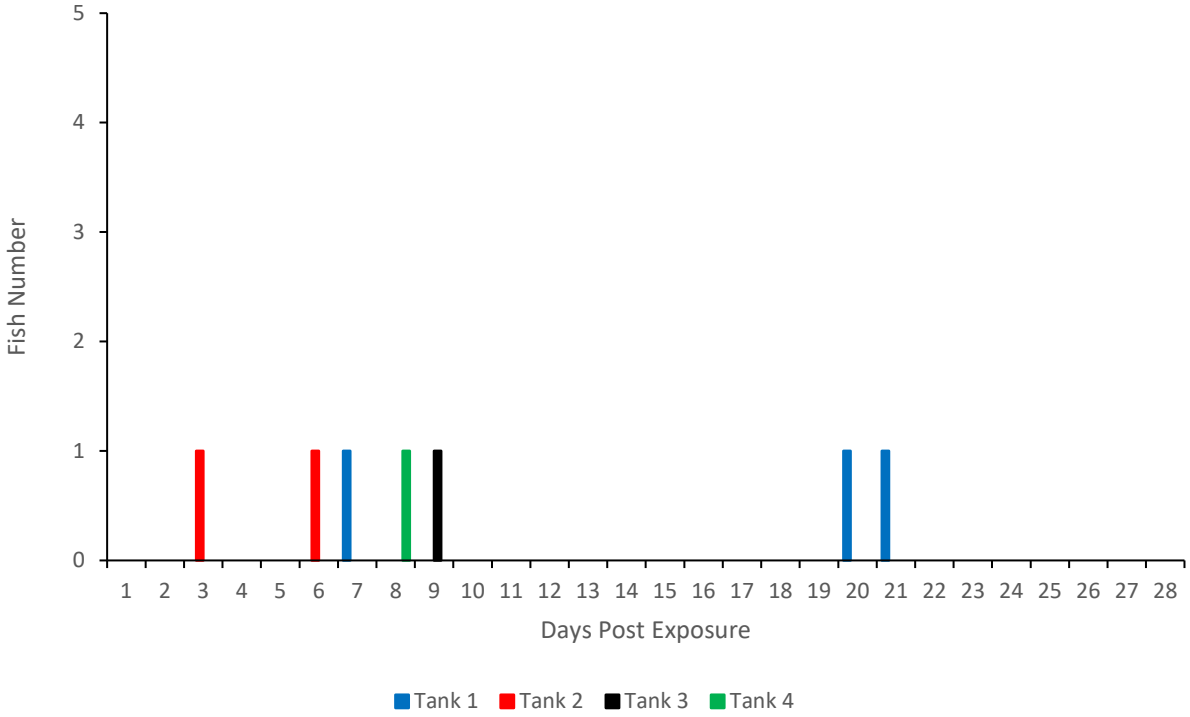

Figure 8b. Daily morbidity in Murray cod after exposure to CyHV-3.

1.2.3 Molecular testing of negative control and CyHV-3-exposed finfish

1.2.3.1 Negative control carp, Murray cod and silver perch

No gill or kidney tissue samples from negative control carp, Murray cod and silver perch were test positive for CyHV-3 using the Gilad CyHV-3 qPCR (Table 6, 7 and 8). As there were no positive detections for CyHV-3 DNA the samples obtained from the negative control fish were not tested for the presence of CyHV-3 mRNA.

Table 6. Gilad CyHV-3 qPCR results for negative control carp

Carp Negative control	Sample type	No of Samples	CyHV-3 Gilad qPCR (Ethanol Fixed)			
			Positive	Indeterminate#	Negative	C_T Range
Tank 1	Gill	16	0	0	16	N/A
	Kidney	16	0	0	16	N/A
	Skin	0	N/A	N/A	N/A	N/A
Tank 2	Gill	16	0	0	16	N/A
	Kidney	16	0	0	16	N/A
	Skin	1	0	0	1	N/A
Tank 3	Gill	16	0	0	16	N/A
	Kidney	16	0	0	16	N/A
	Skin	0	N/A	N/A	N/A	N/A
Tank 4	Gill	16	0	0	16	N/A
	Kidney	16	0	0	16	N/A
	Skin	0	N/A	N/A	N/A	N/A
All tanks		**129**	**0**	**0**	**129**	**N/A**

#Indeterminate = one well positive and one well negative

Table 7. Gilad CyHV-3 qPCR results for negative control Murray cod

Murray cod Negative control	Sample type	No of Samples	CyHV-3 Gilad qPCR (Ethanol Fixed)			
			Positive	Indeterminate#	Negative	C_T Range
Tank 1	Gill	15	0	0	15	N/A
	Kidney	16	0	0	16	N/A
	Skin	0	N/A	N/A	N/A	N/A
Tank 2	Gill	16	0	0	16	N/A
	Kidney	16	0	0	16	N/A
	Skin	0	N/A	N/A	N/A	N/A
Tank 3	Gill	15	0	0	15	N/A
	Kidney	15	0	0	15	N/A
	Skin	0	N/A	N/A	N/A	N/A
Tank 4	Gill	16	0	0	16	N/A
	Kidney	16	0	0	16	N/A
	Skin	0	N/A	N/A	N/A	N/A
All tanks		**125**	**0**	**0**	**125**	**N/A**

#Indeterminate = one well positive and one well negative

Table 8. Gilad CyHV-3 qPCR results for negative control silver perch

Silver perch Negative control	Sample type	No of Samples	CyHV-3 Gilad qPCR (Ethanol Fixed)			
			Positive	Indeterminate#	Negative	C_T Range
Tank 1	Gill	15	0	0	15	N/A
	Kidney	15	0	0	15	N/A
	Skin	0	N/A	N/A	N/A	N/A
Tank 2	Gill	15	0	0	15	N/A
	Kidney	15	0	0	15	N/A
	Skin	0	N/A	N/A	N/A	N/A
Tank 3	Gill	13	0	0	13	N/A
	Kidney	14	0	0	14	N/A
	Skin	0	N/A	N/A	N/A	N/A
Tank 4	Gill	14	0	0	14	N/A
	Kidney	14	0	0	14	N/A
	Skin	0	N/A	N/A	N/A	N/A
All tanks		**115**	**0**	**0**	**115**	**N/A**

#Indeterminate = one well positive and one well negative

1.2.3.2 CyHV-3 exposed carp, Murray cod and silver perch

All samples collected into 80% (v/v) ethanol from CyHV-3-exposed carp, Murray cod and silver perch were initially screened for the presence of CyHV-3 DNA using the CyHV-3 Gilad qPCR. This included samples collected from moribund fish collected during the trial and those humanly killed at the termination of the trial on Day 28. All carp samples collected into RNAlater were tested using the CyHV-3 Gilad qPCR and the AFDL CyHV-3 Terminase RT-qPCR. Due to the low level of mortality and detection of CyHV-3 DNA in initial screening using the CyHV-3 Gilad PCR for ethanol-fixed samples from Murray cod and silver perch, only the corresponding RNALater samples from positive or indeterminate ethanol-fixed samples from these species were tested using the CyHV-3 Gilad qPCR and the AFDL CyHV-3 Terminase RT-qPCR. Summary details of the test results are in Table 9.

Table 9. Summary molecular test results for carp, Murray cod and silver perch exposed to CyHV-3

Species	No of Samples	CyHV-3 Gilad qPCR (Ethanol Fixed)			No of Samples	CyHV-3 Gilad qPCR (RNALater Fixed)			No of Samples	CyHV-3 Terminase RT-qPCR (RNALater Fixed)		
		POS	Indet	NEG		POS	Indet	NEG		POS	Indet	NEG
Carp	152	100	13	39	148	100	7	41	148	65	9	14
Murray cod	128	1	4	124	5	1	1	3	5	0	0	5
Silver perch	125	5	5	115	10	4	0	6	10	0	0	10

#Indeterminate = one well positive and one well negative

Of the 152 ethanol fixed samples collected from CyHV-3-exposed carp, 74.3% were test positive or indeterminate (i.e., not test negative) for CyHV-3 DNA when screened using the CyHV-3 Gilad PCR. In contrast, only 3.9% of CyHV-3-exposed Murray cod and 8% of CyHV-3-exposed silver perch samples tested positive or indeterminate when ethanol-fixed samples were screened for CyHV-3 DNA. Comparative results were obtained when RNALater samples were tested for carp using the CyHV-3 Gilad PCR where 72.3% of samples tested positive of indeterminate. When samples were tested using the CyHV-3 Terminase RT-qPCR to detect CyHV-3 mRNA, indicative of replicating virus, 50% of CyHV-3-exposed carp were test positive or indeterminate. All Murray cod and silver perch samples were test negative for CyHV-

3 mRNA (Table 9). This indicates that replicating CyHV-3 (i.e. an active infection) was detected in exposed carp but not in exposed silver perch and Murray cod.

In carp exposed to CyHV-3, 68.4%, 80.5%, 63.9% and 81.1% of ethanol-fixed samples were test positive or indeterminate using the CyHV-3 Gilad assay across the four replicate tanks. All skin samples were test positive for CyHV-3 DNA. While the CT values ranged from 17.03 to 39.39 (Table 10), the lowest CT values were observed during the first 10 days of the trial (Figure 9) and no CyHV-3 DNA was detected in skin after Day 16 post-exposure as no further skin samples were collected as the disease was reducing in severity and no carp met the criteria for humane killing. Detection of mRNA of CyHV-3 in samples preserved in RNALater was from Day to Day 16 post exposure (Figure 10). No detection of mRNA form Day 17 corresponded with a reduction in clinical signs. Gilad CyHV-3 DNA C_T values are included in Figure 10, as this test was used as a positive control for the mRNA assay, and differences in C_T value to those in Figure 9 are due to a different piece of tissue sampled and the preservative used.

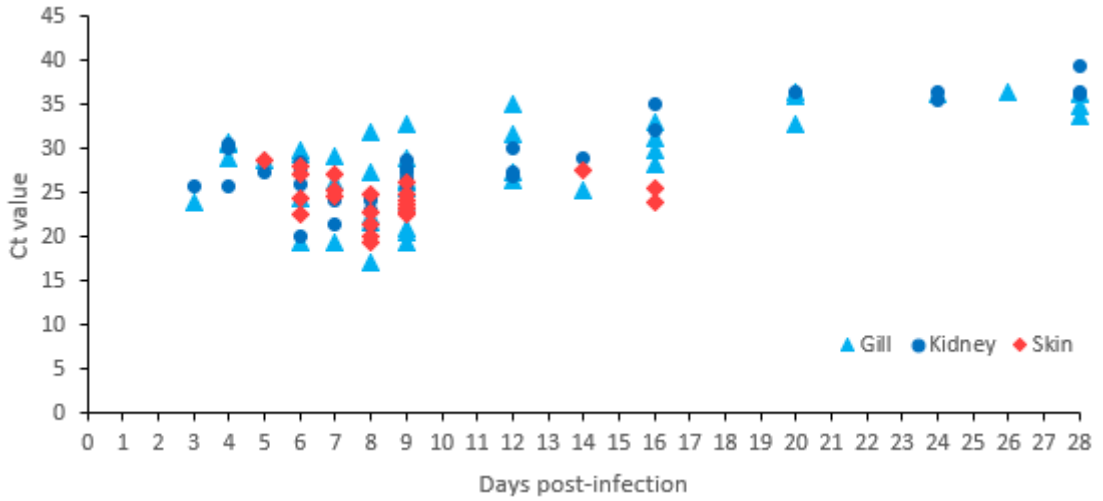

Figure 9. Detection of CyHV-3 DNA in different organs of CyHV-3-exposed carp across all tanks

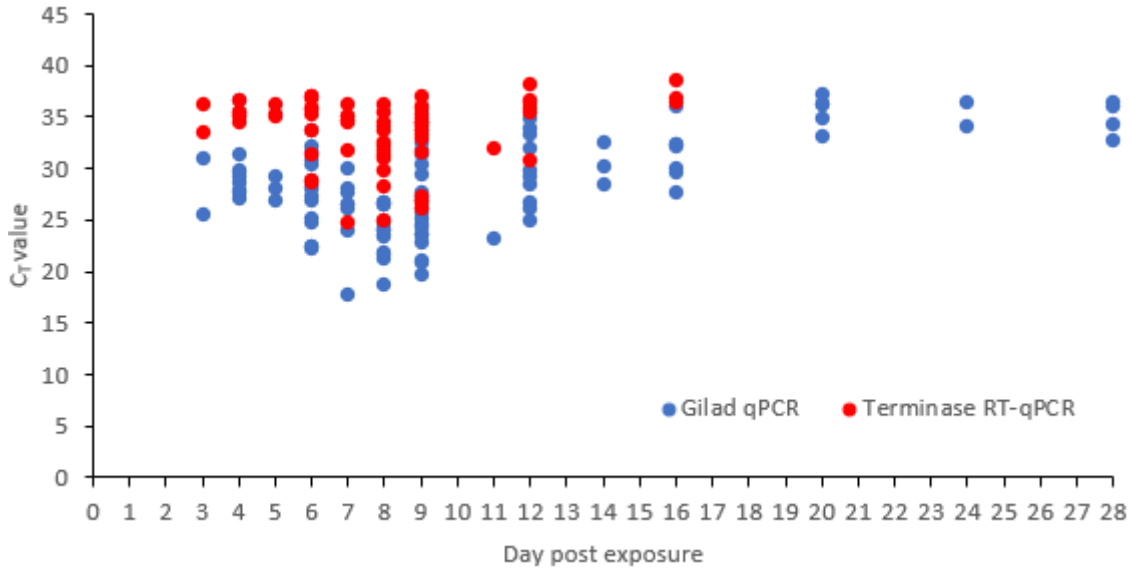

Figure 10. Detection of CyHV-3 DNA (Gilad qPCR) and CyHV-3 mRNA (Terminase RT-PCR) of CyHV-3-exposed carp across all tanks

Table 10. Gilad CyHV-3 qPCR and AFDL CyHV-3 Terminase RT-qPCR results for CyHV-3-exposed carp. Results are mean C_T values of samples tested in duplicate.

Carp CyHV-3 Challenged	Sample type	No of Samples	CyHV-3 Gilad qPCR (Ethanol Fixed)				No of Samples	CyHV-3 Gilad qPCR (RNALater Fixed)				No of Samples	CyHV-3 Terminase RT-qPCR (RNALater Fixed)			
			POS	Indet	NEG	C_T Range		POS	Indet	NEG	C_T Range		POS	Indet	NEG	C_T Range
Tank 1	Gill	16	10	0	6	19.37-35.92	16	9	1	6	22.42-36.21	16	2	0	14	28.07-33.13
	Kidney	16	9	1	6	20.03-39.39	16	9	0	7	22.21-33.28	16	4	2	10	28.65-37.63
	Skin	6	6	0	0	19.38-27.97	6	6	0	0	21.83-30.74	6	5	0	1	29.70-36.35
	TOTAL	**38**	**25**	**1**	**12**	**19.38-39.39**	**38**	**24**	**1**	**13**	**21.83-36.21**	**38**	**11**	**2**	**25**	**28.07-37.63**
Tank 2	Gill	16	12	1	3	17.03-36.10	16	12	0	4	18.67-33.11	16	7	2	7	24.87-36.34
	Kidney	16	10	2	4	23.05-36.29	16	10	2	4	24.71-36.18	16	8	0	8	32.30-36.50
	Skin	9	9	0	0	21.32-27.50	9	9	0	0	22.43-30.17	9	7	1	1	32.04-37.06
	TOTAL	**41**	**31**	**3**	**7**	**17.03-36.29**	**41**	**31**	**2**	**8**	**18-67-36.18**	**41**	**22**	**3**	**16**	**24.87-37.06**
Tank 3	Gill	16	8	2	6	22.23-36.51	16	9	1	6	23.66-34.88	16	6	1	9	31.05-36.76
	Kidney	16	8	1	7	24.17-36.29	14	7	1	6	24.92-37.12	14	4	1	9	30.73-35.39
	Skin	4	4	0	0	24.11-28.57	4	4	0	0	22.83-29.29	4	3	1	0	31.74-36.25
	TOTAL	**36**	**20**	**3**	**13**	**22.23-36.51**	**34**	**20**	**2**	**12**	**22.83-37.12**	**34**	**13**	**3**	**18**	**30.73-36.76**
Tank 4	Gill	16	9	4	3	19.39-36.30	14	10	0	4	21.32-36.06	14	6	0	8	24.78-38.58
	Kidney	15	9	2	4	21.12-36.34	15	9	2	4	23.58-36.67	15	7	1	7	31.25-38.19
	Skin	6	6	0	0	20.03-25.38	6	6	0	0	21.75-26.81	6	6	0	0	32.40-36.00
	TOTAL	**37**	**24**	**6**	**7**	**20.03-36.34**	**35**	**25**	**2**	**8**	**21.32-36.67**	**35**	**19**	**1**	**15**	**27.7-28.58**
All tanks TOTAL		**152**	**100**	**13**	**39**	-	**148**	**100**	**7**	**41**	-	**148**	**65**	**9**	**74**	-

#Indeterminate = one well positive and one well negative

In Murray cod exposed to CyHV-3, 3%, 3%, 3% and 6.3% of ethanol-fixed samples were test positive or indeterminate using the CyHV-3 Gilad assay across the four replicate tanks. No skin samples were tested as no skin lesions were observed in any Murray cod. The C_T values for all positive or indeterminate samples were all greater than 36 (Table 11) indicating a very low relative viral load. While 4 of the 5 CyHV-3 Gilad PCR positive or indeterminate results were obtained from Day 4 to Day 8 post exposure, one kidney sample form a Murray cod humanely killed at the termination of the trial on Day 28 tested indeterminate with a C_T value of 37 (Figure 11).

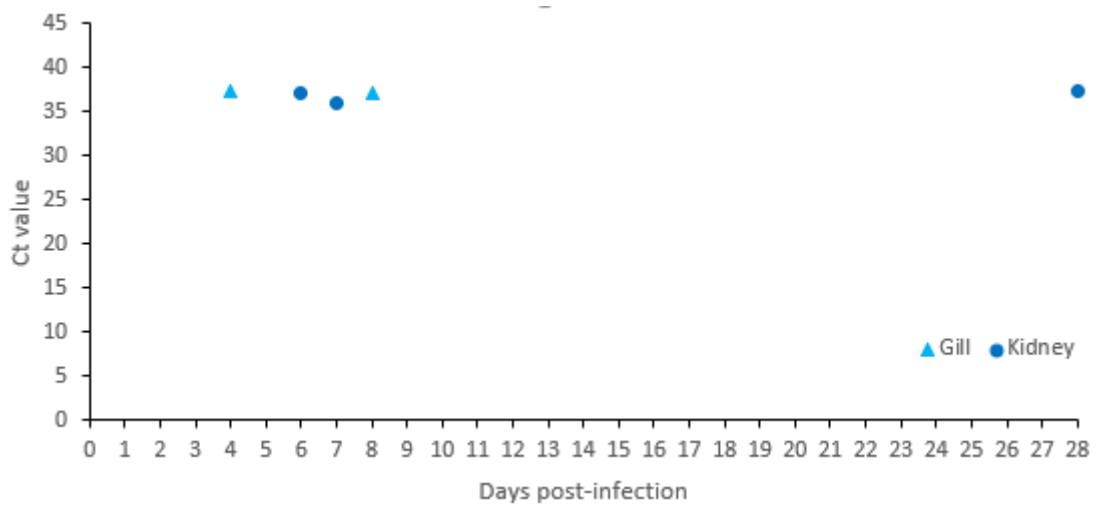

Figure 11. Detection of CyHV-3 DNA in different organs of CyHV-3-exposed Murray cod across all tanks

In silver perch exposed to CyHV-3, 3.3%, 11.1%, 6.25% and 7.4% of ethanol-fixed samples were test positive or indeterminate using the CyHV-3 Gilad assay across the four replicate tanks. No skin samples were tested as no skin lesions were observed in any silver perch. While two C_T values for a gill and kidney sample form two fish were 31.38 and 33.51, the C_T all other positive or indeterminate samples were greater than 35 (Table 12) indicating a very low relative viral load. All CyHV-3 Gilad PCR positive or indeterminate results were obtained from Day 1 to Day 14 post exposure (Figure 12).

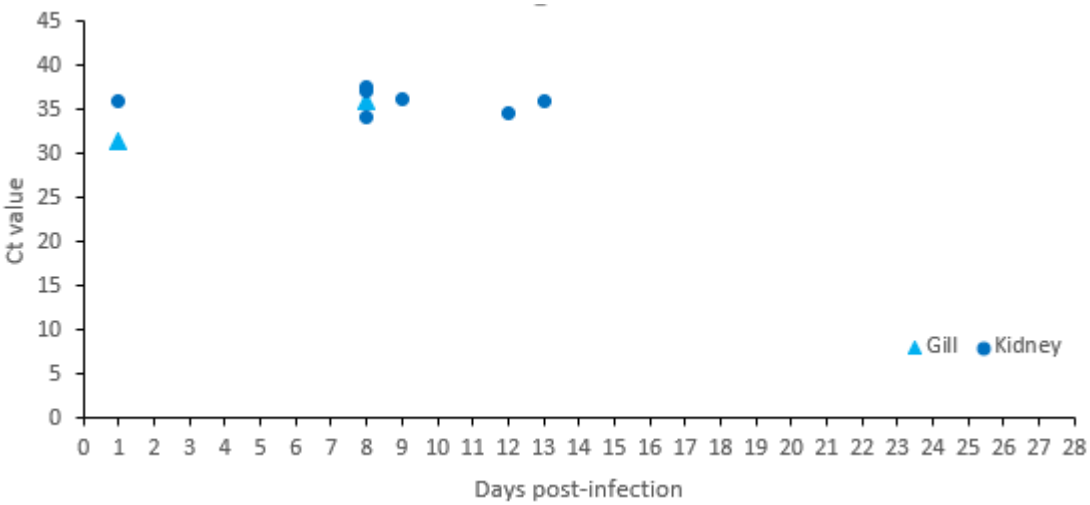

Figure 12. Detection of CyHV-3 DNA in different organs of CyHV-3-exposed silver perch across all tanks

Table 11. Gilad CyHV-3 qPCR and AFDL CyHV-3 Terminase RT-qPCR results for CyHV-3-exposed Murray cod. Results are mean C_T values of samples tested in duplicate.

Murray cod CyHV-3 Challenged	Sample type	No of Samples	CyHV-3 Gilad qPCR (Ethanol Fixed)				No of Samples	CyHV-3 Gilad qPCR (RNALater Fixed)				No of Samples	CyHV-3 Terminase RT-qPCR (RNALater Fixed)			
			POS	Indet	NEG	C_T Range		POS	Indet	NEG	C_T Range		POS	Indet	NEG	C_T Range
Tank 1	Gill	16	1	0	15	37.22	1	1	0	0	35.91	0	0	0	1	-
	Kidney	16	0	0	16	-	0	-	-	-	-	0	-	-	-	-
	Skin	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
	TOTAL	**32**	**1**	**0**	**31**	**37.22**	**0**	**1**	**0**	**0**	**35.91**	**0**	**0**	**0**	**1**	**-**
Tank 2	Gill	16	0	0	16	-	0	-	-	-	-	0	-	-	-	-
	Kidney	16	0	1	15	37.18	1	0	0	1	-	1	0	0	1	-
	Skin	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
	TOTAL	**32**	**0**	**1**	**31**	**37.18**	**1**	**0**	**0**	**1**	**0**	**1**	**0**	**0**	**1**	**-**
Tank 3	Gill	16	0	0	16	-	0	-	-	-	-	0	-	-	-	-
	Kidney	16	0	1	15	37.20	1	0	0	1	-	1	0	0	1	-
	Skin	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
	TOTAL	**32**	**0**	**1**	**31**	**37.20**	**1**	**0**	**0**	**1**	**0**	**1**	**0**	**0**	**1**	**-**
Tank 4	Gill	16	0	1	15	37.10	1	0	1	0	36.03	1	0	0	1	-
	Kidney	16	0	1	15	36.02	1	0	0	1	-	1	0	0	1	-
	Skin	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
	TOTAL	**32**	**0**	**2**	**30**	**36.02-37.10**	**2**	**0**	**1**	**1**	**36.03**	**2**	**0**	**0**	**2**	**-**
All tanks TOTAL		**128**	**1**	**4**	**123**	**-**	**5**	**1**	**1**	**3**	**-**	**5**	**0**	**0**	**5**	**-**

#Indeterminate = one well positive and one well negative

Table 12. Gilad CyHV-3 qPCR and AFDL CyHV-3 Terminase RT-qPCR results for CyHV-3-exposed silver perch. Results are mean C_T values of samples tested in duplicate.

Silver perch CyHV-3 Challenged	Sample type	No of Samples	CyHV-3 Gilad qPCR (Ethanol Fixed)				No of Samples	CyHV-3 Gilad qPCR (RNALater Fixed)				No of Samples	CyHV-3 Terminase RT-qPCR (RNALater Fixed)			
			POS	Indet	NEG	C_T Range		POS	Indet	NEG	C_T Range		POS	Indet	NEG	C_T Range
Tank 1	Gill	16	0	1	15	35.89	1	0	0	1	-	1	0	0	1	-
	Kidney	14	1	0	13	33.51	1	0	0	1		1	0	0	1	-
	Skin	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
	TOTAL	**30**	**1**	**1**	**28**	**33.51-35.89**	**2**	**0**	**0**	**2**	**36.29-37.18**	**2**	**0**	**0**	**2**	**-**
Tank 2	Gill	18	1	0	17	31.38	1	1	0	0	32.70	1	0	0	1	-
	Kidney	18	2	1	15	34-62-37.17	3	2	0	1	34.00-35.78	3	0	0	3	-
	Skin	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
	TOTAL	**36**	**3**	**1**	**32**	**31.38-37-17**	**4**	**3**	**0**	**1**	**32.70-37.18**	**4**	**0**	**0**	**4**	**-**
Tank 3	Gill	16	0	0	16	-	0	-	-	-	-	0	-	-	-	-
	Kidney	16	1	1	14	36.06-34.12	2	1	0	1	35.8	2	0	0	2	-
	Skin	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
	TOTAL	**32**	**1**	**1**	**30**	**36.06-34.12**	**2**	**1**	**0**	**1**	**35.8**	**2**	**0**	**0**	**2**	**-**
Tank 4	Gill	14	0	0	14	-	0	-	-	-	-	0	-	-	-	-
	Kidney	13	0	2	11	36.11-37.46	2	0	0	2	-	2	0	0	2	-
	Skin	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
	TOTAL	**27**	**0**	**2**	**25**	**36.11-37.46**	**2**	**0**	**0**	**2**	**-**	**2**	**0**	**0**	**2**	**-**
All tanks TOTAL		**125**	**5**	**5**	**115**		**10**	**4**	**0**	**6**		**10**	**0**	**0**	**10**	**-**

#Indeterminate = one well positive and one well negative

After exposure of the three species of fish to CyHV-3 by intraperitoneal injection and immersion, positive and indeterminate CyHV-3 Gilad qPCR test results of 74.3%, 3.9% and 8% were obtained for samples from carp, Murray cod and silver perch, respectively. Mixed measures ANOVA using the Bonferroni post-test correction was used to compare the CyHV-3 Gilad qPCR C_T values obtained for carp, silver perch and Murray cod (acknowledging the limited data set of only 5 and 1 positive results for silver perch and Murray cod, respectively) found no statistically significant difference between the C_T values for silver perch and Murray cod ($p>0.9999$). However, there were statistically significant differences between the C_T results for carp and silver perch ($p<0.0001$) and carp and Murray cod ($p=0.0141$). When samples were tested using the CyHV-3 Terminase RT-qPCR to detect CyHV-3 mRNA, indicative of replicating virus, 50% of CyHV-3-exposed carp were test positive or indeterminate. All Murray cod and silver perch samples were test negative for CyHV-3 mRNA.

Based on molecular testing, the high level of CyHV-3 Gilad qPCR and CyHV-3 Terminase RT-qPCR positive and indeterminate results for the carp exposed to CyHV-3, indicates that this species was infected with CyHV-3. The low level of CyHV-3 Gilad qPCR positive and indeterminate results and all samples testing negative using the CyHV-3 Terminase RT-qPCR for the Murray cod and silver perch exposed to CyHV-3, indicates that these species were not infected with CyHV-3. The detection of CyHV-3 DNA in the silver perch and Murray cod may be due to residual CyHV-3 in the environment (e.g., fomites).

Testing samples collected into both ethanol and RNALater for testing for KHV DNA using the CyHV-3 Gilad qPCR indicated that overall test sensitivity was slightly higher for samples collected into ethanol (Table 10). This is consistent with standard practices in place in AFDL and other laboratories and mitigates against the biosecurity risk of using of RNALater where infectious virus has been isolated form tissues fixed in RNALater (Authors pers. obs.)

1.2.4 Histological examination of carp, silver perch and Murray cod.

1.2.4.1 Carp

Four gill and kidney sections were examined by histopathology from negative control carp. All gills had a low-grade, generalised inflammatory cell infiltration throughout the primary lamellae. Low-grade to moderate proliferative changes (hyperplasia & hypertrophy) were evident in the primary lamellar epithelium of all gills. Low-grade or equivocal hyperplasia of secondary lamellar epithelium accompanied by low-grade to moderate hypertrophy was seen in three gills. Notably, one gill set showed no significant hyperplasia/hypertrophy of the secondary lamellar epithelium.

Twenty-four sections gill and mid-posterior kidney were examined from twelve CyHV-3 exposed carp. These represented two humanely killed and one carp surviving at the end of the 28-day trial, from each of the four replicate tanks. All gills showed inflammatory and proliferative lesions. A low-grade, generalised inflammatory cell infiltration of the primary lamellae was present in all gills. All gills show low-grade to moderate hyperplasia and hypertrophy in the primary lamellar epithelium, accompanied by low-grade mucoid metaplasia. Similarly, generally low-grade proliferative and metaplastic changes are present in the secondary lamellae of all gills, with one exception in which the secondary lamellae appeared normal. This was from a clinically normal CyHV-3 exposed carp that was sacrificed at the end of the trial. There was no evidence of degenerative, proliferative or inflammatory changes within the stromal or parenchymal components of the kidney of any CyHV-3 exposed carp.

The only histological difference between negative control and CyHV-3 exposed carp appears to be general low-grade proliferative and metaplastic changes are present in the secondary lamellae of all gills in the CyHV-3 exposed carp. This lack of histological differences is not surprising as the carp exposed to CyHV-3 were humanely killed when moderate clinical signs were observed. These moderate clinical signs were most commonly the observation of skin lesions

The lack of histological changes the kidney and gills of carp exposed to CyHV-3 is not surprising given the humane-endpoint was the appearance of moderate clinical signs, persisting for 24 hours. The most observed moderate clinical sign was skin discolouration with raised scales. Where histological changes have been reported in the gill and kidney of fish exposed to CyHV-3, these were from fish that were exhibiting severe clinical signs, including erosive skin lesions and exophthalmia or endophthalmia (Miyazaki et al 2008)

1.2.4.2 Silver perch

Gill and kidney tissue from 12 silver perch (2 x pre-trial, 5 x negative control and 5 x CyHV-3 exposed) were examined histologically. Low-grade to moderately severe generalised proliferative changes characterised by hyperplasia and hypertrophy of the primary and secondary lamellae were present in all silver perch, regardless of treatment (pre-trial, negative control and CyHV-3 exposed). There was no evidence of histopathological changes in the anterior kidney of any silver perch.

1.2.4.3 Murray cod

Gill and kidney tissue from 14 Murray cod (2 x pre-trial, 6 x negative control and 6 x CyHV-3 exposed) were examined histologically. The gills showed histopathological changes like the silver perch, and while generally more severe, were present in all Murray cod, regardless of treatment (pre-trial, negative control and CyHV-3 exposed). Posterior kidney was present in four fish, one of which showed low-grade generalised tubular dilation, the cause of which is not apparent. Anterior kidney was also present from four (different) fish with no histopathological changes evident.

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Discussion

The NCCP had commissioned a review of best practice in non-target species susceptibility (Pyecroft, 2018). While the review acknowledged that the work undertaken by McColl et al. (2016) used the most advanced techniques available at the time, it was recommended that some species which had experienced unexpected mortalities in the former study be reassessed using the most recent methodological recommendations.

Therefore, the objective of this work was to determine the susceptibility of rainbow trout (*Oncorhynchus mykiss*), silver perch (*Bidyanus bidyanus*) and Murray cod (*Maccullochella peelii*) to infection with CyHV-3. Carp (*Cyprinus carpio*) were also exposed concurrently to act as a positive infection control as a known susceptible species. Due to issues with maintenance of rainbow trout, this species was excluded from the exposure trial, so only silver perch, Murray cod and carp were exposed to CyHV-3 by injection and immersion.

The results presented in this report demonstrate that carp are susceptible to infection with CyHV-3, based on;

1. the appearance of mild clinical signs (from Day 2 post exposure) progressing to moderate clinical signs, when carp where humanely killed for animal welfare reasons, up to Day 13. From Day 13, the clinical signs diminished as the fish recovered from the infection and form Day 18 to the termination of the trial on Day 28, no clinical signs of disease were observed. Cumulative morbidity ranged from 50% to 62.5%.

2. detection of CyHV-3 mRNA (indicative of replicating virus) from Day 3 to Day 16 post exposure. The mRNA negative test results from Day 17 onwards correlated with the disappearance of clinical signs of disease in the carp and was indicative of the absence of replicating CyHV-3.

The pattern of disease observed in CyHV-3 exposed carp, particularly in terms of clinical signs and detection of CyHV-3 mRNA, is indicative of infection, development of disease, decline and recovery.

Disease was not observed in silver perch and Murray cod and no CyHV-3 mRNA was detected in either of these species after exposure to CyHV-3 by injection and immersion. Compared to carp, where CyHV-3 DNA was detected in 74.3% of all samples tested, CyHV-3 DNA was only detected in 8% of silver perch samples and 4% of Murray cod samples. C_T values for both silver perch and Murray cod were all >30, compared to carp where C_T values as low as 17.03 occurred. The high CT values, low percentage of positive samples, negative detections of CyHV-3 mRNA and absence of disease indicate that silver percha and Murray cod are not susceptible to infection with CyHV-3.

Morbidity, based on the humane endpoint of moderate clinical signs, for the work described in this report was 50% to 62.5%. This is lower than the mortality of 9350% to 100% reported by McColl et al (2016). This article is a summary of nine infectivity trials undertaken and insufficient detail is provided to compare with the results reported herein. It should be noted that while 29.6% of the negative control carp were KHV qPCR positive in the McColl et al (2016) article, no non-exposed fish of any species were KHV qPCR test positive for the current project. Similarly, while morbidity/mortality was observed in the non-target species, there was no significant statistical difference between treatments (CyHV-3 exposed and non-exposed) for either silver perch or Murray cod. Also in contrast to the McColl et al (2016) article, where selected samples were tested by KHV qPCR, all fish (carp, silver perch and Murray cod) exposed to CyHV-3 were tested using the Gilad CyHV-3 qPCR.

Conclusions

Based on observed clinical signs, morbidity, and presence or absence of CyHV-3 DNA and mRNA (Table 13), the results demonstrate that carp are susceptible to infection with CyHV-3 and silver perch and Murray cod are not susceptible to infection with CyHV-3.

Table 13. Summary of observations and test results combined for each species (CyHV-3 exposed and negative control fish).

Species	Treatment	Clinical Signs of CyHV-3D	Morbidity	CyHV-3 qPCR* (DNA)	CyHV-3 RT-qPCR (mRNA)	Histology indicative of CyHV-3D	Interpretation
Carp	CyHV-3-exposed	Yes	36/64 (56.3%)	113/152 (74.3%)	74/148 (50.0%)	No	Susceptible
	Negative control	No	0/64 (0%)	0/128 (0%)	0/128 (0%)	No	
Silver perch	CyHV-3-exposed	No	11/64 (20.3%)	10/125 (8.0%)	0/10 (0%)	No	Not susceptible
	Negative control	No	7/64 (10.9%)	0/115 (0%)	0/115 (0%)	No	
Murray cod	CyHV-3-exposed	No	7/64 (10.9%)	5/124 (4.0%)	0/5 (0%)	No	Not susceptible
	Negative control	No	7/64 (10.9%)	0/125 (0%)	0/125 (0%)	No	

*Positive and Indeterminate results included

Implications

While the results presented in this report are based on 28-day experimental infection in an artificial environment, they indicate that if CyHV-3 were released into the environment, there would be no infection and no disease event caused by CyHV-3 in wild or farmed silver perch or Murray cod.

Recommendations

Morbidity, based on the humane endpoint of moderate clinical signs, for this experiment was 50% to 62.5%. This is lower than the mortality of 95% and 100% and 50% to 100% reported by Sunarto et al 2011) and McColl et al (2016) and is more consistent with the morbidity reported by Sunarto et al (2020) for FRDC 2017-135 which ranged from 40.4% to 51.4%.

Based on the differences in morbidity/mortality reported in this report and by McColl et al (2016), it is important to confirm the expected mortality in common carp after exposure to CyHV-3 using death as an endpoint. The morbidity observed in this project is not a useful indicator of mortality as it is unknown how many of the fish that were humanely killed for animal welfare reasons, when moderate clinical signs were observed, would have recovered from the disease, or progressed to death.

A study using death as an endpoint should be the first experiment undertaken for investigations into the pathogenicity of newly identified pathogens, or exotic pathogens being considered for use as a biological control agent. This will enable complete documentation of clinical signs, to inform appropriate intervention points for subsequent experiments, and determine mortality to inform risk assessments as to the significance of a newly identified pathogen or the potential of a pathogen as a biological control agent.

Extension and Adoption

Due to the global COVID-19 pandemic, no extension has been undertaken. The information in this report will contribute to decision making regarding release of CyHV-3 as a biological control for carp.

References

McColl KA, Sunarto A, Slater J, Bell K, Asmus M, Fulton W, Hall K, Brown P, Gilligan D, Hoad J, Williams LM and Crane MStJ (2016) Cyprinid herpesvirus 3 as a potential biological control agent for carp (*Cyprinus carpio*) in Australia: susceptibility of non-target species. Journal of Fish Diseases doi:10.1111/jfd.12591

Miyazaki T, Kuzuya Y, Yasumoto S, Yasuda M and Kobyashi T (2008) Histopathological and ultrastructural features of Koi herpesvirus (CyHV-3)-infected carp *Cyprinus carpio*, and the morphology and morphogenesis of CyHV-3. Diseases of Aquatic Organisms 80: 1-11

Moody NJG, Cummins DC, Mohr PM, Williams LM, Hoad J, Valdeter S, Slater J and Crane MStJ (2021) Aquatic Animal Health Subprogram: Development of stable positive control material and development of internal controls for molecular tests for detection of important endemic and exotic pathogens, CSIRO Australian Centre for Disease Preparedness. FRDC 2014-002 Final Report, ISBN 978-1-925994-19-3

Pyecroft SB (2018) NCCP: defining best practice for viral susceptibility testing of non-target species to Cyprinid herpesvirus 3 -a discussion paper based on systematic quantitative literature reviews. FRDC 2017-127 Final Report.

Sunarto A, McColl K A, Crane, MStJ, Sumiati T, Hyatt A D, Barnes AC and Walker PJ (2011). Isolation and characterization of koi herpesvirus (KHV) from Indonesia: identification of a new genetic lineage. Journal of Fish Diseases, 34(2), 87-101. doi:10.1111/j.1365-2761.2010.01216.x

Sunarto A, Moody NJG and Durr PA (2020) Essential studies on cyprinid herpesvirus 3 (CyHV-3) prior to release of the virus in Australian waters: Seasonality studies. FRDC. Canberra. FRDC 2017-135 Final Report

Appendices

Appendix 1. List of Project staff

Name	Position	Organisation
Dr Nick Moody	Group Leader – AFDL	ACDP Fish Diseases Laboratory
Dr Peter Mohr	Team Leader – Aquatic Diagnostic Capability	ACDP Fish Diseases Laboratory
Dr David Cummins	Team Leader – Aquatic Disease Research	ACDP Fish Diseases Laboratory
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Ms Lynette Williams	Research Technician	ACDP Fish Diseases Laboratory
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Ms Stacey Valdeter	Research Technician	ACDP Fish Diseases Laboratory
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Mr Reuben Klein	Research Technician	ACDP Fish Diseases Laboratory
Dr Agus Sunarto	Senior research Scientist	Health and Biosecurity
Dr Kiran Krishnankuttynair	Research Technician	Health and Biosecurity

Appendix 2. Details of mortalities observed in silver perch and Murray cod exposed to CyHV-3

Negative control Murray cod

Tank	Health Status	Number of fish	Description	PCR result (Gill, Kidney, skin)
1	Dead	2	8DPI: NCSO	Negative
			25DPI: flared opercula. Roughened skin, tail rot.	Negative
	Moribund	0		
2	Dead	1	7DPI: flared opercula.	Negative
	Moribund	0		
3	Dead	0		
	Moribund	0		
4	Dead	1	8DPI: NCSO	Negative
	Moribund	3	9DPI: side swimming, darkened skin.	Negative
			9DPI: side swimming, darkened skin.	Negative
			28DPI: side swimming	Negative
TOTAL	**Dead**	**4**		
	Moribund	**3**		

NCSO: No clinical signs observed. DPI: Days Post Infection.

KHV challenged Murray cod

Tank	Health Status	Number of fish	Description	PCR result (Gill, Kidney, skin)
1	Dead	3	7DPI: NCSO	Negative
			20DPI: Mechanical damage	Negative
			21DPI: NCSO	Negative
	Moribund	0		
2	Dead	1	3DPI: fin/tail rot, tail discolouration, distended abdomen.	Negative
	Moribund	1	6DPI: Side swimming, empty gut.	**Indeterminate** (kidney 37.18)
3	Dead	0		
	Moribund	1	9DPI: tail discolouration, flared opercula.	Negative
4	Dead	1	7DPI: NCSO	**Indeterminate** (kidney 36.02)
	Moribund	0		
TOTAL	**Dead**	**5**		
	Moribund	**2**		

NCSO: No clinical signs observed. DPI: Days Post Infection.
PCR Ct values shown for positive or indeterminate (one positive and one negative result when tested in duplicate) samples.

Negative control Silver Perch

Tank	Health Status	Number of fish	Description	PCR result (Gill, Kidney, skin)
1	Dead	1	12DPI: NCSO	Negative
	Moribund	0		
2	Dead	4	1DPI: NCSO	Negative
			2DPI: NCSO	Negative
			8DPI: NCSO	Negative
			9DPI: flared opercula.	Negative
	Moribund	0		
3	Dead	1	11DPI: NCSO	Negative
	Moribund	0		
4	Dead	0		
	Moribund	1	5DPI: incoordination, lethargic, tail rot.	Negative
TOTAL	**Dead**	**6**		
	Moribund	**1**		

NCSO: No clinical signs observed. DPI: Days Post Infection.

KHV challenged Silver Perch

Tank	Health Status	Number of fish	Description	PCR result (Gill, Kidney, skin)
1	Dead	4	8DPI: NCSO	**Indeterminate** (gill 35.86)
			9DPI: darkened skin.	Negative
			13DPI: NCSO	Negative
			15DPI: Flared opercula.	Negative
	Moribund	0		
2	Dead	4	1DPI: NCSO	**POSITIVE** (gill 31.37; kidney 36.16)
			8DPI: NCSO	**Indeterminate** (kidney 37.17)
			9DPI: reddening around opercula.	Negative
			16DPI: NCSO	Negative
	Moribund	0		
3	Dead	2	8DPI: NCSO	**POSITIVE** (kidney 34.12)
			11DPI: NCSO	Negative
	Moribund	1	13DPI: side swimming, twitching.	**Indeterminate** (kidney 36.06)
4	Dead	1	8DPI: NCSO	Negative
	Moribund	1	9DPI: side swimming.	**Indeterminate** (kidney 36.11)
TOTAL	**Dead**	**11**		
	Moribund	**2**		

NCSO: No clinical signs observed. DPI: Days Post Infection.
PCR Ct values shown for positive or indeterminate (one positive and one negative result when tested in duplicate) samples.

Appendix 3. Details of CyHV-3 Gilad qPCR positive and negative detections in CyHV-3 exposed carp.

KHV Infected Carp

KHV Challenged Carp TANK 1					
Days Post Infection	**No of samples tested**	**Health Status**	**PCR Result**		
			Kidney	**Gill**	**Skin**
4	1	Healthy	POSITIVE	POSITIVE	NSC
6	4	Moribund	POSITIVE	POSITIVE	POSITIVE
		Moribund	POSITIVE	POSITIVE	POSITIVE
		Moribund	POSITIVE	POSITIVE	POSITIVE
		Moribund	POSITIVE	POSITIVE	POSITIVE
8	1	Moribund	POSITIVE	POSITIVE	POSITIVE
9	1	Moribund	POSITIVE	POSITIVE	POSITIVE
12	1	Moribund	POSITIVE	POSITIVE	NSC
16	1	Healthy	POSITIVE	POSITIVE	NSC
20	1	Moribund	Negative	POSITIVE	NSC
24	1	Healthy	Negative	Negative	NSC
28	5	Healthy	Negative	Negative	NSC
		Healthy	**Indeterminate**	Negative	NSC
		Healthy	Negative	Negative	NSC
		Healthy	Negative	Negative	NSC
		Healthy	Negative	Negative	NSC
	Total = 16		9x POSITIVE 1x Indet 6x Negative	10x POSITIVE 6x Negative	6x POSITIVE

NSC = No sample collected
NT = Not tested

KHV Challenged Carp TANK 2					
Days Post Infection	**No of samples tested**	**Health Status**	**PCR Result**		
			Kidney	**Gill**	**Skin**
4	1	Moribund	POSITIVE	POSITIVE	NSC
6	1	Moribund	POSITIVE	POSITIVE	POSITIVE
8	2	Moribund	POSITIVE	POSITIVE	POSITIVE
		Moribund	POSITIVE	POSITIVE	POSITIVE
9	4	Moribund	POSITIVE	POSITIVE	POSITIVE
		Moribund	POSITIVE	POSITIVE	POSITIVE
		Moribund	POSITIVE	POSITIVE	POSITIVE
		Moribund	POSITIVE	POSITIVE	POSITIVE
12	1	Moribund	POSITIVE	POSITIVE	NSC
14	1	Moribund	POSITIVE	POSITIVE	POSITIVE
16	1	Healthy	**Indeterminate**	POSITIVE	NSC
20	1	Healthy	**Indeterminate**	POSITIVE	NSC
24	1	Healthy	Negative	Negative	NSC
28	3	Healthy	Negative	**Indeterminate**	NSC
		Healthy	Negative	Negative	NSC
		Healthy	Negative	Negative	NSC
	Total = 16		10x POSITIVE 2x Indet 4x Negative	12x POSITIVE 1x Indet 3x Negative	8x POSITIVE

NSC = No sample collected
NT = Not tested

KHV Challenged Carp TANK 3					
Days Post Infection	**No of samples tested**	**Health Status**	**PCR Result**		
			Kidney	**Gill**	**Skin**
3	1	Moribund	**Indeterminate**	POSITIVE	NSC
4	1	Moribund	POSITIVE	POSITIVE	NSC
5	1	Moribund	**Indeterminate**	POSITIVE	POSITIVE
7	1	Moribund	**Indeterminate**	POSITIVE	POSITIVE
8	1	Moribund	POSITIVE	POSITIVE	POSITIVE
9	1	Moribund	POSITIVE	POSITIVE	POSITIVE
12	1	Moribund	POSITIVE	POSITIVE	NSC
16	1	Moribund	POSITIVE	POSITIVE	NSC
20	1	Healthy	Negative	**Indeterminate**	NSC
24	1	Healthy	**Indeterminate**	Negative	NSC
28	6	Healthy	Negative	Negative	NSC
		Healthy	Negative	Negative	NSC
		Healthy	Negative	Negative	NSC
		Healthy	Negative	Negative	NSC
		Healthy	Negative	Negative	NSC
		Healthy	Negative	Negative	NSC
	Total = 16		5x POSITIVE 4x Indet 7x Negative	8x POSITIVE 1x Indet 7x Negative	4x POSITIVE

NSC = No sample collected
NT = Not tested

KHV Challenged Carp TANK 4					
Days Post Infection	**No of samples tested**	**Health Status**	**PCR Result**		
			Kidney	**Gill**	**Skin**
4	2	Moribund	POSITIVE	POSITIVE	NSC
		Dead	Negative	Negative	NSC
7	2	Moribund	POSITIVE	POSITIVE	POSITIVE
		Moribund	POSITIVE	POSITIVE	POSITIVE
8	1	Moribund	POSITIVE	POSITIVE	POSITIVE
9	3	Moribund	POSITIVE	POSITIVE	POSITIVE
		Moribund	POSITIVE	POSITIVE	POSITIVE
		Moribund	POSITIVE	POSITIVE	NSC
12	1	Moribund	POSITIVE	**Indeterminate**	NSC
16	1	Healthy	NSC	POSITIVE	POSITIVE
20	1	Healthy	Negative	Negative	NSC
24	1	Healthy	POSITIVE	**Indeterminate**	NSC
26	1	Dead	Negative	**Indeterminate**	NSC
28	3	Healthy	**Indeterminate**	**Indeterminate**	NSC
		Healthy	**Indeterminate**	POSITIVE	NSC
		Healthy	Negative	Negative	NSC
	Total = 16		9x POSITIVE 2x Indet 4x Negative 1x NSC	9x POSITIVE 4x Indet 3x Negative	6x POSITIVE

NSC = No sample collected
NT = Not tested

Appendix 4. Development of the CyHV-3 Terminase RT-qPCR for the detection of CyHV-3 mRNA

AFDL Internal Research Summary: KHV Terminase RT-PCR and RT-qPCR – Evaluations

BACKGROUND

The FRDC Project 2019-176 "NCCP: Determination of the susceptibility of silver perch, Murray cod and rainbow trout to infection with CyHV-3" involved immersion and injection exposure of carp, Murray cod and silver perch with KHV (CyHV-3) to determine the susceptibility of these species to infection with KHV. If KHV DNA was detected by PCR in samples from any non-target species (Murray cod or silver perch), the same samples required testing with a KHV mRNA specific PCR. A positive detection of KHV mRNA would infer that the virus was actively replicating in the non-target species.

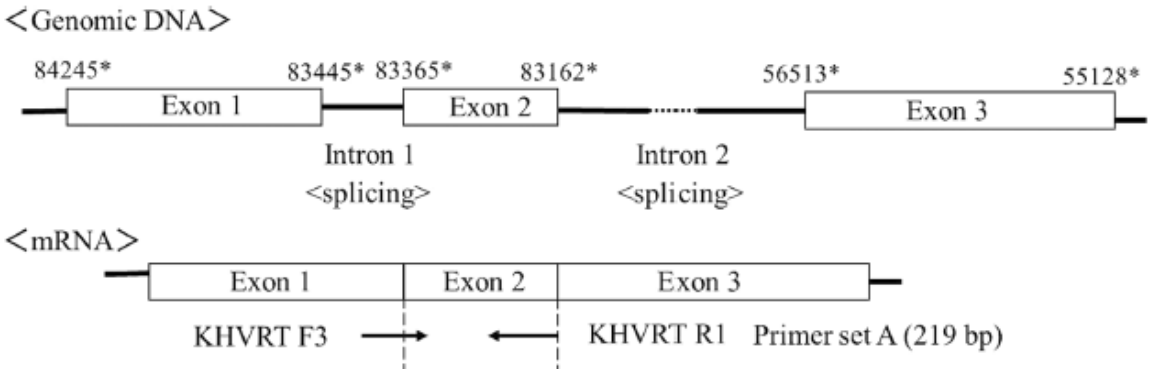
- A KHV qPCR, based on Gilad et al. (2004), is in routine use for the detection of KHV by AFDL [hereafter described as KHV Gilad DNA qPCR]
- KHV mRNA has previously been detected at ACDP with a KHV RT-PCR, based on Yuasa et al. (2012) [hereafter described as KHV Yuasa mRNA RT-PCR]

A criticism of previous non-target species KHV trials conducted at ACDP (i.e. McColl et al. 2017) was that the KHV Yuasa mRNA RT-PCR could lack sensitivity for detecting low levels of KHV mRNA in samples that were positive for KHV DNA (detected by the KHV Gilad DNA qPCR). Therefore, as part of the current project, a real-time RT-qPCR for detection of KHV mRNA was designed and evaluated as no published KHV mRNA TaqMan probe-based RT-qPCR assays were available. In addition, the development of a real-time RT-qPCR would enable more efficient high-throughput testing of samples.

KHV Yuasa mRNA RT-PCR

The KHV Yuasa mRNA RT-PCR assay contains a forward primer that crosses an exon-intron boundary (exon 1/exon 2) of the KHV ORF33 - terminase gene and therefore only amplifies exon 2 mRNA (Figure 1). The terminase gene encodes an enzyme involved in packaging viral DNA into capsids and viral multiplication. The KHV Yuasa mRNA RT-PCR assay has previously been used in research activities at ACDP (McColl et al. 2017) and its analytical characteristics were further assessed during this evaluation.

Figure 1: KHV Yuasa mRNA RT-PCR primer annealing sites to ORF33 - terminase gene (Yuasa et al. 2012)



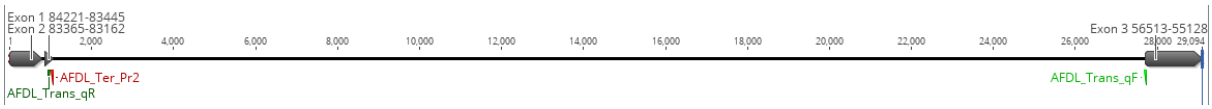
AFDL KHV Terminase RT-qPCR - Design

As the KHV Yuasa mRNA RT-PCR is the current method for determining if KHV mRNA was present in non-target species, with a positive result indicative of replicating virus (i.e. Kim et al. 2019), mRNA of the same gene (KHV terminase) was used to design a real-time RT-qPCR. A 26,648 bp intron separates KHV

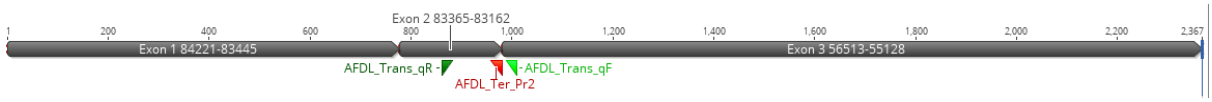
terminase exon 2 and exon 3. Therefore, the AFDL KHV Terminase RT-qPCR was designed with a reverse primer (AFDL_Trans_qR) and TaqMan probe (AFDL_Ter_Pr2) annealing to exon 2 and a forward primer (AFDL_Trans_qF) annealing to exon 3 (Figure 2). The large intron between exon 2 and exon 3 eliminates the possibility of KHV DNA being amplified by the assay and ensures that only KHV mRNA is amplified. By targeting the same gene as the KHV Yuasa mRNA RT-PCR, analytical sensitivity differences between the two assays could also be compared.

Figure 2: AFDL KHV Terminase RT-qPCR primer and probe annealing sites to ORF33 - terminase gene

Genomic DNA



mRNA



ANALYTICAL SENSITIVITY

To determine and compare the analytical sensitivity of both the KHV Yuasa mRNA RT-PCR and AFDL KHV Terminase RT-qPCR assays, a sample containing KHV mRNA was required. Therefore, nucleic acid was extracted from a KHV C07-infected KF-1 cell pellet, cultured in a 75 cm^2 tissue culture flask (2102-19-1000), with the QIAGEN RNeasy mini kit. A 10-fold dilution series of KHV 2103-01-1201 nucleic acid was prepared in TE Buffer (pH 8.0) containing 50 ng/µl yeast tRNA.

KHV Yuasa mRNA RT-PCR

At the same time as assessing the analytical sensitivity of the KHV Yuasa mRNA RT-PCR assay, an optimisation of the extension temperature to align with that recommended for the Superscript III One-Step RT-PCR with Platinum Taq DNA Polymerase was also undertaken. The original PCR cycling conditions described by Yuasa et al. (2012) were modified by Kim et al. (2019) during a study to assess the susceptibility of silver crucian carp to KHV. The AFDL PCR cycling conditions adopted for this project were the same as those described by Yuasa et al. (2012) but adopting the increase in cycles to 40 as per Kim et al. (2019) and reducing the extension steps from 72°C to 68°C (Table 1).

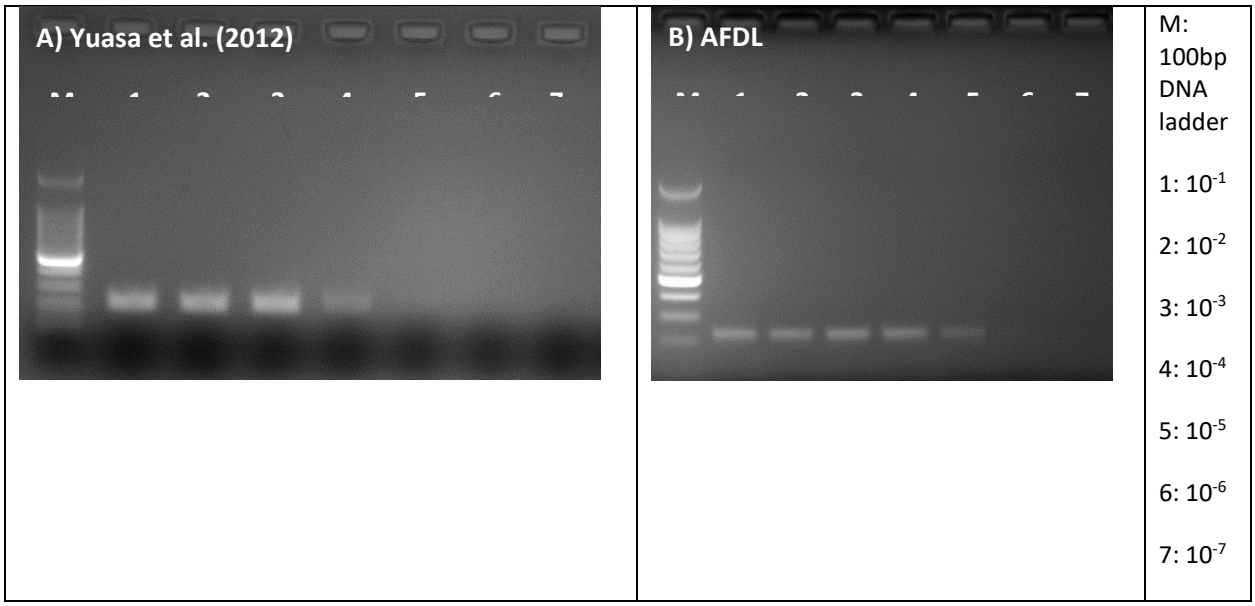
Table 1. PCR cycling conditions for the KHV Yuasa mRNA RT-PCR

PCR Conditions	Yuasa et al. (2012)	Kim et al. (2019) modification	AFDL modification
Reverse transcription	55°C for 30 min	55°C for 30 min	55°C for 30 min
Initial denature	94°C for 2 min	94°C for 2 min	94°C for 2 min
Cycles	35	40	40
Denature	94°C for 30 sec	94°C for 30 sec	94°C for 30 sec
Annealing	60°C for 30 sec	65°C for 30 sec	60°C for 30 sec
Extension	72°C for 30 sec	72°C for 30 sec	68°C for 30 sec
Final Extension	72°C for 7 min	72°C for 7 min	68°C for 7 min

The KHV Yuasa mRNA RT-PCR was tested on the 10-fold dilutions of KHV nucleic acid with Yuasa et al. (2012) and AFDL PCR cycling conditions. The limit of detection (LOD) for the KHV Yuasa mRNA RT-PCR was dilution 10^{-4} when tested with the Yuasa et al. (2012) PCR cycling conditions (Figure 3A). In contrast the LOD was dilution 10^{-5} for the same assay using the modified AFDL PCR cycling conditions (Figure 3B). The

AFDL PCR cycling conditions were adopted for this project due to the 10-fold increase in analytical sensitivity.

Figure 3. Detection of KHV 10-fold dilutions with KHV Yuasa mRNA RT-PCR assay A) Yuasa et al. (2012) and B) AFDL PCR cycling conditions. Each dilution was tested in singlicate with the expected amplicon size 219 bp.



AFDL KHV Terminase RT-qPCR

The LOD for the AFDL KHV Terminase RT-qPCR was determined to be dilution 10^{-4} when testing the KHV 10-fold dilution series (Table 2). The same LOD was obtained for this assay when testing of the dilution series was repeated.

To ensure that the AFDL KHV Terminase RT-qPCR amplified only KHV mRNA, the PCR master mix, AgPath-ID One-Step (containing a reverse transcriptase enzyme) was changed to TaqMan Universal (lacking a reverse transcriptase enzyme) to test the KHV 10-fold dilution series. When tested with the TaqMan Universal PCR master mix, all dilutions tested negative with the AFDL KHV Terminase RT-qPCR, positive controls performed as per expected (data not shown). This result confirmed that the AFDL KHV Terminase RT-qPCR only amplified KHV mRNA.

Table 2. Detection of KHV 10-fold dilutions with AFDL KHV Terminase RT-qPCR

KHV Nucleic Acid Dilutions	AFDL KHV Terminase RT-qPCR			
	Rep 1 Cт	Rep 2 Cт	Rep 3 Cт	Cт Mean
2103-01-1201 10^{-1}	22.65	22.79	22.64	22.69
2103-01-1201 10^{-2}	26.76	26.86	26.84	26.82
2103-01-1201 10^{-3}	30.09	30.14	30.25	30.16
2103-01-1201 10^{-4}	32.79	33.24	33.56	33.19
2103-01-1201 10^{-5}	-	-	-	-
2103-01-1201 10^{-6}	36.47	37.81	-	37.14
2103-01-1201 10^{-7}	-	-	-	-
2103-01-1201 10^{-8}	-	-	-	-
2103-01-1201 10^{-9}	-	-	-	-
2103-01-1201 10^{-10}	-	-	-	-

Rep = replicate, - = not detected, LOD (lowest dilution with 100% positive reactions detected) is highlighted in blue, cycle threshold 0.1

The LOD for the AFDL KHV Terminase RT-qPCR was also determined by testing ten-fold dilutions (prepared in TE Buffer (pH 8.0) with containing 50 ng/µl yeast tRNA) of a synthetic plasmid containing the KHV terminase gene nucleotide sequence specific for the assay (partial exon 2 and partial exon 3 with no intron in between). The LOD was 0.2 copies per reaction which demonstrated that the AFDL KHV Terminase RT-qPCR assay was highly sensitive and could detect as low as a single plasmid copy per reaction (Table 3).

Table 3. Detection of KHV terminase plasmid 10-fold dilutions with AFDL KHV Terminase RT-qPCR

KHV Terminase Plasmid Copies/Reaction	AFDL KHV Terminase RT-qPCR			
	Rep 1 Cᴛ	Rep 2 Cᴛ	Rep 3 Cᴛ	Cᴛ Mean
20,000,000	9.29	9.42	9.40	9.37
2,000,000	12.55	12.58	12.79	12.64
200,000	15.92	16.16	16.28	16.12
20,000	19.41	19.63	19.67	19.57
2,000	22.62	22.71	22.78	22.70
200	26.22	26.17	26.21	26.20
20	29.45	29.49	29.28	29.41
2	32.46	32.16	32.75	32.45
0.2	36.31	36.19	38.22	36.91
0.02	-	-	-	-

Rep = replicate, - = not detected, LOD (lowest dilution with 100% positive reactions detected) is highlighted in blue, cycle threshold 0.1

Analytical Sensitivity Summary - KHV Terminase RT-PCR and RT-qPCR

The LOD for the KHV Yuasa mRNA RT-PCR was 10-fold more sensitive than the AFDL KHV Terminase RT-qPCR when testing the KHV 10-fold dilution series. The reduced analytical sensitivity of the AFDL KHV Terminase RT-qPCR could have resulted from mRNA degradation during the multiple freeze-thaws of the dilution series between the testing of the two assays.

To determine the presence of KHV DNA within the KHV 10-fold dilution series, each dilution was also tested with the KHV Gilad DNA qPCR. The KHV Gilad DNA qPCR LOD for DNA detection was dilution 10^{-6} (Table 4). The higher sensitivity of the KHV Gilad DNA qPCR in comparison to the KHV Yuasa mRNA RT-PCR and AFDL KHV Terminase RT-qPCR assays likely reflects the presence of more KHV DNA than mRNA in the dilution series tested.

Table 4. LOD comparison for DNA and mRNA detection in KHV 10-fold dilutions

KHV Nucleic Acid Dilutions	KHV Gilad DNA qPCR (Cᴛ Mean)	AFDL KHV Terminase RT-qPCR (Cᴛ Mean)	KHV Yuasa mRNA RT-PCR (singlicate)
2103-01-1201 10^{-1}	16.20	22.69	+
2103-01-1201 10^{-2}	20.64	26.82	+
2103-01-1201 10^{-3}	23.81	30.16	+
2103-01-1201 10^{-4}	27.05	33.19	+
2103-01-1201 10^{-5}	30.46	-	+
2103-01-1201 10^{-6}	33.72	37.14 (2/3)	-
2103-01-1201 10^{-7}	37.05 (2/3)	-	-
2103-01-1201 10^{-8}	-	-	-
2103-01-1201 10^{-9}	-	-	-
2103-01-1201 10^{-10}	-	-	-

- = not detected, LOD (lowest dilution with 100% positive reactions detected) is highlighted in blue for each KHV mRNA assay and highlighted in orange for the KHV DNA assay, cycle threshold 0.1

ANALYTICAL SPECIFICITY - Inclusivity

KHV Yuasa mRNA RT-PCR

To ensure that the KHV Yuasa mRNA RT-PCR was amplifying the terminase gene from KHV C07 an amplicon of the expected size was gel-purified and sequenced. The primer trimmed sequence shared 100% nucleotide identity with KHV-U terminase gene (DQ657948). As KHV C07 was the isolate to be used in the NTS project analytical specificity-inclusivity was not further assessed with other KHV isolates for this assay.

AFDL KHV Terminase RT-qPCR

To ensure that the AFDL KHV Terminase RT-qPCR assay was amplifying the terminase gene from KHV C07 a conventional RT-PCR was performed with forward and reverse primers for the assay. An amplicon of the expected size (148 bp) was gel-purified and sequenced. The primer trimmed sequence shared 100% nucleotide identity with KHV-U terminase gene (DQ657948). As KHV C07 was the isolate to be used in the NTS project analytical specificity-inclusivity was not further assessed with other KHV isolates for this assay.

ANALYTICAL SPECIFICITY - Exclusivity

KHV Yuasa mRNA RT-PCR

The analytical specificity of the KHV Yuasa mRNA RT-PCR to exclude detection of terminase mRNA from closely related cyprinid herpesviruses, CyHV-1 and CyHV-2, was demonstrated by Yuasa et al. (2012). These authors undertook this work with freshly cultured CyHV-1 and CyHV-2 viruses with the identity of each virus confirmed by specific PCR. *In silico* analysis of KHV Yuasa mRNA RT-PCR primers with CyHV-1 and CyHV-2 reference nucleotide sequences (Figure 4) demonstrated that;

- Primer KHV RT F3 had two and four nucleotide mismatches with CyHV1 (NC_019491 strain NG-J1) and CyHV2 (NC_019495 strain ST-J1) reference sequences respectively.
- Primer KHV RT R1 had four nucleotide mismatches with both CyHV1 (NC_019491 strain NG-J1) and CyHV2 (NC_019495 strain ST-J1) reference sequences.

Figure 4. KHV Yuasa mRNA RT-PCR primer annealing sites to ORF33 - terminase genes of CyHV-1, CyHV-2 and CyHV-3 (KHV)

As amplicons generated with this RT-PCR assay when testing carp, Murray cod or silver perch samples during the FRDC KHV NTS project will be sequenced, no further evaluation of testing known KHV negative samples for these species was undertaken.

AFDL KHV Terminase RT-qPCR

When designing the AFDL KHV Terminase RT-qPCR the analytical specificity of detecting closely related cyprinid herpesviruses (i.e. CyHV-1 and CyHV-2) was not initially considered during the design of this assay as it was developed specifically for testing during the FRDC KHV NTS project. Retrospective *in silico* analysis with CyHV-1 and CyHV-2 reference nucleotide sequences (Figure 5) demonstrated that;

- Primer AFDL_Trans_qF had one and three mismatches with CyHV1 (NC_019491 strain NG-J1) and CyHV2 (NC_019495 strain ST-J1) reference sequences respectively.

- Primer AFDL_Trans_qR had three mismatches with both CyHV1 (NC_019491 strain NG-J1) and CyHV2 (NC_019495 strain ST-J1) reference sequences.
- TaqMan probe AFDL_Ter_Pr2 had four and five mismatches with CyHV1 (NC_019491 strain NG-J1) and CyHV2 (NC_019495 strain ST-J1) reference sequences respectively.

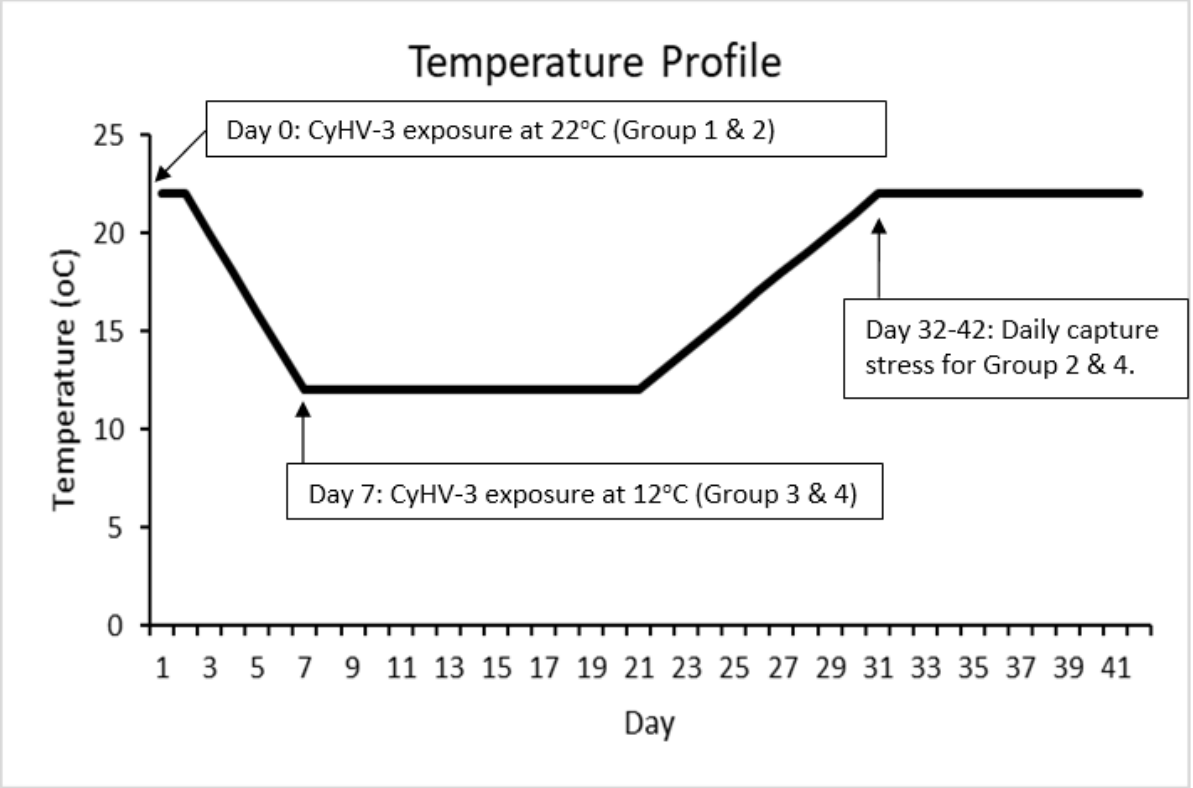
Figure 5. AFDL KHV Terminase RT-qPCR primer and probe annealing sites to ORF33 - terminase genes of CyHV-1, CyHV-2 and CyHV-3 (KHV)

To ensure that the AFDL KHV Terminase RT-qPCR did not generate non-specific amplifications from known KHV negative fish samples, 16 samples (8 gill and 8 kidney) were tested in duplicate from negative control carp, Murray cod and silver perch collected during the FRDC KHV NTS project. All 48 known KHV negative fish samples tested negative with the AFDL KHV Terminase RT-qPCR.

DIAGNOSTIC CHARACTERISTICS

To initially assess how the KHV Yuasa mRNA RT-PCR and AFDL KHV Terminase RT-qPCR assays performed when testing fish samples, a sub-set of KHV infected samples from the FRDC KHV Trojan carp project were selected for testing. Eight samples (19-04629-0119 to -0126) with KHV Gilad DNA qPCR mean C_T values ranging from 17.78 to 24.42 and eight samples (19-04629-0147 to -154) with KHV Gilad DNA qPCR mean C_T values ranging from 24.60 to negative were tested (Table 5). The samples represented kidney, gill or skin samples collected from carp infected by injection exposure with KHV C07. The tissue had been collected from dead, moribund, healthy or sacrificed animals in November and December 2019 and added to 80% (v/v) ethanol and stored at room temperature. Carp in room C4 were Group 1; infected with KHV at 22°C before the temperature was decreased by 2°C a day to 12°C by 6 dpi until 21 dpi when the temperature was increased by 1°C a day to 22°C until 32 dpi (Figure 6). The experiment was ended at 42 dpi and capture stress was not applied to the carp in this room. Nucleic acid was extracted from the samples in early 2020 and stored at -80°C until being moved to -20°C for approximately 1 year.

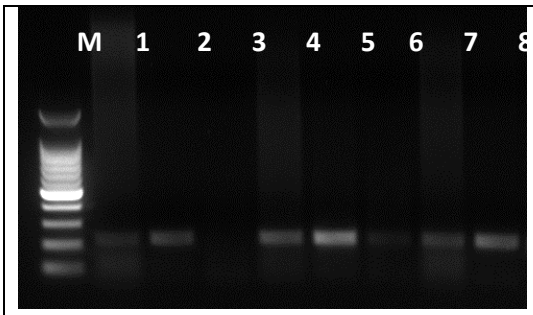
Figure 6. FRDC KHV Trojan carp project chronology

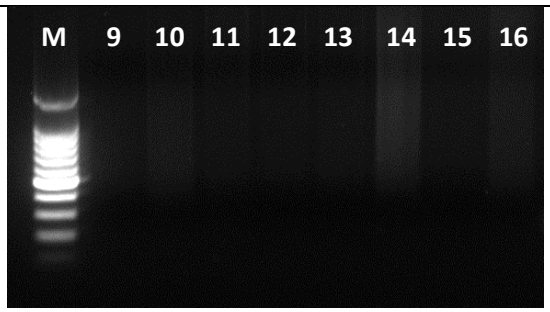


KHV Yuasa mRNA RT-PCR

Seven of the 16 samples tested were positive by the KHV Yuasa mRNA RT-PCR (Figure 7 and Table 5). Each of the samples with a KHV Gilad DNA qPCR mean C_T of 24.42 or below were positive except 19-04629-0121 gill sample, which had a mean C_T of 20.61.

Figure 7. Testing of FRDC KHV Trojan carp samples with KHV Yuasa mRNA RT-PCR assay. Expected amplicon size 219 bp.





M: 100bp DNA ladder 1: 19-04629-0119 2: 19-04629-0120 3: 19-04629-0121 4: 19-04629-0122 5: 19-04629-0123 6: 19-04629-0124 7: 19-04629-0125 8: 19-04629-0126	M: 100bp DNA ladder 9: 19-04629-0147 10: 19-04629-0148 11: 19-04629-0149 12: 19-04629-0150 13: 19-04629-0151 14: 19-04629-0152 15: 19-04629-0153 16: 19-04629-0154

AFDL KHV Terminase RT-qPCR

Eight of the 16 samples tested were positive by the AFDL KHV Terminase RT-qPCR with mean C_T values of 29.33 to 43.31 (Table 5). Each of the seven samples testing positive with the KHV Yuasa mRNA RT-PCR, which had a corresponding KHV Gilad DNA qPCR mean C_T of 24.42 or below, were also positive by the AFDL KHV Terminase RT-qPCR. Gill sample 19-04629-0121, with a KHV Gilad DNA qPCR mean C_T of 20.61, that was negative by KHV Yuasa mRNA RT-PCR, was positive by AFDL KHV Terminase RT-qPCR with a mean C_T of 43.31. An additional kidney sample (19-04639-0149) tested indeterminate (one replicate positive and the other negative) with the AFDL KHV Terminase RT-qPCR. Based on testing this small sub-set of samples, the AFDL KHV Terminase RT-qPCR was at least as sensitive as the KHV Yuasa mRNA RT-PCR.

Several factors should be considered when making comparisons between the sensitivity of the KHV Yuasa mRNA RT-PCR, AFDL KHV Terminase RT-qPCR and KHV Gilad DNA qPCR assays in this initial evaluation:

1) KHV DNA will be detected by the KHV Gilad DNA qPCR assay in tissues when the virus is present. KHV mRNA will only be detected by the KHV Yuasa mRNA RT-PCR and AFDL KHV Terminase RT-qPCR assays when the virus was actively replicating.
2) The FRDC KHV Trojan carp project involved modulation of temperature below the permissive temperature for the virus. The carp in room C4 were inoculated at a permissive temperature (22°C) but by 6 dpi this was reduced to 12°C and maintained until 21 dpi when temperature was increased by 1°C a day to reach 22°C at 32 dpi. The samples tested were collected from 29 to 42 dpi as a result of death, humanely killed due to mild clinical signs, humanely killed due to welfare reasons or sacrificed at the end of the trial.
3) Tissues from this experiment were collected into ethanol and nucleic acid after extraction stored at -20°C for an extended period after initially being stored at -80°C. It is possible that KHV mRNA degradation had occurred during both of these processes.

Table 5. Comparison of KHV DNA and mRNA detection from FRDC KHV Trojan carp samples

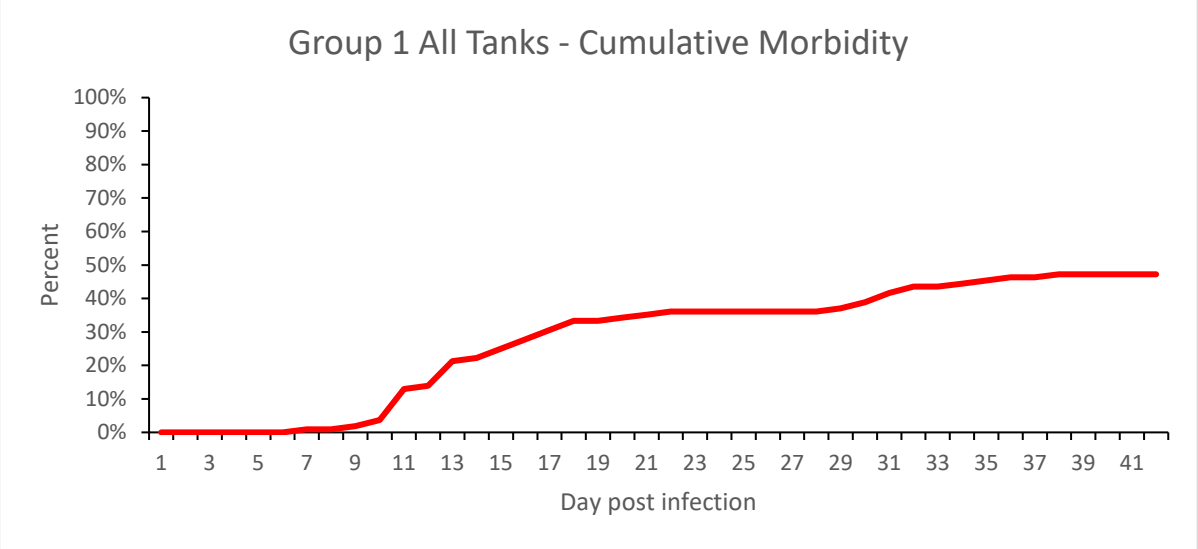
Sample ID.	Fish Information	Tissue	DPI	Water Temp. (°C)	KHV Gilad DNA qPCR (C_T Mean)	AFDL KHV Terminase RT-qPCR (C_T Mean)	KHV Yuasa mRNA RT-PCR (Singlicate)
19-04629-0119	T5 #40 dead	Kidney	29	20	17.78	33.00	+
19-04629-0120	T5 #40 dead	Skin	29		20.90	32.58	+
19-04629-0121	T1 #41 dead	Gill	30	21	20.61	43.31	-
19-04629-0122	T1 #41 dead	Kidney	30		16.41	31.34	+
19-04629-0123	T1 #41 dead	Skin	30		19.20	29.33	+
19-04629-0124	T1 #42 dead	Gill	30	21	19.47	35.71	+
19-04629-0125	T1 #42 dead	Kidney	30		22.93	32.72	+
19-04629-0126	T1 #42 dead	Skin	30		24.42	31.32	+
19-04629-0147	T1 #49 moribund	Skin	35	22	29.17	-	-
19-04629-0148	T3 #50 moribund	Gill	35	22	24.60	-	-
19-04629-0149	T3 #50 moribund	Kidney	35		27.09	-/38.54	-
19-04629-0150	T3 #50 moribund	Skin	35		28.51	-	-
19-04629-0151	T4 #51 healthy	Gill	38	22	32.88	-	-
19-04629-0152	T4 #51 healthy	Kidney	38		34.68	-	-
19-04629-0153	T4 #51 healthy	Skin	38		32.71	-	-
19-04629-0154	T1 #52 sacrificed	Gill	42	22	-	-	-

T = Tank, - = not detected, samples tested with qPCR and RT-qPCR assays in duplicate, cycle threshold 0.1

As the samples initially tested were collected predominately due to deaths or morbidity in FRDC KHV Trojan carp Group 1, after water temperature was returned to the permissive temperature, the samples

collected earlier in the experiment from this group were subsequently tested with the AFDL KHV Terminase RT-qPCR. This additional set of 118 samples were predominately collected from humanely killed, moribund or dead carp from 7 to 22 dpi (Figure 8) during the acute phase and moving into the declining, or recovery phase of the disease, which included time when the carp were being held at 12°C, well below the permissive temperature for expression of disease.

Figure 8. FRDC KHV Trojan carp project group 1 cumulative morbidity



Forty-eight of the 118 samples tested were positive by the AFDL KHV Terminase RT-qPCR (Table 6). In total, 14 gill, 17 kidney and 17 skin samples tested positive for KHV mRNA. In general, the majority of gill, kidney and skin testing positive for KHV mRNA in carp were sampled from 7 to 12 dpi. From 13 to 21 dpi the samples were generally negative for KHV mRNA with only nine of 69 testing positive. Based on these results, and if KHV mRNA had not degraded in the samples after storage at -20°C for over 12 months, it appeared that KHV continued to replicate for several days after the water temperature was lowered below the permissive temperature for the virus. However, after 6 d at 12°C KHV replication essentially ceased until after the water temperature neared 20°C later in the experiment.

Table 6. Comparison of KHV DNA and mRNA detection from FRDC KHV Trojan carp samples

Sample ID.	Fish Information	DPI	Water Temp. (°C)	Tissue	KHV Gilad DNA qPCR (C_T Mean)	AFDL KHV Terminase RT-qPCR (C_T Mean)
19-04629-0001	T1 #1 Dead	7	12	Gill	21.72	27.99
19-04629-0002				Kidney	19.31	25.30
19-04629-0003				Skin	22.50	31.35
19-04629-0004	T4 #2 Dead	9	12	Gill	28.54	36.82
19-04629-0005				Kidney	26.46	33.22
19-04629-0006				Skin	30.90	-
19-04629-0007	T2 #3 Moribund	10	12	Gill	29.07	35.70
19-04629-0008				Kidney	24.91	29.43
19-04629-0009				Skin	24.97	33.22
19-04629-0010	T4 #4 Moribund	10	12	Gill	34.73	-
19-04629-0011				Kidney	28.96	-/35.24
19-04629-0012				Skin	28.76	36.66
19-04629-0013	T4 #5 Dead	11	12	Gill	28.83	-/38.99
19-04629-0014				Kidney	27.62	32.16
19-04629-0015				Skin	24.41	32.70
19-04629-0016	T6 #6 Moribund	11	12	Gill	22.57	27.42
19-04629-0017				Kidney	20.97	26.25
19-04629-0018				Skin	22.33	32.09
19-04629-0019	T6 #7 Moribund	11	12	Gill	19.65	27.43
19-04629-0020				Kidney	15.92	23.37
19-04629-0021				Skin	18.01	28.66
19-04629-0022	T1 #8 Moribund	11	12	Gill	26.38	31.01
19-04629-0023				Kidney	24.07	29.71
19-04629-0024				Skin	27.51	33.64
19-04629-0025	T3 #9 Moribund	11	12	Gill	32.50	-
19-04629-0026				Kidney	27.37	33.95
19-04629-0027				Skin	23.35	34.92
19-04629-0028	T3 #10 Moribund	11	12	Gill	28.30	35.12
19-04629-0029				Kidney	24.55	31.34
19-04629-0030				Skin	23.87	32.71
19-04629-0031	T4 #11 Moribund	11	12	Gill	23.23	28.37
19-04629-0032				Kidney	21.34	26.71
19-04629-0033				Skin	24.73	30.78
19-04629-0034	T4 #12 Moribund	11	12	Gill	31.57	-
19-04629-0035				Kidney	30.69	-/38.41
19-04629-0036				Skin	23.79	32.29
19-04629-0037	T6 #13 Moribund	11	12	Gill	19.32	24.88
19-04629-0038				Kidney	14.26	20.65
19-04629-0039				Skin	22.45	28.59
19-04629-0040	T6 #14 Moribund	11	12	Gill	20.79	25.85
19-04629-0041				Kidney	17.40	22.96
19-04629-0042				Skin	25.51	31.42
19-04629-0043	T4 #15 Moribund	12	12	Gill	21.42	26.41
19-04629-0044				Kidney	14.49	24.59
19-04629-0045				Skin	21.56	-
19-04629-0046	T2 #16 Moribund	13	12	Gill	27.60	-
19-04629-0047				Kidney	25.75	-
19-04629-0048				Skin	26.67	-
19-04629-0049	T3 #17 Moribund	13	12	Gill	27.74	-
19-04629-0050				Kidney	28.40	-
19-04629-0051				Skin	25.88	-
19-04629-0052	T3 #18 Moribund	13	12	Gill	26.68	-
19-04629-0053				Kidney	27.08	-
19-04629-0054				Skin	25.42	-
19-04629-0055	T3 #19 Moribund	13	12	Gill	33.25	-

19-04629-0056				Kidney	31.86	-
19-04629-0057				Skin	27.11	-
19-04629-0058	T4 #20 Moribund	13	12	Gill	29.85	-
19-04629-0059				Kidney	23.95	-
19-04629-0060				Skin	25.44	37.23
19-04629-0061	T4 #21 Moribund	13	12	Gill	31.14	-
19-04629-0062				Kidney	28.83	-
19-04629-0063				Skin	27.77	-
19-04629-0064	T4 #22 Moribund	13	12	Gill	20.43	-
19-04629-0065				Kidney	21.21	34.13
19-04629-0066				Skin	24.85	40.13
19-04629-0067	T5 #23 Moribund	13	12	Gill	28.34	-
19-04629-0068				Kidney	23.82	-
19-04629-0069				Skin	21.03	32.36
19-04629-0070	T4 #24 Moribund	14	12	Gill	28.15	-
19-04629-0071				Kidney	26.43	-
19-04629-0072				Skin	27.34	-
19-04629-0073	T3 #25 Moribund	15	12	Gill	30.01	-
19-04629-0074				Kidney	25.38	34.20
19-04629-0075				Skin	28.91	-
19-04629-0076	T4 #26 Moribund	15	12	Gill	20.31	-
19-04629-0077				Kidney	25.56	-
19-04629-0078				Skin	31.36	-
19-04629-0079	T5 #27 Moribund	15	12	Gill	23.04	-
19-04629-0080				Kidney	21.69	-
19-04629-0081				Skin	22.09	-
19-04629-0082	T4 #28 Moribund	16	12	Gill	33.91	-
19-04629-0083				Kidney	26.63	-
19-04629-0084				Skin	28.06	-
19-04629-0085	T4 #29 Moribund	16	12	Gill	31.81	-
19-04629-0086				Kidney	27.48	-
19-04629-0087				Skin	30.26	-
19-04629-0088	T4 #30 Moribund	16	12	Gill	22.80	-
19-04629-0089				Kidney	19.03	38.81
19-04629-0090				Skin	26.17	-
19-04629-0091	T3 #31 Moribund	17	12	Gill	23.21	37.86
19-04629-0092				Kidney	23.60	-
19-04629-0093				Skin	26.80	-
19-04629-0094	T3 #32 Moribund	17	12	Gill	22.04	-
19-04629-0095				Kidney	17.27	34.10
19-04629-0096				Skin	27.71	-
19-04629-0097	T4 #33 Moribund	17	12	Gill	20.22	36.34
19-04629-0098				Kidney	23.33	-
19-04629-0099				Skin	23.51	-
19-04629-0100	T2 #34 Moribund	18	12	Gill	29.81	-
19-04629-0101				Kidney	26.73	-
19-04629-0102				Skin	30.13	-
19-04629-0103	T3 #35 Moribund	18	12	Gill	28.00	-
19-04629-0104				Kidney	26.15	-
19-04629-0105				Skin	25.85	-
19-04629-0106	T6 #36 Dead	18	12	Gill	27.22	-
19-04629-0107				Kidney	25.92	-
19-04629-0108				Skin	32.40	-
19-04629-0109	T3 #37 Moribund	20	12	Gill	29.07	-
19-04629-0110				Kidney	25.05	-
19-04629-0111				Skin	31.37	-
19-04629-0112	T5 #38 Moribund	21	12	Gill	26.93	-
19-04629-0113				Kidney	26.56	-
19-04629-0114				Skin	24.01	-
19-04629-0115	T5 #39 Moribund	22	13	Gill	19.70	33.92

19-04629-0116				Kidney	20.46	-/39.43
19-04629-0117				Skin	19.37	33.28
19-04629-0118	T5 #40 Dead	29	20	Gill	17.74	43.52/-

T = Tank, - = not detected, samples tested with qPCR and RT-qPCR assays in duplicate, cycle threshold 0.1

WHAT IS KNOWN ABOUT KHV TERMINASE mRNA TEMPORAL EXPRESSION IN CARP?

Testing of the above sub-set of FRDC KHV Trojan carp samples demonstrated that the AFDL KHV Terminase RT-qPCR assay could detect mRNA in a range of carp samples. However, prior to using the assay to test samples in the FRDC KHV NTS project, it is important to understand what is known about the temporal expression of KHV terminase mRNA in carp.

The following exerts briefly capture the information available in the literature when testing carp with the KHV Yuasa mRNA RT-PCR:

- *Yuasa et al. 2012*; KHV terminase mRNA was detected in KHV-exposed carp 1 and 2 dpi.
- *McColl et al. 2017*; The stability of KHV terminase mRNA in unfixed gill and kidney samples was monitored over a ~24h period at 21-23°C post humane killing and found to be positive in all 11 fish at 16 hpe. 79.6% (39/49 over 9 trials) of KHV qPCR positive viral-challenged carp were positive for viral mRNA [dpi and tissue type information (kidney, spleen or gill) for each carp was not provided].
- *Kim et al. 2019*; KHV terminase mRNA was detected in KHV-exposed koi carp 3, 5 and 7 dpi in both gills/fins and spleens/kidneys. Of note is that 1-2 faint RT-PCR products were detected at 3 dpi in crucian carp (non-target species).
- *Tolo et al. 2021*; KHV terminase mRNA was detected in KHV-exposed S-carp 6 dpi from brain, gill, kidney and skin samples.

RECOMMENDATION

Based on the evaluation undertaken to date, the AFDL KHV Terminase RT-qPCR is suitable for use for the detection of KHV mRNA for FRDC 2019-175 “Determination of the susceptibility of silver perch, Murray cod and rainbow trout to infection with CyHV-3.” Due to the limited information available regarding the temporal expression of KHV terminase mRNA in carp for experiments longer than a week in duration, results obtained from KHV exposed carp collected during the FRDC KHV NTS project will be critical to interpreting the results when testing samples from the non-target species.

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 Microbiology* **5,** 1–9.

Bergmann SM, Riechardt M, Fichtner D, Lee P and Kempter J (2010) Investigation on the diagnostic sensitivity of molecular tools used for the detection of koi herpesvirus. Journal of Virological Methods 163, 229-233.

Kim HJ, Kwon SR, Olesen NJ, Yuasa K (2019) The susceptibility of silver crucian carp (*Carassius auratus langsdorfii*) to infection with koi herpesvirus (KHV). Journal of Fish Diseases 42(10), 1333-1340.

Mc Coll KA, Sunarto A, Slater J, Asmus M, Fulton W, Hall K, Brown P, Gilligan D, Hoad J, Williams LM, Crane MStJ (2017) Cyprinid herpesvirus 3 as a potential biological control agent for carp (*Cyprinus carpio*) in Australia: susceptibility of non-target species. Journal of Fish Diseases 40, 1141-1153.

OIE Manual of Diagnostic Tests for Aquatic Animals (2017) Chapter 2.3.7 KOI HERPESVIRUS DISEASE. World Organisation for Animal Health, Paris.

Tolo IE, Padhi SK,l Williams K, Singh V, Halvorson S, Mor SK, Phelps NBD (2021) Susceptibility of *Pimephales promelas* and *Crassius auratus* to a strain of koi herpesvirus isolated from wild Cyprinus carpio in North America. Nature Research 11, 1985.

Yuasa K, Kurita J, Kawana M, Kiryu I, Oseko N, Sano M (2012) Development of mRNA-specific RT-PCR for the detection of koi herpesvirus (KHV) replication stage. Disease of Aquatic Organisms 100, 11-18.

Yuasa K, Sano M, Kurita J, Ito T, Iida T. 2005. Improvement of a PCR method with the Sph 1–5 primer set for the detection of koi herpesvirus (KHV). *Fish Pathology* **40,** 37–39.

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