RESEARCH 11 2017-127

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

NATIONAL CARP CONTROL PLAN

Defining best practice for viral susceptibility testing of non-target species to Cyprinid herpesvirus 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

Determining best practice for testing the resistance of non-target species (NTS) to Cyprinid herpesvirus 3 (CyHV-3) infection as part of the National Carp Control Program

A discussion paper based on systematic quantitative literature review

Stephen B. Pyecroft and Ben Jones

December 2019

FRDC Project No **2017/217**

Determining best practice for testing the resistance of non-target species to Cyprinid herpesvirus 3 (CyHV-3) infection as part of the National Carp Control Program - A discussion paper based on systematic quantitative literature review

2017/217

2019

Ownership of Intellectual property rights

Creative Commons licence

Disclaimer

Researcher Contact Details

Name:	S.B. Pyecroft
Address:	University of Adelaide Roseworthy, South Australia, 5371
Phone:	+61 8 83137823
Fax:	
Email:	stephen.pyecroft@adelaide.edu.au

FRDC Contact Details

Address:	25 Geils Court Deakin ACT 2600
Phone:	02 6285 0400
Fax:	02 6285 0499
Email:	frdc@frdc.com.au
Web:	www.frdc.com.au

In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

Tables

Figures

Table of Contents

1. Introduction

1.1. Cyprinid Herpesvirus 3 and the National Carp Control Plan

Cyprinid Herpesvirus 3 (CyHV-3) is the aetiological agent of a highly pathogenic disease causing mass mortality in wild and captive populations of common carp (*Cyprinus carpio*) and its ornamental variety, koi. Disease outbreaks associated with this virus are reported from Europe, the Middle East, North America, Asia and South Africa. Mortality rates for captive common carp are typically in the range of 50-95% while mortality events in wild populations can involve tens to hundreds of thousands of common carp deaths (e.g. Matsui et al., 2008; OIE, 2017b; Thresher, Allman, & Stremick-Thompson, 2018). CyHV-3 was first identified as a pathogenic agent in mortalities of common carp in Germany in 1997 and in Israel and the USA in 1998. However, subsequent analysis of samples from a mass mortality event in the UK in 1996 also detected the DNA of CyHV-3 (OIE, 2017b). Following these initial mortality events, the virus spread rapidly and now has a vast geographical range, having been positively identified in at least 28 countries (OIE, 2017b).

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

Common carp are a highly successful invasive fish species in Australia that have been reported as causing serious ecological impacts, and for which there are no currently effective, broad-scale control measures. Consideration of the use of CyHV-3 as a biological control agent for common carp in Australia began with a study, funded by the Invasive Animals Cooperative Research Centre and undertaken by the CSIRO Animal Health Laboratory, examining the specificity of CyHV-3 to common carp and the susceptibility of a range of non-target species (NTS) to infection by the virus (McColl et al. 2016). This study reported that none of the 21 NTS assessed were susceptible to CyHV-3 infection.

The findings of McColl et al. (2016) that the NTS assessed were not susceptible to CyHV-3 infection, together with the ability of the virus to cause mass mortality in common carp populations, suggested that CyHV-3 might be an effective biological control agent for common carp in Australia. On the basis of this potential opportunity, the Australin Government initiated the development of the NCCP.

1.2. Host specificity of Cyprinid Herpesvirus 3

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 (OIE, 2017a).

Because of the global economic importance of common carp and the widespread occurrence of CyHV-3 and its severe effects on carp, the virus and its interactions with a range of species has been extensively studied. More than 60 species have been investigated for their susceptibility to CyHV-3 infection or for the potential for the virus to be detected in their tissues, with results from studies reported in at least 41 published articles (see Appendix 1). These studies were usually performed for either of two reasons: to identify strains of common carp or hybrids of this species that are resistant

to CyHV-3 infection, for aquaculture purposes; or to determine the potential for other species to carry the virus and act as a vector in the spread of CyHV-3 amongst common carp.

There is growing evidence that CyHV-3 can persist in a number of cyprinid and non-cyprinid species, all be it without causing major observable clinical signs during tank trials or in the field, and that a number of these species can transmit the virus to naïve common carp. CyHV-3 DNA has been detected in a range of non-common carp species including goldfish (*C. auratus*), grass carp (*Ctenopharyngodon idella*), ide (*Leuciscus idus*), ornamental catfish (*Ancistrus sp.*), Russian sturgeon (*Acipenser gueldenstaedtii*), Atlantic sturgeon (*A. oxyrinchus*), stone loach (*Barbatula barbatula*), hybrids between sterlet (*Acipenser ruthenus*) and beluga (*Huso huso*), topmouth gudgeon (*Pseudorasbora parva*), Prussian carp (*Carassius gibelio*), silver carp (*Hypophthalmichthys molitrix*), rudd (S*cardinius erythrophthalmus*), ruffe (*Gymnocephalus cernua*), rainbow trout (*Oncorhynchus mykiss*), common bream (*Abramis brama*), common barbell (*Barbus barbus*), crucian carp (*Carassius carassius*), common nase (*Chondrostoma nasus*), spined loach (*Cobitis taenia*), gudgeon (*Gobio gobio*), belica (*Leucaspius delineates*), common dace (*Leuciscus leuciscus*), common roach (*Rutilus rutilus*), European chub (*Squalius cephalus*), tench (*Tinca tinca*), vimba bream (*Vimba vimba*), northern pike (*Esox lucius*), European perch (*Perca fluviatilis*), zander (*Sander lucioperca*), European bullhead (*Cottus gobio*), and sheatfish (*Ictalurus melas*) (See Chapter 7: S. Bergmann et al., 2009; S. M. Bergmann, Stumpf, Schütze, et al., 2007; M. El-Matbouli, Saleh, & Soliman, 2007; Fabian, Baumer, & Steinhagen, 2013; Ilouze, Davidovich, Diamant, Kotler, & Dishon, 2011; J. Kempter & Bergmann, 2007; J Kempter et al., 2009; Kempter., Kielpinski, Panicz, & Sadowski, 2008; OIE, 2017b; Pospichal, Piackova, Pokorova, & Vesely, 2016; Pospichal, Pokorova, Vesely, & Piackova, 2018; Sadler, Marecaux, & Goodwin, 2008).

These studies have often used aspects of the World Organisation for Animal Health (OIE) criteria for listing a species as susceptible to infection by a specific pathogen (OIE, 2017a). The primary application of the OIE criteria is to determine the susceptibility of a subject species to natural infection by a pathogen. The decision to list a species as susceptible to infection under these criteria must be based on a finding of definite evidence. While this approach is a powerful tool in confirming that a species is susceptible to infection by a specific pathogen, it may be less suitable when seeking to determine that a species is definitively *not* susceptible, that is, *resistant* to infection. In this case, additional factors may need to be taken into account to determine the true resistance-status of a subject species. This may include assessing the resistance of all life stages to infection and assessing resistance to infection under adverse or stressful conditions.

In addition to this rigorous system of defining resistance to a pathogen by a target species commonly the definition of resistance may imply simply the lack of obvious clinical signs (*affect*) post pathogen exposure to the host rather than infection.

1.3. Biological Control and Non-Target Species

Biological control is the process of controlling a pest species by using other organisms. This can involve the use of a pathogen to control the abundance of an invasive pest species. Where a biological control program seeks to introduce an exotic pathogen into a naïve ecosystem, several fundamental questions must first be answered. Two of the most important questions involve the susceptibility of the target species by the pathogen and the resistance-status of any NTS likely to be exposed to the pathogen.

It is obviously critical that the targeted pest species is vulnerable to the pathogen being used. This can be readily determined by assessing the pathogen and target species against the criteria listed by

the OIE (OIE, 2017a). However, of equal importance is that any NTS likely to be exposed to the pathogen are resistant to the infection, even under adverse conditions that may promote pathogenic infection and clinical effects. This will prevent the pathogen from having a negative effect on NTS, which could have catastrophic consequences.

Designing protocols to test NTS for resistance to infection by a specific pathogen may require a different approach compared to assessing a target species for susceptibility to infection. When assessing for susceptibility to infection, the focus of the study is often to provide definite evidence that the target species can be infected and/or that clinical signs can be induced by the pathogen. However, when assessing a NTS for resistance, it may be necessary to provide definite evidence that the pathogen cannot infect the NTS under any conditions likely to be encountered by the NTS. In this latter case, there may be a need to achieve a greater level of confidence to safeguard against the pathogen infecting and causing disease in NTS.

This project (Determining best practice for testing the resistance of non-target species (NTS) to Cyprinid herpesvirus 3 (CyHV-3) infection as part of the National Carp Control Program) As noted above, McColl et al. (2016) and McColl et al, (2017)undertook a series of studies to determine the susceptibility of NTS to CyHV-3 infection, the findings of which were a key driver for the NCCP. These studies, performed *in vitro* and *in vivo*, were undertaken to predict *in situ* outcomes and are the most comprehensive examination of susceptibility to CyHV-3 infection in aquatic and terrestrial species that has been reported in the scientific literature. Published in *Journal of Fish Diseases*, the work presents compelling data and reported that none of the 21 species assessed were susceptible to CyHV-3 infection.

Despite the compelling evidence of non-susceptibility of NTS to CyHV-3 infection presented by McColl et al. (2016), a number of questions as to the confidence of their findings have been raised by stakeholders during the development of the NCCP. It was felt by some stakeholders that while the study provided much compelling data, it fell short of showing definitively that all NTS likely to be exposed to CyHV-3, if it were to be released into Australian waters as a biological control agent for common carp , are resistant to the virus. In addition the Science Advisory Group of the NCCP raised questions about the testing of NTS using stress such as anoxia, and the testing of particularly vulnerable life stages. Whilst the project is not intended to be an appraisal of the work of McColl et al. (2016), some reference to this study must be made for contextual clarity.

Six key concerns raised by stakeholders warranted further investigation and were reviewed by this project:

1. Techniques used to diagnose CyHV-3 infection were perceived to be unreliable.
2. Mortalities occurring in NTS exposed to CyHV-3 during trial work, but reported to be unrelated to CyHV-3, were not adequately investigated or explained..
3. ‘False positive’ results reported for some Polymerase Chain Reaction (PCR) tests of NTS exposed to CyHV-3, were not adequately explained.
4. Immuno-competency status and stress were not considered in susceptibility studies.
5. Only mature or advanced juvenile fish were assessed for susceptibility to CyHV-3 infection in the NTS testing.
6. The NTS investigated for susceptibility to infection by CyHV-3 were limited in extent of species and did not include a number of species that may be exposed to CyHV-3 if it were to be intentionally released into Australian waters.

To address these concerns and further inform the development of the NCCP a review of the published scientific literature related to CyHV-3 was undertaken in the period February- June 2018

The review process is presented below in Chapter 2. *General Methodology*. This chapter sets out the clearly identifiable and repeatable process by which applicable scientific papers were identified and selected for inclusion into the review. Chapters 3 to 7 draw on this body of literature to review previous studies with respect to specific stakeholder-raised concerns. These chapters each contain specific background information, place the review into context and outline the process by which studies were categorised against specific stakeholder concerns. Each chapter is then a review and discussion of the literature gathered and provides specific recommendations to guide future resistance testing of NTS to CyHV-3 infection, in the NCCP.

Chapter 3. *What is the most effective diagnostic approach to determine the resistance status of NTS to CyHV-3 infection in the NCCP?* discusses techniques previously used to diagnose CyHV-3 infection and identifies the capabilities and deficiencies with the specific tests. This section then provides recommendations as to the most appropriate suite of diagnostic tests that can be used to ensure reliability and accuracy of detecting CyHV-3 infection in the context of resistance testing of NTS in the NCCP. It highlights the concept of 'Fit for Purpose' selection of diagnostic test methods.

Chapter 4. *Overcoming undiagnosed mortalities & false positives through the development of a diagnostic matrix that considers all available diagnostic information* explores how previous studies have dealt with undiagnosed mortalities and false positive diagnostic results within trial exposure studies and discusses the uncertainties that arise for poorly or undiagnosed mortalities.. A recommended framework is then presented that gives confidence in assessing resistance to CyHV-3 infection in exposed NTS.

Chapter 5. *Should stressors be deliberately imparted when assessing the susceptibility/resistance of fish to CyHV-3 infection in the NCCP?* provides a review of previous studies that have applied, assessed or discussed the effects of applying an external stressor to target species during CyHV-3 exposure and its effects on infection dynamics. The review assesses immunocompentency as an influential factor in CyHV-3 exposure testing. Specific recommendations are made as to the application of stressors in resistance testing of NTS in the NCCP.

Chapter 6. *Should all life stages of fish be evaluated in studies that investigate the resistance of NTS to CyHV-3 infection in the NCCP?* discusses the effect of life stage on the susceptibility to infection of fish to a range of globally important viruses and viruses closely related to CyHV-3 and CyHV-3 infection in common carp. Recommendations are made on the testing of different life stages of endemic Australian fish to assess the potential of CyHV-3 infection post exposure.

Chapter 7. *What is the potential for NTS beyond those previously investigated, to become infected by CyHV-3? Should future CyHV-3 resistance testing include a wider range of NTS?* discusses the suitability of Australian ecosystems for the survival and persistence of CyHV-3 and provides a summary of the factors that have previously been implicated in the geographic spread of this virus globally and within countries where the virus is now endemic. The resistance status and susceptibility of different fish species around the world to CyHV-3 is also summarised, with special reference to the effects of taxonomy. The information presented can be used to inform the selection of further endemic Australian fish species for exposure trials for the assessment of CyHV-3 infection susceptibility.

In the absence of novel experimental data, this review provides critical information based on the experimental work of others. This approach is limited in its ability to comprehensively demonstrate the resistance status of NTS to CyHV-3. However, by drawing together literature from a wide-range of sources, it can provide critical insights into the infection dynamics of CyHV-3 and provide some recommendations as to: the likely resistance status and susceptibility of NTS to CyHV-3; the need for further NTS resistance testing; and protocols to optimise future resistance testing, if required. This should be seen as a critical step towards guiding future actions of the NCCP.

The objectives of the review was to provide some recommendations about NTS CyHV-3 exposure testing in the context of the NCCP, so that there is more confidence in the results of experimentally derived results.

OBJECTIVES

1 The overarching objective of this project is to investigate potential problems, limitations and concerns related to viral susceptibility testing of NTS to CyHV-3 in the context of the NCCP and to define what constitutes best practice in viral susceptibility testing.

2 Review and discuss the implications of immune-competency status in pathogen susceptibility testing of aquatic organisms.

3 Review the range of techniques for assessing viral infection in aquatic animals and evaluate their accuracy and reliability.

4 Discuss the occurrence and implications of undiagnosed mortalities and □false positives□□in pathogen susceptibility studies.

5 Determine, based on existing literature, potential differences in the susceptibility of larval, juvenile and mature fish to viruses.

6 Determine, based on existing literature, the potential for fish and other aquatic organisms beyond those previously investigated to become infected by CyHV-3 as a result of actions of the NCCP.

2. General Methodology

The literature reviews undertaken for this report applied methods adapted from Pickering and Byrne (2014) and elements of the guidelines outlined by the Preferred Reporting Items for Systematic Reviews (PRISMA) recommendations (Moher, et al.,2009). PRISMA statements 1 – 5 graphically represent how the reviews were conducted.

To identify studies that investigated CyHV-3, a review of the literature was undertaken to identify original published research studies and reviews that: (1) referred to Cyprinid Herpesvirus 3 (CyHV-3), Koi Herpesvirus (KHV), Koi Herpesvirus Disease (KHVD) or Carp Interstitial Nephritis and Gill Necrosis Virus (CNGV); and (2) included a positive identification of CyHV-3, where the virus was observed; and (4) were published after 1996; and (5) were published in English.

Eligible studies were identified by conducting a systematic search of online databases. During February–June 2018, Web of Science, Scopus and Google Scholar were searched using a string of the following search terms: Cyprinid Herpesvirus 3 (CyHV-3), Koi Herpesvirus (KHV), Koi Herpesvirus Disease (KHVD), Carp Interstitial Nephritis, Gill Necrosis Virus (CNGV). Different combinations of these search terms were used depending on the requirements and limitations of each database. For example, the search string used in Scopus was: ("Cyprinid Herpesvirus 3" OR CyHV-3 OR "Koi Herpesvirus" OR KHV OR "Koi Herpesvirus Disease" OR KHVD OR "Carp Interstitial Nephritis and Gill Necrosis Virus" OR CNGV). This was repeated for each database.

The studies identified were then screened to ensure that only studies relevant to the topic of review were included. Titles and/or abstracts were read and studies were excluded where they failed to mention CyHV-3 or its synonyms. After this screening process, 451 of an original 759 studies were identified as meeting the criteria for review.

The studies identified were then screened against two discrete sets of criteria.

1. To investigate the most suitable diagnostic tools for determining CyHV-3 infection in fish (see Chapter 3), titles and/or abstracts were read and studies were excluded where they failed to: present novel techniques for the diagnosis of CyHV-3; or discuss, assess or review existing methodology involved in the diagnosis of CyHV-3 infection. After this screening process, 152 studies of an original 465 were retained. This screening process is graphically represented in PRISMA Statement 1 (Figure 1). These studies were subjected to additional screening to identify those studies that directly appraised diagnostic techniques. This process is detailed in *Chapter 3. What is the most suitable diagnostic approach to determine CyHV-3 infection in NTS in the NCCP?*
2. For the remaining topics of review, studies were excluded where they failed to directly assess the susceptibility or resistance of at least one potential target species to CyHV-3 infection. After this round of screening, 41 studies, of an original 451 were retained. The number of studies identified in the initial search and those removed at each screening step are shown in PRISMA Statement 2 (Figure 2).

For the 41 articles that assessed the susceptibility or resistance of at least one subject species to CyHV-3 infection, key information was extracted and is presented in Appendix 1. This information is intended to provide a snapshot of the body of knowledge relating to susceptibility/resistance testing of fish to CyHV-3.

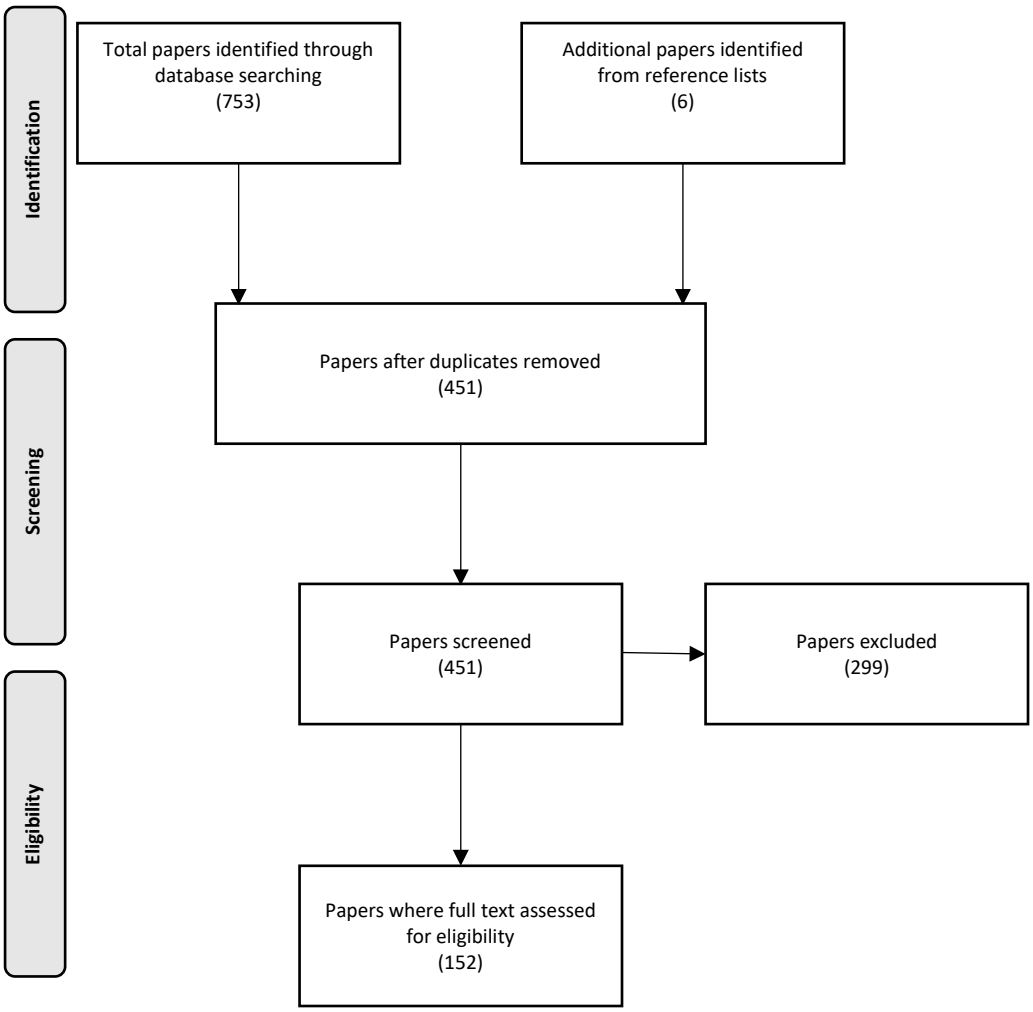

Figure 1. PRISMA statement 1. Screening process used to identify articles that present novel techniques for the diagnosis of CyHV-3 infection or discuss, assess or review existing methodology involved in the diagnosis of CyHV-3 infection.

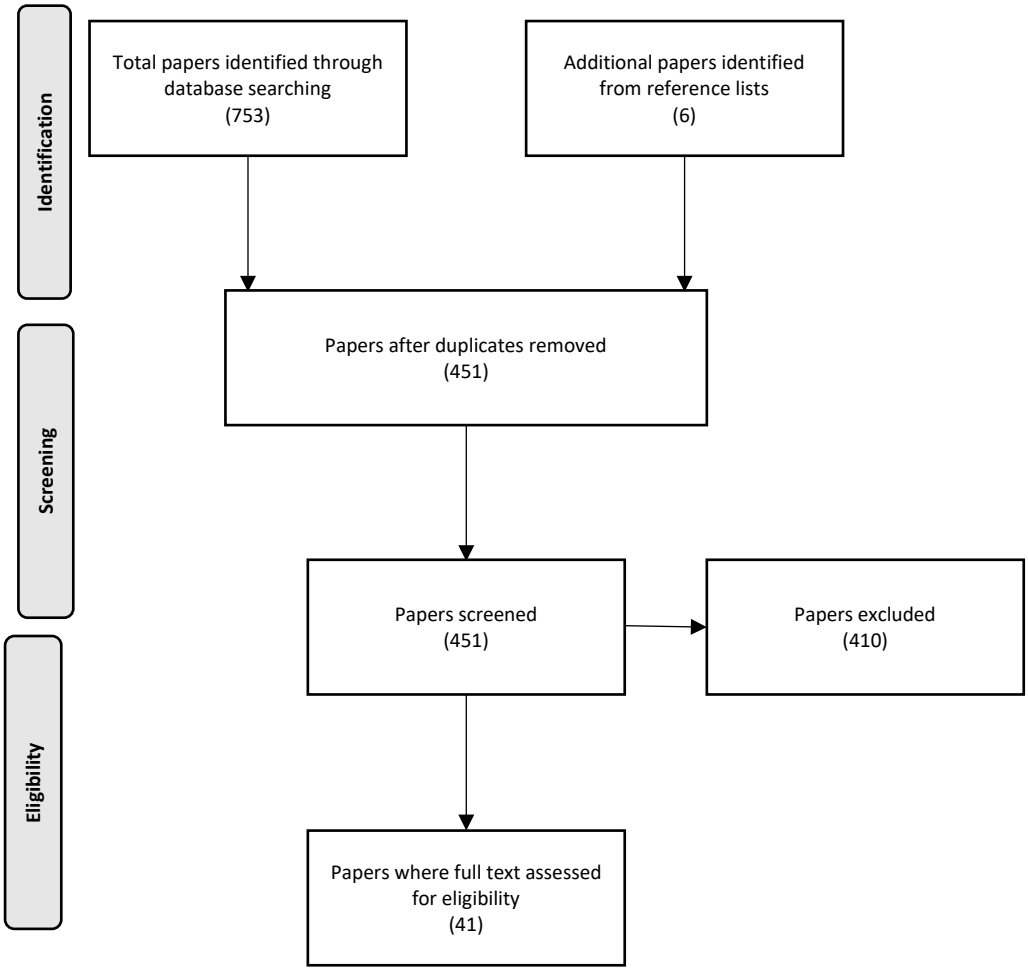

Figure 2. PRISMA statement 2. Screening process used to identify articles that assessed the susceptibility or resistance of at least one potential target species to CyHV-3 infection.

The 41 articles identified in this initial systematic, quantitative review were used: to explore how previous work has dealt with undiagnosed mortalities and false positive diagnostic results; to review previous investigations that have applied, assessed or discussed the effects of applying an external stressor to subject fish on CyHV-3 infection dynamics; and to explore the resistance status and vulnerability of different fish species around the world to viral infections. Where necessary, additional screening was performed to exclude studies that were not relevant to each of these topics of review. Details of specific literature review methodology, are presented in Chapters 4, 5 and 7.

The systematic, quantitative literature review process (Pickering & Byrne, 2014) proved to be sub-optimal for categorically identifying literature related to specific patterns in the susceptibility/resistance of different life stages of fishes to viruses. As a consequence, Chapter 6. *Should all life stages of fish be evaluated in studies that investigate the resistance of NTS to CyHV-3 in the NCCP?* is presented as a conventional narrative review. The details of the specific review process are presented in Chapter 6.

Where it was deemed necessary, additional information was gathered from studies that investigated a broader range of pathogens. This ensured that the recommendations provided are widely informed, robust and able to withstand the scrutiny of stakeholder groups. Employing a systematic,

quantitative approach to this multifaceted review has ensured that the results obtained provide the most appropriate recommendations possible, at this time, while ensuring transparency in the review process and eliminating the potential for unintended bias.

3. What is the most suitable diagnostic approach to determine the resistance status of NTS to CyHV-3 infection?

Ensuring that NTS are resistant to infection by a pathogen is a key requirement in assessing whether that pathogen is suitable to use as a biological control agent. In testing the resistance of NTS to infection by a specific pathogen, it is necessary that diagnostic tools are fit for purpose and perform within a diagnostic protocol to identify infection and hence allow assessment of resistance in NTS after exposure to CyHV-3 virus. This chapter addresses concerns raised about the reliability of techniques used to diagnose CyHV-3 infections in NTS susceptibility undertaken by McColl et al. 2017.

3.1. Defining viral infection and resistance to viral infection in the context of the NCCP

K. McColl et al. (2017), in their investigation into the susceptibility of NTS to CyHV-3 in the NCCP stated that "*the best biocontrol agents will preferably not infect, and certainly not affect, any non-target species (NTS)*". This statement demonstrates the fundamental cautiousness required when assessing the resistance of NTS in a biological control program. However, some ambiguity remains. It is uncertain whether the approach of McColl et al. (2017) was designed to investigate the potential for CyHV-3 to *infect* or *affect* NTS.

Moving forward it is important to clearly define infection and resistance to infection, so that a decision on the use of CyHV-3 as a biological control agent can be made on robust transparent data gained from any NTS susceptibility testing.

Viral infection can be defined as the presence of a multiplying or otherwise developing or latent viral agent in a host. It may cause no clinical signs (subclinical infection) or it may cause signs that are clinically apparent. These clinical signs of viral infection reflect the potential for a pathogen to affect the host and can be highly variable and range from severe to benign. The most severe and conspicuous clinical signs is mortality, while benign clinical signs may include mild lethargy, reduced food consumption, etc. Organisms may also act as carriers of a pathogen, developing no clinical signs after infection, but hosting viable, replicating virus and having the ability to transfer the pathogen to other susceptible hosts.

Resistance to viral infection can be defined as:

- The inability of a viral agent to infect a species i.e. there can be no presences of that multiplying or otherwise developing or latent viral agent in that species; or
- The inability of a viral agent to affect a species i.e. cause clinical signs.

Clinical signs reflect the potential for a virus to affect a species. In the context of NTS, it is the potential ability of CyHV-3 to affect NTS, leading to negative impacts on ecological communities, that is a primary concern of the use of the virus as a biological control agent.

However relying on clinical signs alone as definitive evidence of resistance of NTS to CyHV-3 infection post exposure is problematic. CyHV-3 has the potential to infect fish latently, or sub-clinically, with clinical signs developing only when conditions are favourable for viral activation (e.g. Reed et al., 2017). Furthermore, specific clinical signs caused by viral infection can be difficult to detect during laboratory trials, and some of these clinical signs may have critical implications on wild populations of fish. For example, pathogenic infection may cause reduced fecundity, which although difficult to assess in short term laboratory trials, has the potential to severely impact wild

populations of fish (Reno, P.W., 1998). Additionally if CyHV-3 is able to infect NTS (actively, subclinically or latently), the potential for spill over infections or species jumps is likely be increased (Flanagan et al., 2012).

While it is true that infection with a pathogen may not affect a host animal, it is also true that the inability of a pathogen to infect NTS precludes its ability to affect those NTS directly. For this reason, the NCCP is being developed on the basis of the first definition of resistance i.e. that the virus cannot infect non-target species.

Working under this definition, it is essential to use a diagnostic approach that gives the highest possible level of confidence that all NTS tested for resistance to CyHV-3 infection are truly resistant to infection, in which case, the ability for the virus to affect those NTS becomes irrelevant.

Providing a prescriptive experimental design for resistance testing of NTS to CyHV-3 infection in the NCCP, was largely beyond the scope of this project, a review of techniques used to diagnose CyHV-3 infection is presented below. Recommendations are made as to the most appropriate diagnostic approach to be used in future resistance testing protocols in the NCCP.

3.2. Diagnostic tools used to identify viral infections with specific reference to teleost fish

Diagnosing diseases in fish can be undertaken using a range of diagnostic tools. The OIE Manual of Diagnostic Tests for Aquatic Animals is a valuable resource in this regard, and provides detailed protocols for a range of techniques used to diagnose various specific pathogens (OIE, 2017b). These tools can be categorised by their mode of identifying the viral pathogen and include:

- Field diagnostic methods
  - Clinical signs
  - Behaviour
- Clinical methods
  - Gross pathology
  - Clinical chemistry
  - Microscopic pathology
  - Wet mounts
  - Smears
  - Electron microscopy
  - Cytopathology
- Direct detection methods
  - Microscopy
  - Cell culture
  - Antibody based antigen detection methods
  - Molecular techniques
- Serological methods

When deciding on the most appropriate diagnostic approach in a given situation, it is critical that the reason for seeking a diagnosis is clearly defined. The performance characteristics of the diagnostic tools must also be carefully considered. Important aspects are the diagnostic sensitivity and specificity and the analytical sensitivity and specificity of the diagnostic tool. Diagnostic sensitivity and specificity refer directly to the likelihood of a test experiencing false negative or false positive results respectively. Analytical sensitivity refers to the concentration at which a pathogen becomes

detectable in the host animal, while analytical specificity refers to the ability of the test to only recognise the target pathogen and not other, similar pathogens.

Diagnostic sensitivity refers to the accuracy of a test to correctly identifying the presence of a specific pathogen in an infected animal. If a test has 100% sensitivity, it will return a positive result in all tests on animals which are truly infected by the pathogen (i.e. always identify the pathogen when it is present). A test with 90% sensitivity will return a positive result in 90% of tests on animals that are truly infected (i.e. identify the pathogen in 90% of tests of animals that are carrying the pathogen). The remaining 10% of tests of animals that are actually infected with the pathogen will be negative for the pathogen (i.e. fail to identify the pathogen in 10% of tests where the host is actually infected). These failed tests are termed “false negatives” and reflect an inherent and unavoidable risk in many diagnostic procedures.

Diagnostic specificity refers to the ability of a test to correctly identify those hosts subjects that are not infected with a specific pathogen. A test with 100% specificity will return a negative result in all tests on subjects in which are not infected by the pathogen is truly absent. A test with 90% specificity will return a negative result in 90% of tests on subjects that are actually not infected with the pathogen. The remaining 10% of tests on subjects that are not actually not infected with the pathogen will return a “false positive” result. These failed tests are termed “false positives”.

While diagnostic sensitivity and specificity appear to be simple concepts, these factors can be difficult to determine and can be expressed in different ways. Sensitivity and specificity can be evaluated statistically or relatively. To evaluate a diagnostic test statistically requires the true infection status of all subjects to be known. For this to occur, there must be a tool available that can determine, absolutely, if the subject is infected. In many cases, this may not be possible, especially when responding to new infectious agents for which diagnostics are in the early stages of development.

Where a gold-standard for detecting true infection-status does not exist, relative sensitivity is often used to evaluate diagnostic tests. For example, alternate tests may be compared with one-another to determine the number of subjects that return a positive result from each test. Where one test returns more positive results than the other, it can be considered to be more sensitive. However, in this instance, the specificity of the test remains unknown and some of the positive results returned may in fact be false positives. While this approach may seem unsatisfactory, it is useful where the consequences of experiencing false negatives are great, as some reduction in specificity may be tolerable.

Analytical sensitivity is also important. It is important that a diagnostic test can identify infection by a pathogen even when it is present in very low abundance. This becomes more critical for pathogens that can establish low-level persistent or latent infections. In this case, it is desirable that a diagnostic test has very good analytical sensitivity so that it can detect the pathogen not only during active infection, when the pathogen load is high, but also when very few individual pathogenic units are present.

To avoid confusion between the terms diagnostic sensitivity and analytical sensitivity, the term analytical responsiveness or just responsiveness is used hereafter to refer to analytical sensitivity.

Analytical specificity is also sometimes used in infection diagnostics. Rather than referring directly to the number of false positives occurring from a test, analytical specificity refers to whether or not a test will detect a similar pathogen and mistake it for the target pathogen. To determine analytical

specificity, a diagnostic test for a specific pathogen is performed on samples known to also contain similar pathogens. If it fails to identify these non-target pathogens, then the test is considered to have good analytical specificity.

Ideally, a test would be 100% accurate. This is rarely the case and critical decisions must be made to ensure that the test/s used in a given situation provide a level of diagnostic sensitivity and specificity and have sufficient analytical responsiveness to suitably manage the risks associated with returning "false negative" and "false positive" results.

The implications of false negative results are greatest where the consequences of failing to identify an infection are profound. This is the case when assessing the resistance of a NTS to a pathogenic infection in a biological control program. In this case, failing to identify infection by a pathogen when it is actually present may lead to NTS being considered to be resistant to infection when this is not actually the case. This has the potential to expose a NTS to a pathogen to which it is not resistant, which may have serious, irreversible consequences. To limit the occurrence of false negatives it is important that the diagnostic approach has high diagnostic sensitivity and high analytical responsiveness. This will safeguard against false negative results, even when the infection occurs with the pathogen present at low abundance.

The implications of "false positives" are less profound but have the potential to confuse results if they are not adequately addressed. A false positive result would identify a NTS as not resistant to infection by a pathogen when in fact that NTS has not been actually infected. Where false positives are suspected, additional diagnostic procedures may be performed, or exposure trials repeated to ensure that the true resistance status of NTS is known. A more detailed discussion of false positives is presented in *Chapter 4.* (Bergman et al. 2009; Kempton et al, 2009)

3.3. Fit-for-purpose diagnostic protocols

The diagnostic protocol used to determine the susceptibility/resistance of a target species to a specific pathogen infection is a key concern and careful consideration must be given to ensure that the sensitivity and specificity of the diagnostic approach meets the requirements of the study. This will ensure that the diagnostic approach is fit-for-purpose.

To optimise the accuracy of overall results (i.e. increase sensitivity and specificity of the diagnostic outcome), a range of tests may be applied together in a broader diagnostic protocol. By applying a range of tests, the level of confidence that the diagnostic protocol is delivering an accurate finding is increased (OIE, 2017b).

In resistance testing of NTS to infection by CyHV-3, a diagnostic protocol that is fit-for-purpose must be used; a protocol that has the power to determine if NTS are resistant to CyHV-3 infection and that the virus cannot establish multiplying or otherwise developing or latent CyHV-3 infections within them..

To achieve this, the diagnostic approach should have the highest possible diagnostic sensitivity and analytical responsiveness. This may require running a number of diagnostic procedures in combination to minimise the risk of experiencing false negatives. By applying a range of tests the level of confidence that the diagnostic protocol is delivering an accurate finding is increased (OIE, 2017b).

The greatest care must also be taken to avoid false positives during resistance testing of NTS to CyHV-3 infection as these have the potential to complicate findings and cast doubt over the validity

of the study. False positives can often be managed against by undertaking a range of diagnostic tests with varying degrees of sensitivity and specificity. It may also be advantageous to undertake diagnostic procedures that have the ability to provide information on the presence of alternate pathogens, diseases, injuries or distress that may confuse the findings of the study.

A number of studies have been published that compare the accuracy and reliability of a wide range of diagnostic techniques for assessing CyHV-3 infection in fish (Appendix 2). These publications provide a valuable resource, against which various diagnostic tools can be assessed for their suitability in the resistance testing of NTS to CyHV-3 in the NCCP. To provide well-informed recommendations, developed through a rigorous and transparent process, *FRDC Project 2017-127* has reviewed this published literature. The process and results of this review are presented below.

3.4. Literature review methodology

The 152 articles identified through the systematic, quantitative review process outlined above (in Chapter *2. General Methodology*), and graphically represented in PRISMA Statement 1 (Figure 1) were subjected to an additional screening process. This was performed to identify those articles that provided a comparison of diagnostic techniques. Studies were excluded where they failed to compare at least two different techniques used in the diagnosis of CyHV-3 infection. After screening, 27 articles of the original 152 were identified as meeting the criteria for inclusion in the review. The full text of each article was then obtained and reviewed in detail. Of each of the relevant publications identified, the reference lists were checked to identify additional relevant publications.

After this process, 27 Studies were included for final review. Limited information from these studies is presented in Appendix 2 providing a snapshot of research work that include important information on diagnosing CyHV-3 infection.

Molecular tests are the most widely used techniques for the diagnosis of CyHV-3. These tests regularly outperform alternative diagnostic procedures (OIE, 2017b). However, a range of different molecular assays have been developed with varying performance characteristics. Because of the importance of molecular tools in the diagnosis of CyHV-3 infection.

The 27 articles identified were subsequently screened to identify those that examined experimental procedures to directly compare the efficacy of various molecular diagnostic tests. Articles were excluded where they failed to undertake experimental procedures to evaluate and compare the effectiveness of at least two different molecular diagnostic tests. After this round of screening, six articles were identified that directly compared a range of different molecular assays for the diagnosis of CyHV-3 infection. This process is graphically represented in PRISMA Statement 3 (Figure 3.1). The studies included for review are discussed in section *3.10. Molecular methods for the diagnosis of CyHV-3 in the NCCP*.

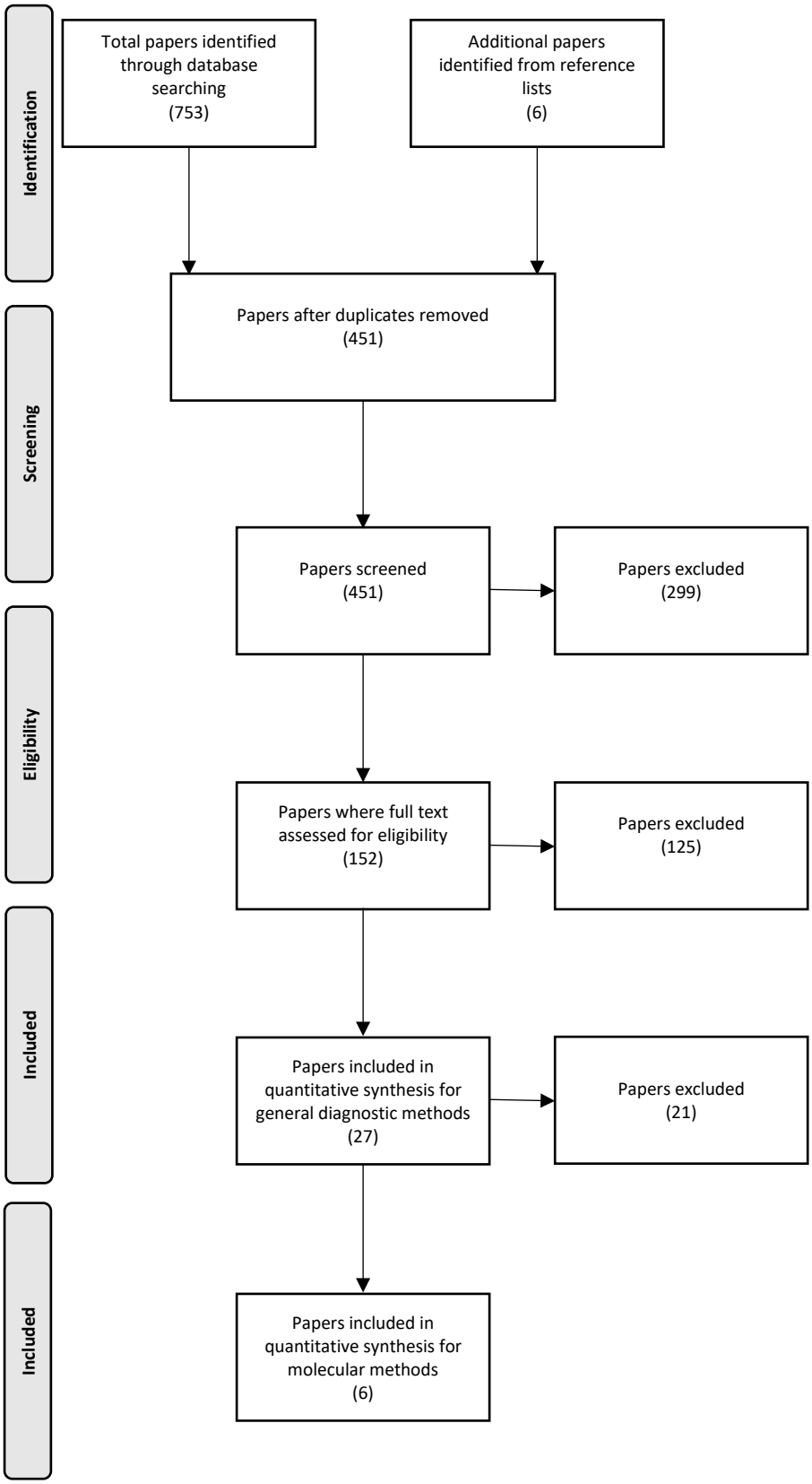

Figure 3. PRISMA Statement 3. Screening process used to identify studies that compare different techniques used in the diagnosis of CyHV-3.

3.5. Appraisal of methods used to diagnose CyHV-3 infection.

The *OIE – Manual of Diagnostic Tests for Aquatic Animals – Chapter 2.3.7. – Koi Herpesvirus Disease* provides important information on a range of tests used in the diagnosis of CyHV-3 infection (OIE, 2017b). This important document is a valuable resource that not only discusses the accuracy and reliability of a range of diagnostic tests but also provides detailed methodology to optimise performance. The information compiled by the OIE (2017b) is often regarded as the current standard for diagnosing CyHV-3 infection and whilst it is used in this review as the foundation for the development of a diagnostic protocol to be applied in future resistance testing of NTS the NCCP presents a unique opportunity for aquatic viral resistance testing and the diagnostic tests presented by the OIE (2017b) can be further appraised to develop a 'Fit for purpose' matrix of diagnostics for CyHV-3 infection.

Diagnostic methods reported by the OIE (2017b) are presented in Table 1, recreated from OIE (2017b). These techniques are rated by the OIE for their suitability of use for targeted surveillance, presumptive diagnosis and confirmatory diagnosis. Resistance testing of NTS to CyHV-3 in the NCCP doesn't fit clearly into any of these categories. As a consequence, the most appropriate diagnostic methods must be selected to satisfy the unique requirements of the NCCP.

The diagnostic protocol used in the resistance testing of NTS in the NCCP, must be able to identify the presence of CyHV-3 infection in NTS where a multiplying or otherwise developing or latent infection is occurring. It must be able to detect the infection where no clinical signs are apparent (subclinical infection) and where clinical signs are evident. The procedure should also ensure that an accurate diagnosis can be made where any clinical signs are observed, even when CyHV-3 virus is not the cause. This will ensure that the true cause of clinical clinical signs or mortalities can be identified, thus providing supporting evidence that CyHV-3 is not the causative agent. Because of the need to satisfy these complex and diverse needs, the diagnostic approach should include aspects of targeted surveillance, presumptive diagnosis and confirmatory diagnosis of CyHV-3 infection in fish.

Targeted surveillance is performed when attempting to identify the presence of CyHV-3 infection in apparently healthy fish. While targeted surveillance is usually applied in ecological or aquaculture settings to determine if there is an underlying presence of CyHV-3 infection, aspects of this approach may also be appropriate when assessing the resistance of NTS to infection in a biological control plan. PCR and antibody detection assays (serology) are considered appropriate for targeted surveillance as these are standard methods with good diagnostic sensitivity and specificity (OIE, 2017b).

A presumptive diagnosis provides indicative evidence of infection only. This approach is useful to identify the possible occurrence of a specific pathogenic infection but lacks the sensitivity and specificity required to conclusively diagnose the pathogen. The OIE lists a number of techniques with good diagnostic sensitivity and specificity for providing a presumptive diagnosis of CyHV-3 infection. These are: gross signs, direct light microscopy, histopathology, isolation in cell culture, transmission electron microscopy, antibody-based virus detection assays, in situ DNA probes, PCR and antibody detection assays (serology). In real terms, many of the techniques in this category have low specificity for providing a confirmatory diagnosis of CyHV-3 infection. As such, when used in

isolation, they are prone to false positive results and confirmatory diagnosis is required to assess the true infection-status of fish.

Despite the fact that many of the techniques suggested for presumptive diagnostics have low sensitivity and specificity, they can still form a critical part of a diagnostic protocol for assessing resistance of NTS to CyHV-3 infection.. These techniques should be applied routinely when seeking a diagnosis, and can be used to generate a broader understanding of the infection-status of healthy, moribund or dead fish. In the case of assessing the resistance-status of NTS to CyHV-3, this can be extremely important as it may prompt further investigation – leading to confirmatory diagnosis of CyHV-3 infection, or it may provide supporting evidence that CyHV-3 infection is not present, or not responsible for mortalities. Published literature related to these techniques, as it pertains to the diagnosis of CyHV-3 infection will be reviewed.

Confirmatory diagnostic procedures are used to confirm the presence of a specific pathogen in sub-clinical, clinically affected, moribund or dead fish. PCR and sequencing methods are presented by the OIE as the recommended methods for confirmatory diagnosis of CyHV-3 virus presence for reasons of availability, utility, and diagnostic specificity and sensitivity (OIE, 2017b). For resistance-testing in the NCCP, these techniques may be used to confirm the presence of CyHV-3 infection when it occurs in common carp or NTS.

PCR and other molecular diagnostic assays can have high sensitivity and specificity. However, a suite of different PCR assays have been developed for diagnosing CyHV-3 presence, with varying levels of sensitivity, specificity and suitability for CyHV-3 diagnosis in different situations. Additionally the specific characteristics of PCR assays, directed to a known pathogen can determine the interpretations that can be made from the results of those assays. Incorrect interpretation of results lead to inappropriate diagnostic outcomes. A key point of note when developing the most appropriate PCR and molecular assays and protocols for determining the resistance status of NTS to CyHV-3 infection.

The sequencing of CyHV-3, when it occurs in fish, is useful to confirm its presence. However, this method relies on the successful cultivation of the virus in cell cultures, prior to it being sequenced. While cell-culturing techniques, coupled with DNA sequencing may be the only way to truly show that an animal is infected with viable CyHV-3, a number of studies have demonstrated considerable difficulties in reliably culturing CyHV-3 in a number of different cell-lines. Despite this, it is important that the applicability of sequencing, in combination with virus isolation in cell culture, be reviewed to determine its applicability in resistance testing studies in the NCCP.

Table 1. Methods for targeted surveillance and diagnosis of CyHV-3 infection (OIE, 2017b). Diagnostic methods recommended, for reasons of availability, utility, and diagnostic specificity and sensitivity, for confirmatory diagnosis of CyHV-3 infection are highlighted in yellow while standard methods with good diagnostic sensitivity and specificity for targeted surveillance are highlighted in green and for presumptive diagnosis are highlighted in blue.

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	c	c	b	d
Direct LM	d	d	c	c	b	d
Histopathology	d	c	c	c	b	c
Isolation in cell culture	d	d	d	d	b	d
Transmission EM	d	d	d	d	b	c
Antibody-based virus detection assays	d	d	c	c	b	b
***In situ* DNA probes**	d	d	c	c	b	b
PCR	d	b	b	b	a	a
Sequence	NA	NA	NA	NA	NA	a
Antibody detection assays (serology)	d	d	c	b	b	d
Bioassay	NA	NA	NA	NA	NA	NA

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; PCR = polymerase chain reaction; NA = Not applicable.

a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose.

As stated previously the OIE (2017b) lists: gross signs, direct light microscopy, histopathology, isolation in cell culture, transmission electron microscopy, antibody-based virus detection assays, in situ DNA probes, PCR and antibody detection assays (serology) as techniques with variable diagnostic sensitivity and specificity for identifying CyHV-3 infection. Each will now be specifically discussed.

3.6. Clinical signs, histopathology and electron microscopy

The clinical presentation of common carp infected with CyHV-3 is typified by pale discoloration or reddening of the skin, the development of a rough, sand-paper like texture on the skin, focal or total loss of the epidermis, over or underproduction of mucus on the skin and gills, pale discoloration of the gills, sunken eyes, haemorrhages on the skin and base of fins and fin erosion (OIE, 2017b; Pikarsky et al., 2004). Infected fish may also become lethargic, loose equilibrium or orientation, become hyperactive, separate from the school, seek areas of higher dissolved oxygen, and gasp at the surface of the water (Boutier et al., 2015; OIE, 2017b; Pikarsky et al., 2004).

Histopathology and microscopy of CyHV-3 infected fish can reveal erosion of primary lamellae, fusion of secondary lamellae, and swelling at the tips of the primary and secondary lamellae. Inflammation and necrosis of gill tissue is observed consistently. The gills are also frequently affected by hyperplasia and hypertrophy of branchial epithelium and adhesion of gill filaments. Nuclear swelling of branchial epithelial cells and leucocytes may also occur, along with margination of chromatin to give a signet ring appearance, while pale diffuse eosinophilic intranuclear inclusions are commonly observed. The kidney, spleen, pancreas, liver, brain, gut and oral epithelium may also be affected by inflammation, necrosis and nuclear inclusions (Boutier et al., 2015; OIE, 2017b; Pikarsky et al., 2004; Pokorova, Vesely, Piackova, Reschova, & Hulova, 2005).

While these clinical signs and histological changes follow a general pattern, they do not present consistently and have attributes shared by a range of other pathogenic agents, thus they are not pathognomonic (Boutier et al., 2015; OIE, 2017b). Furthermore, clinical signs in CyHV-3 infected NTS may vary from those observed in common carp and are currently unknown. As a result, clinical signs and histopathology have low diagnostic sensitivity and specificity, which precludes their use in providing an accurate confirmatory diagnosis of CyHV-3 infection. Despite this, clinical signs may be used in resistance testing protocols for two important purposes.

Firstly, the assessment of clinical signs can give a presumptive diagnosis of CyHV-3 infection. This can provide a rapid indication of CyHV-3 infection and trigger the application of additional confirmative diagnostic procedures. It is equally important that these confirmative procedures have the power to either confirm the presence of CyHV-3 infection or its absence. Secondly, by assessing clinical signs, it may be possible to identify a range of other causative factors that may be causing disease, injury or distress in experimental fish. This is especially important where clinical signs, that are analogous with CyHV-3 infection, or mortality are observed. In these cases it is critical that the true cause of clinical signs is determined, as without this information, it may be inappropriate to rule-out CyHV-3 as the causative agent.

Electron microscopy has also had varying degrees of success in the diagnosis of CyHV-3 infection and relies on a high abundance of CyHV-3 in sample tissue. As a consequence, electron microscopy may have some limited application for confirming the presence of CyHV-3 in heavily infected fish but it is not considered to be a reliable diagnostic method overall (OIE, 2017b; Pikarsky et al., 2004).

3.6.1. Conclusions on the use of clinical signs, histopathology and electron microscopic diagnostic methods to determine CyHV-3 infection.

An assessment of clinical signs and histopathology should be undertaken for all carp and NTS assessed during resistance testing in the NCCP. While CyHV-3 infection is not categorised by pathognomonic signs, it is important to identify and record clinical signs and histological observations from all NTS exposed to the virus and in negative controls. This is a critical part of any diagnostic protocol as it may facilitate the building of a diagnostic picture for confirmation of the presence of CyHV-3 infection, where it occurs. However, where alternative pathogens, injury or distress can be identified, this may assist by providing compelling evidence for the absence of CyHV-3.

Providing evidence for the absence of CyHV-3 infection in NTS exposed to the virus is equally as important as making an attempt to identify viral infection. This is of greatest importance where clinical signs or mortality occur in exposed animals. In these instances, it is critical that the true cause of disease, morbidity or mortality is identified. In doing so, compelling evidence for the absence of CyHV-3 infection may be developed, thus providing clarity around the resistance-status of NTS.

3.7. Virus isolation in cell culture and identification by DNA sequencing

Virus isolation in cell culture is often considered to be the most effective method of confirmatory diagnosis for a range of viral pathogens. When undertaken successfully it is able to conclusively demonstrate the presence of viable virus, the identity of which can then be subsequently confirmed by electron microscopy , appropriate PCR or DNA sequencing analysis.

The isolation of CyHV-3 has been attempted in cell culture using a range of cell lines: common carp brain, CCB (Neukirch et al., 1999); common carp fin-derived cell line, KF-1 (Hedrick et al., 2000); koi caudal fin-derived cell line, KCF-1 (Dong et al., 2011); koi tail cell line, KT-2 (Hedrick et al., 2000); and koi snout cell line, KS (Wang et al., 2015). While diagnosis of CyHV-3 infection by virus isolation in cell culture can be achieved, these cell lines are often difficult to handle (OIE, 2017b).

Diagnosis of CyHV-3 by cell culture isolation is also constrained by other limitations associated with the technique for this virus. Virus isolation in cell culture is not effective when frozen samples of infected fish are assessed and is not suitable for identifying healthy, carrier fish (O. Gilad et al., 2002). As a consequence, virus isolation in cell culture is not considered to be as sensitive as PCR to diagnose this virus (Clouthier et al., 2017; Haenen, et al, 2004).

Recently a new cell line was established from koi brain, KB (Wang et al., 2018) for use in KHV epidemiological characterization, pathogenesis and diagnosis. The new KB cell line is yet to be validated by a third party but the authors claim that it is easy to handle and susceptible to CyHV-3 infection, displaying typical cytopathic effect after exposure to the virus (Wang et al., 2018). If this new cell line can be validated as a reliable tool for virus isolation and infection diagnosis, it may prove to be a suitable tool to assess the resistance status of NTS to CyHV-3.

Sequencing methods provide the most robust and accurate tool to confirm the presence of viable CyHV-3 in common carp. However, while sequencing is the most robust method for confirmative diagnosis of CyHV-3, it is limited in its practical application. Sequencing methods rely upon successful virus cultivation to generate a sufficient quantity of viral DNA. This can be difficult, with cell lines from common carp and koi often being difficult to handle and virus cultivation having variable success. As such, while sequencing of the virus, when isolated in cell culture, provides confirmation of the presence of viable CyHV-3, the method is prone to false negatives, when used as a diagnostic

tool. This occurs at an unacceptable rate, as the virus frequently fails to proliferate in cell culture, despite the occurrence of active infection in fish (Clouthier et al., 2017).

3.7.1. Conclusions on the use of virus isolation in cell culture and DNA sequencing to determine resistance of NTS to CyHV-3 infection.

Viral isolation in cell culture is generally not considered to be a suitable and reliable diagnostic tool for the diagnosis of CyHV-3 infection. However an appraisal of various cell lines, including the newly developed KB cell line (Wang et al., 2018) should be undertaken to determine if virus isolation in cell culture can be used to diagnose CyHV-3 infection in carp and NTS. If successful, virus isolation and identification could be used to demonstrate the presence of viable virus in NTS post exposure. Currently however, using presently available cell culturing techniques, a negative result from cell culture cannot be considered an accurate reflection of resistance of NTS to CyHV-3 infection.

3.8. Serological diagnosis of CyHV-3 infection.

Serological methods rely upon a seroconversion by the exposed target species against the specific virus and have been developed for diagnosing CyHV-3 infection in common carp. These methods are characterised by good sensitivity and specificity. However, the OIE (2017b) points out that there is insufficient knowledge on the serological response of fish to viral infections and the detection of fish antibodies to viruses is not currently accepted as a reliable screening method. Despite this, serological methods may be a useful diagnostic tool for diagnosing CyHV-3 infection in sub-clinically or latently infected fish, where a serological response may continue to be detectable after CyHV-3 DNA in fish tissue has fallen below the detectable limit of PCR assays.

The presence of a serological response in NTS may not provide evidence of susceptibility/resistance to CyHV-3. Following exposure to CyHV-3, the immune system of NTS may mount a serological response, regardless of the ability of CyHV-3 to establish a multiplying or otherwise developing or latent infection. Thus, while serology may be useful to demonstrate that fish have been exposed to CyHV-3, it may not provide useful information related to the resistance-status of NTS..

3.9. Molecular methods for the diagnosis of CyHV-3 infection.

Molecular diagnostic techniques are used to directly detect nucleic acid (NA) sequences that are specific to a pathogen. These techniques include PCR assays and isothermal amplification assays, including loop mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) assays. Well developed and performed molecular techniques have high sensitivity and specificity and can be effectively used for confirmatory diagnosis, presumptive diagnosis and targeted surveillance (OIE, 2017b).

PCR is a molecular technique used in the diagnosis of a range of pathogenic agents, including bacteria and viruses. Pathogen-specific PCR assays are developed to directly identify the presence of a specific region of pathogenic DNA in a sample of host tissue, blood or bodily products, or in the environment. The technique works by repeatedly replicating a specific segment of DNA, from the target pathogen, across several orders of magnitude, enabling it to be more readily detected in the sample. PCR assays are a reliable and routinely applied technique used in the diagnosis of a wide range of human and animal pathogens.

Well designed and performed PCR assays can have high sensitivity and specificity. However, considerable care is required when performing these techniques as they can be prone to contamination, which can yield false-positive results. PCR results must also be interpreted with care as the presence of a fragment of pathogenic DNA is not necessarily a reliable indicator of true

infection, but may only indicate exposure to the pathogen. The specific segment of DNA targeted by the PCR assay is also important and can affect the sensitivity and specificity of the test, both at the time of its development and also as the DNA sequence of the pathogen changes over time.

Isothermal amplification assays are a relatively new diagnostic procedure. LAMP techniques were first described by Notomi et al.,(2000) and the RPA method was first described by Piepenburg, et al., (2006). These techniques provide a rapid procedure for the amplification of DNA without the need for specialised equipment. These techniques may facilitate rapid, sensitive and specific diagnosis of CyHV-3 presence in fish tissues.

3.9.1. PCR assays for the diagnosis of CyHV-3

While PCR assays must be performed and interpreted with care, this technique is a recommended method for presumptive and confirmatory diagnosis of CyHV-3 infection in fish for reasons of availability, utility and diagnostic specificity and sensitivity. PCR is also considered to be a standard method with good diagnostic sensitivity and specificity for targeted surveillance of CyHV-3 (OIE, 2017b; Piepenberg, et al., 2006). A range of PCR assays for the diagnosis of CyHV-3 infection have been published. These methods vary with respect to diagnostic sensitivity and specificity and have varying applicability depending on the situation in which the test is being performed (i.e.. Fit for purpose).

Early conventional PCR methods targeting different regions of the CyHV-3 genome were published by Gilad et al.,(2002), Gray, et al.,(2002), Pikarsky et al. (2004) and Ishioka et al., (2005). The Gilad c-PCR was later improved by Yuasa, et al., (2005) to yield higher sensitivity and specificity. Bercovier et al. (2005) also developed a PCR assay targeting thymidine kinase (TK) gene which was more sensitive than previously developed PCR methods. A quantitative PCR (qPCR) assay, able to detect very low copy numbers of target nucleic acid sequences was also developed by Gilad et al. (2004), and subsequently a quantitative quenching probe PCR was developed with similar performance characteristics (Kamimura et al., 2007). These early PCR assays have high sensitivity and specificity for diagnosing CyHV-3 infection in clinically affected fish, but are less reliable in diagnosing its presence in apparently healthy fish that are carrying the virus (Yuasa & Sano, 2009).

A small number of studies: Bercovier et al. (2005); Pokorova et al. (2010); Bergmann et al, (2010); Monaghan, et al (2015); Clouthier et al. (2017); and Meyer, et al (2012) have appraised and directly compared the sensitivity and specificity of a range of PCR techniques used to diagnose CyHV-3 in fish. These studies will be reviewed to provide an overview of the PCR methods available and to compare their accuracy and suitability under different conditions.

The first published study to evaluate different molecular assays for the diagnosis of CyHV-3 was undertaken by Bercovier et al. (2005). This study compared a newly developed Bercovier c-PCR with existing Gilad c-PCR and Gray c-PCR. The new Bercovier c-PCR was as sensitive as virus isolation in cell culture for diagnosing CyHV-3 and more sensitive than Gilad c-PCR or Gray c-PCR, detecting CyHV-3 in a greater number of samples. This new assay was also more responsive than previously developed c-PCRs. The new c-PCR was able to identify CyHV-3 DNA in all fish during active CyHV-3 infection but was less effective in identifying CyHV-3 DNA in healthy fish that had survived CyHV-3 exposure. This may have been due to the viral load decreasing to below the analytical threshold of the new c-PCR assay.

Pokorova et al. (2010) compared the sensitivity of various conventional single-round PCR assays: Gilad c-PCR (O. Gilad et al., 2002); Yuasa c-PCR (Gray et al., 2002; Yuasa et al., 2005); and Bercovier

c-PCR (Bercovier et al., 2005), and nested PCR assays: Bergman n-PCR (Bergmann, et al, 2006; Gilad et al., 2002) and CEFAS n-PCR (Bercovier et al., 2005; CEFAS, Unpublished).

Similar diagnostic responsiveness (i.e. level at which template DNA can be detected) was observed for all protocols when applied to homogenated tissue samples that had been spiked with a known amount of CyHV-3 DNA with the exception of the Yuasa c-PCR assay which was less responsive (i.e. relatively high minimum level of template present before detection by assay).

When field samples were collected from fish with a suspected history of exposure to CyHV-3 and selected preferentially for clinical signs, the various PCR assays showed widely varying diagnostic sensitivities. The CEFAS n-PCR assay was relatively more sensitive than the other methods assessed, returning 47 positive results from the 106 samples tested. The Bercovier c-PCR and Yuasa c-PCR returned 36 and 34 positive results respectively while the Bergmann n-PCR returned 31 positive results. The Gilad c-PCR had the lowest diagnostic sensitivity, returning 8 positive PCR results.

Bergmann, et al. (2010) assessed 12 different molecular tests used in the diagnosis of CyHV-3 Infection, including 11 PCR and one LAMP method. This included the description of a newly-developed semi-nested PCR (Bergmann sn-PCR). The first aim of the study was to determine the concentration of viral DNA, in diluted virus stock, required to produce a positive signal in each molecular test. In this respect, the most responsive techniques were: an adapted real-time TaqMan PCR (Gilad q-PCR) (Oren Gilad et al., 2004), Bergmann n-PCR (Bergmann et al., 2006) and the newly developed Bergmann sn-PCR (Bergmann, et al., 2010).

The diagnostic sensitivity of these techniques was also assessed by investigating samples from 18 common carp that were previously determined to have died from CyHV-3 infection. The adapted Gilad q-PCR returned a positive result in 60% of samples, the Bergmann n-PCR in 67% of samples and the newly developed Bergmann sn-PCR returned positive results in 100% of samples assessed.

This study also found that pooling samples from multiple carp reduced the sensitivity of the Gilad q-PCR technique. While 60% of samples were determined to be CyHV-3 positive when ten individual fish were assessed, this fell to 25% when five pools of two of these carp were assessed and 0% when two pools of five carp were assessed.

Finally, Bergmann, et al. (2010) assessed gills swabs, leukocytes and pools of gill and kidney tissue from healthy-appearing koi carp that had survived a CyHV-3 outbreak. The Gilad q-PCR and Bergmann sn-PCR techniques identified CyHV-3 DNA most often, returning positive results in all gills swabs and tissue pools and in two out of three leukocyte samples. The Bergmann n-PCR was the only other PCR to identify CyHV-3 DNA, returning positive results in two out of three gill swabs, leukocyte samples and tissue pools.

The collective findings presented by Bergmann, et al. (2010) show wide variability in results obtained from a range of molecular diagnostic methods. Clear differences are reported in: the concentration of DNA that is detectable by the various techniques; the frequency of returning positive results in fish suspected to have died from CyHV-3; and the results obtained from fish that have survived an occurrence of CyHV-3 but appear to be healthy. The choice of molecular test used can, therefore, have a considerable impact on the findings of a diagnostic protocol. The observation that pooling samples from multiple fish reduces diagnostic sensitivity is also important and has significant implications to future diagnostic endeavours.

According to Bergmann,et al. (2010) the newly developed Bergmann sn-PCR had superior responsiveness and diagnostic sensitivity relative to most other PCR assays assessed. The Gilad q-PCR and Bergmann n-PCR also performed well relative to the remaining PCR methods.

Monaghan, et al., (2015) also evaluated a number of PCR assays for the detection of CyHV-3 DNA in carp. This study was designed to evaluate the ability of various PCR methods to diagnose CyHV-3 in the early stages of infection – the first hours and days post challenge. This study highlighted the difficulties in diagnosing CyHV-3 infection during the early stages of infection, when there is a low concentration of viral DNA in tissues. False negatives were reported from a range of PCR tests with the proportion of false-negatives being associated with the period of time that had elapsed post challenge and the tissue sampled. The inefficiency of PCR assays to identify early CyHV-3 infection were of particular concern given the rapid onset of mortality occurring in some of the fish assessed – in many cases PCR was unable to detect CyHV-3 in fish despite mortalities occurring. However, the study also observed that swabs of skin mucus were an efficient site for the detection of CyHV-3 DNA during early infection.

The time period allowed to elapse post-challenge, before diagnosis was attempted, had a significant impact on the CyHV-3 DNA load in a range of tissues (gill, skin, spleen, kidney, gut, liver, brain and leukocytes), as measured by Gilad q-PCR (Bergmann, et al., 2010; Gilad et al., 2004). This relationship was most profound for tissue samples collected by lethal biopsy. At <5 days post challenge (dpc) CyHV-3 DNA was rarely detected in kidney, gut, liver or brain samples. While in the same treatment tanks, CyHV-3 DNA was frequently detected at high levels in these tissues at ≥5 dpc. Similar trends were observed for gill, skin and spleen biopsies, although CyHV-3 DNA was frequently detected in these tissues at <5 dpc, albeit at moderate levels. However, when swab samples (fin base, skin and gill) and leukocytes were collected by non-lethal sampling methods, Gilad q-PCR was able to detect CyHV-3 DNA at high levels at <5 dpc, with further significant increases observed in samples collected ≥5 dpc. This highlights the inefficiency of Gilad q-PCR to detect CyHV-3 DNA during the early stages of infection, especially when performed on internal organs, while skin swabs appear to be an effective site to detect CyHV-3 DNA during early infection.

While the use of skin swabs was more efficient in detecting CyHV-3 DNA by Gilad q-PCR during early infection, some questions remain as to whether the DNA observed was actually a true indication of active infection. It could be argued that the high DNA concentration observed by Gilad q-PCR in skin swabs was a result of the bath-challenge-inoculum attaching to the skin mucus of the fish, and not a result of viral infection and replication. This is acknowledged by the authors (Monaghan, et al,2015). However, in the single fish that survived the challenge experiment, CyHV-3 DNA was detected in skin swabs after 70 dpc. This suggests that the virus may be continuously excreted through the skin by sub-clinically infected fish, as has been observed previously for channel catfish infected with the closely related *Ictalurid herpesvirus* 1 (Kancharla & Hanson, 1996). If this is confirmed to be the case, then the skin may be considered as a useful sampling location for PCR methods targeting CyHV-3, even in laboratory exposure trials. This warrants further investigation.

The relative diagnostic sensitivity of seven PCR assays for the detection of CyHV-3 DNA during early infection was also assessed by Monaghan et al., (2015). Individual fish were exposed to CyHV-3 by bath immersion. Subsequently, a range of tissues (gill, skin, spleen, kidney, gut, liver, brain and leukocytes) were analysed individually by various PCR techniques. Fish were considered to be CyHV-3 positive where a positive PCR result was observed in any one tissue. When this methodology was applied to fish during early infection (<5 dpc), PCR assays returned variable results. The highest diagnostic sensitivity was observed for Gilad q-PCR which returned positive results in 11 of the 14

fish assessed. A conventional single round PCR according to the method of Bergmann, et al (2010) (Bergmann c-PCR) and Bergmann sn-PCR identified ten fish as being CyHV-3 positive while CEFAS n-PCR and Bergmann n-PCR assays each identified seven positive fish. Other conventional single round PCR assays, Bercovier c-PCR and Gilad c-PCR were less sensitive, returning positive results for six and three fish respectively. In later infection (≥5 dpc) all PCR assays returned positive results in all fish. This study not only highlights how variable the diagnostic sensitivity of different PCR assays can be, but also the prevalence of false negatives in PCR tests, especially during early infection.

Monaghan et al. (2015) considered the prevalence of false negatives returned by PCR assays to be a result of low-levels of CyHV-3 DNA in the tissues samples. In this instance, low levels of DNA appeared to be related to the limited time that had elapsed since the fish were challenged. However, reduced sensitivity may also be expected whenever a low level of CyHV-3 DNA exists in sample tissue, as may be the case in sub-clinically infected fish. Limited diagnostic sensitivity of PCR assays was also observed for the lone surviving fish in this experiment, with only the CEFAS n-PCR and the Bergman c-PCR returning positive results. When individual tissues were assessed by these PCR assays, only 3 of the seven tissues assessed were positive for CyHV-3 DNA.

Taken in the broader context of diagnostic protocols for CyHV-3, the reduced sensitivity of PCR assays that occurs as the concentration of CyHV-3 DNA decreases, has important implications. Not only can these methods be expected to yield more false negatives when low concentrations of CyHV-3 DNA is present, but the pooling of samples prior to PCR analysis may also reduce diagnostic sensitivity (S. J. Monaghan et al., 2015).

Relative to all other PCR assays assessed by Monaghan et al. (2015), Gilad q-PCR had the highest diagnostic sensitivty, detecting CyHV-3 DNA in the most fish assessed at < 5dpc (11 positive samples out of 14 assessed). Bergmann c-PCR and Bergmann sn-PCR had similar diagnostic sensitivity, identifying CyHV-3 DNA in 10 of 14 fish assessed. All other PCR assays, CEFAS n-PCR, Bergman n-PCR, Bercovier c-PCR and Gilad c-PCR were less sensitive, returning positive results for seven or less fish.

While a number of studies have assessed the performance of a range of PCR assays for the diagnosis of CyHV-3 infection, until recently the performance characteristics of PCR tests had not been validated to the standards of the OIE. This was recently addressed by Clouthier et al. (2017) who used ring-testing across multiple laboratories to assess the diagnostic sensitivity and specificity of two PCR tests: a slightly modified Bercovier c-PCR; and the adapted Gilad q-PCR (Bergmann,et al., 2010). Both methods were reported to have a diagnostic sensitivity of 99% and specificity of 93% and were considered to be suitable tools for surveillance, presumptive diagnosis and to certify individual fish and populations as CyHV-3 free (Clouthier et al., 2017). The adapted Gilad q-PCR was able to detect lower concentrations of CyHV-3 DNA in samples (greater analytical responsiveness) and may be more effective when assessing healthy individuals, with a low pathogen load (Clouthier et al., 2017). Clouthier et al. (2017) also suggests conducting a second q-PCR, targeting a different nucleic acid sequence to confirm the presence of CyHV-3 where the concentration of CyHV-3 DNA is below the detection threshold of the Gilad q-PCR.

Despite the high diagnostic sensitivity and specificity reported by Clouthier et al. (2017), false positives were reported from individual laboratories, while fish with low pathogen loads may have returned some false negative results. False positives were considered to be caused by cross-contamination of samples with CyHV-3 nucleic acid (NA) due to methodological factors. These are discussed in the study and techniques for overcoming cross-contamination are put forward. False negatives may have occurred due to low virus loads in apparently healthy carp assessed during the

study. This was even the case for the adapted Gilad q-PCR, which is considered to be among the most analytically responsive methods, where non-repeatable pairs from same-fish samples were only observed in fish from a lightly-infected carp population.

The accuracy of PCR methods assessed by Clouthier et al. (2017) may also have been affected by subjectivity in the electrophoretic gel reading step of the Bercovier c-PCR method. This difficulty would be confounded at low virus loads. Conversely, samples with high virus load also reduced the precision of some PCR tests. This was suggested to be a result of unequal distribution of the virus in organs sampled (Clouthier et al., 2017).

While the diagnostic sensitivity and specificity of both PCR methods were reported to be high (Clouthier et al., 2017), these parameters were assessed in the absence of a perfect reference test (as a perfect reference test for CyHV-3 has not yet been developed). Instead, the study used gold-standard negative and positive reference populations of fish and a statistical model (latent class model) to determine diagnostic sensitivity and specificity. A positive reference population with low prevalence was also used but was essentially condensed into the gold-standard negative reference population due to the low prevalence of CyHV-3 observed in these fish. The authors justify this by providing evidence that both PCR methods were able to identify CyHV-3 positive fish in the positive reference population even when prevalence and virus load were low. While this may be a reasonable approach within the constraints of the study, it fails to take into account the potential for sub-clinically and latently infected common carp to exist with very low prevalence of CyHV-3 in tissues (below the analytical responsiveness of the test). Thus, while both PCR tests assessed had high diagnostic sensitivity and specificity under the conditions of this study, sensitivity and specificity may be reduced when such tests are applied to common carp or non-carp species carrying very low concentrations of CyHV-3 DNA. This was acknowledged by the authors (Clouthier et al., 2017).

The potential for diagnostic sensitivity and specificity to change with the health status of the population is acknowledged by Clouthier et al. (2017). The authors state that diagnostic sensitivity and specificity would be high with naïve fish and with fish during an outbreak and potentially lower with convalescent fish following and outbreak of CyHV-3.

Clouthier et al. (2017) highlights a further factor that must be taken into account when using molecular methods for the diagnosis of viral pathogens. That is, that molecular diagnostic techniques detect DNA from intact virions and nascent genomes as well as residual NA fragments. As such, positive results can only be seen as evidence that a fish has been exposed to the virus and do not imply active replication or infection. The study also refers to Fisheries Oceans Canada (DFO) Laboratories protocols that require additional diagnostic testing wherever DNA amplification is detected by PCR in some but not all technical replicates. This highlights the importance of adopting a broader diagnostic protocol that includes a range of diagnostic tests, which can be interpreted in conjunction with one another.

While the Bercovier c-PCR and Gilad q-PCR assays assessed by Clouthier et al. (2017) had equivalent diagnostic sensitivity and specificity, the most analytically responsive test was the adapted Gilad q-PCR. This assay was up to 100 times more responsive, being able to detect as little as 5 copies of CyHV-3 DNA in a sample (Clouthier et al., 2017).

When performing PCR diagnostics, the laboratory faces critical technical decisions with respect to the NA extraction procedure and the PCR assay to be used. Meyer et al. (2012) investigated a range of different PCR assays using different combinations of primer sets and DNA polymerase.

Meyer et al. (2012) found that the reliability of PCR assays, in addition to the primer set, also depends on the DNA polymerase used. From this study, it was recommended that PCR protocols for the diagnosis of CyHV-3 should include a silica column-based DNA extraction method and a PCR protocol that combines Platinum *Taq* DNA polymerase (or a comparable product in quality and performance) with a sensitive primer set, such as those presented by Bercovier et al. (2005) or S. Bergmann et al. (2006). Meyer et al. (2012) also recommended that single-round PCR assays using these primer sets would be most useful for detecting CyHV-3 in clinically infected common carp while more sensitive PCR techniques such as q-PCR and nested PCR may be more suitable for the detection of CyHV-3 DNA in latently infected common carp, presumably due to the low prevalence of CyHV-3 DNA.

The PCR techniques evaluated by Clouthier et al. (2017), and those appraised by; Pokorova et al. (2010); S. M. Bergmann, M. Riechardt, et al. (2010); Monaghan, et al (2015); and Meyer et al. (2012) can be used to provide evidence that fish have been exposed to CyHV-3. But these techniques do not necessarily imply active, replicating CyHV-3 infection in target fish tissues. This is of little consequence for standard targeted surveillance procedures when the aim is to determine if a population of fish has been exposed to the virus. However, when assessing the resistance of fish to CyHV-3 infection during laboratory exposure trials, the inability to distinguish between exposure and infection has considerable implications. To overcome this, alternate PCR assays, designed to amplify genes expressed only during active replication, may be considered as a useful tool in a broader diagnostic protocol.

Reverse transcription-PCR (rt-PCR) assays have been developed by Mansour El-Matbouli and Soliman (2011) and Yuasa et al. (2012). Rt-PCR is technique that is used to detect gene expression. The assays developed by Mansour El-Matbouli and Soliman (2011) and Yuasa et al. (2012) detect the replicating stage of CyHV-3 by targeting messenger RNA (mRNA) associated with viral replication. Thus, while conventional PCR assays can be used to show evidence of the presence of a specific fragment of CyHV-3 DNA in a sample, rt-PCR techniques can provide evidence of a specific fragment of RNA that is only expressed by active, replicating CyHV-3. The methods reported by Mansour El-Matbouli and Soliman (2011) and Yuasa et al. (2012) have not been appraised or validated against other, more frequently used, PCR assays for the detection of CyHV-3 DNA. Papers that directly compare and evaluate these techniques are not available for inclusion in this review, nevertheless a brief discussion of these techniques is presented below.

Mansour El-Matbouli and Soliman (2011) developed and reported an rt-PCR method for the amplification of mRNA associated with replication of CyHV-3. This technique was used to demonstrate that CyHV-3 could replicate in gold fish. While the authors identified mRNA in gold fish and observed that gold fish could transmit CyHV-3 to naïve carp, questions have been raised over the accuracy of the rt-PCR method developed. This is because the rt-PCR developed by Mansour El-Matbouli and Soliman (2011) relies upon DNase treatment to remove viral DNA and ensure specific amplification of mRNA. However, the use of DNase treatment has been shown to be ineffective in removing all CyHV-3 DNA, when present in high amounts (Yuasa et al., 2012). As a consequence, the Mansour El-Matbouli and Soliman (2011) method may amplify residual, undigested CyHV-3 DNA from contaminating virus rather than only amplifying mRNA. Thus the method may not be a true indicator of viral replication.

Yuasa et al. (2012) developed an alternate rt-PCR assay using primers spanning the exon junction of a spliced putative terminase gene in CyHV-3. The advantage of this method is that it does not rely on the use of DNase to remove contaminating CyHV-3 DNA. As well as preventing the detection of viral

DNA, this has the added advantage of preventing the unintended loss of RNA that may occur due to the use of DNase. The primer sets used by Yuasa et al. (2012) are advantageous as they were shown to amplify mRNA but not genomic DNA, thus eliminating the need to use DNase.

The rt-PCR developed by Yuasa et al. (2012) was used by McColl et al. (2017) in their investigation of the susceptibility of NTS to CyHV-3 in the NCCP. The rt-PCR assay was applied to samples that had previously tested positive to CyHV-3 by q-PCR. The rationale for applying the rt-PCR in this manner was to show that positive results returned by q-PCR were due to contamination and not a true indicator of infection. When the rt-PCR testing was performed, no samples returned a positive result, indicating that viral replication was not occurring.

This approach used by McColl et al. (2017) is reasonable, however, such an approach relies not only on the rt-PCR having high diagnostic sensitivity, but also that it has high analytical responsiveness – being able to detect mRNA at low concentration. This is currently uncertain, hence the potential issue in this approach is that infected NTS were not identified if the concentration of mRNA was below the detectable limit for the test.

While low concentrations of mRNA, and for that matter DNA, may be expected only during persistent sub-clinical and latent infections, this is not always the case. Monaghan, et al, (2015) observed a high rate of false negative PCR results, apparently associated with low concentrations of CyHV-3 DNA, during early, acute infections, despite high levels of mortality. While these false-negatives were associated with PCR assays targeting viral DNA, this finding highlights the potential for clinical signs and mortality to occur, even in the absence of high concentrations of CyHV-3 DNA in tissue. Furthermore, the analytical responsiveness of the rt-PCR assay to detect CyHV-3 at low concentrations is currently unknown and requires validation. It was also acknowledged by Yuasa et al. (2012) that while their rt-PCR assay was useful to demonstrate viral replication, it may be less suitable for diagnosis of CyHV-3 infection under some conditions, partly due to the unstable nature of mRNA in fish tissues (Yuasa et al., 2012). Sample preservation protocols are therefore also critical when using some PCR assays.

3.9.2. Diagnostic methods using Isothermal amplification assays

Along with PCR assays, isothermal amplification assays have been developed to directly detect CyHV-3 at the molecular level, by amplifying specific DNA segments.

Loop mediated isothermal amplification (LAMP) represents a potential molecular diagnosis technique for the identification of CyHV-3 in fish. LAMP techniques have been published by Gunimaladevi, et al (2004), Soliman and El-Matbouli (2005), Yoshino, et al (2009) and combined with lateral flow strip diagnosis by Soliman and El-Matbouli (2010).

Recombinase polymerase amplification (RPA) assays have also been developed for the diagnosis of CyHV-3 infection, with published methods presented by Prescott, et al (2016) and Soliman and El-Matbouli (2018).

These methods have not been directly appraised and their sensitivity and specificity have not been compared with alternate molecular diagnostic techniques by a third party. However, it is important to discuss these techniques here as they are promising techniques, with sensitivity and specificity that are comparable with PCR techniques. As such, they may play an important role in any future studies that assess the resistance-status of NTS to CyHV-3 infection.

LAMP is based on the principle of autocycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase for the detection of a specific DNA sequence (Notomi et al., 2000).

Gunimaladevi et al. (2004) developed a LAMP assay targeting the TK gene and compared the analytical responsiveness and sensitivity of this method with a PCR assay (also targeting the TK gene). Both the LAMP assay and the PCR assay detected CyHV-3 DNA in 10^{-6} dilutions of CyHV-3 DNA extracted from common carp gills, indicating equivalent diagnostic responsiveness of the two techniques. The diagnostic sensitivity of the LAMP assay was compared with PCR by running both assays on nine fish known to be infected with CyHV-3, one healthy fish and 14 fish with unknown infection status. The LAMP assay returned more positive results, detecting CyHV-3 DNA in all nine infected fish and two unknown fish, compared to the PCR assay which identified CyHV-3 DNA in eight and zero fish respectively. This indicates that the LAMP assay was slightly more sensitive than the PCR assay used. However, in the absence of a gold-standard test for infection status, it is impossible to know if all CyHV-3-positive fish were identified by the test. Furthermore, the true diagnostic sensitivity of the PCR assay used is also unknown.

Yoshino et al. (2009) also reported on the potential for LAMP assays to be used to diagnose CyHV-3 infection. Two LAMP assays, using different primer sets were developed. The analytical responsiveness of these assays was assessed by comparing their ability to detect CyHV-3 DNA in serial dilutions of CyHV-3 suspension with Gilad c-PCR and Yuasa c-PCR. The detection limits of both LAMP assays were lower than that of the Gilad c-PCR and equal to the more responsive Yuasa c-PCR. When used to assess clinical samples, both LAMP assays exceeded the Gilad c-PCR, in terms of number of samples testing positive to CyHV-3 DNA and were almost identical to the Yuasa c-PCR technique.

Soliman and El-Matbouli (2005) also developed a LAMP assay for the detection of CyHV-3 DNA. This method was further developed and adapted to include the use of a lateral flow test strip (Soliman & El-Matbouli, 2010). The adapted method, using newly designed LAMP primers, was far more responsive than previous LAMP assays presented by Gunimaladevi et al. (2004) and Soliman and El-Matbouli (2005), being able to detect CyHV-3 DNA in 100 and ten fold higher dilutions of CyHV-3 DNA respectively. The new LAMP assay was also used to detect CyHV-3 DNA in clinical samples of common carp that had been previously tested by the Bercovier c-PCR assay. The results of this comparison were not presented but the authors claim that the results from the LAMP assay were comparable to Bercovier c-PCR results.

RPA assays have recently been developed for the diagnosis of CyHV-3 infection (Soliman & El-Matbouli, 2018) including in latently infected fish (Prescott et al., 2016). RPA diagnosis utilises three main enzymes to amplify DNA template of target pathogens (Piepenburg et al., 2006).

The first use of RPA to diagnose CyHV-3 infection was performed on white blood cells of latently infected fish. Prescott et al. (2016) were able to show that the RPA assay applied was more sensitive than Gilad q-PCR, which is considered to be among the most sensitive PCR methods. When twelve fish, previously determined to be latently infected with CyHV-3 by PCR, were assessed by RPA, all returned positive results. In contrast, only one was determined to be CyHV-3 positive by Gilad q-PCR.

More recently, Soliman and El-Matbouli (2018) developed an additional RPA assay for the detection of CyHV-3 DNA in fish. Clinical samples that had previously been assessed using the Bercovier c-PCR assay were retrospectively assessed using the newly developed RPA assay. When 100 samples were assessed, this RPA assay returned identical results to those obtained by Bercovier c-PCR.

Furthermore, when DNA was extracted from clinical samples and used in serial dilutions, the RPA assay performed as well as the PCR assay. However, it was less responsive than the RPA assay presented by Prescott et al. (2016) which could detect CyHV-3 DNA at lower concentration.

3.9.3. Summary of diagnostic sensitivity and analytical responsiveness of PCR for detection of CyHV-3.

Based on the results presented by Bercovier et al. (2005); Pokorova et al. (2010); Bergmann, et al. (2010); Monaghan, et al(2015); and Clouthier et al. (2017) the relative diagnostic sensitivity of PCR assays can vary widely. When viewed collectively, these studies can be used to evaluate the relative diagnostic sensitivity of a range of PCR assays.

According to Monaghan et al. (2015), the Gilad q-PCR adapted by Bergmann, et al. (2010), was slightly more sensitive when assessing fish during early infection (<5 dpc), relative to the other PCR assays assessed. This test returned 11 positive results out of the 14 fish tested compared to ten out of 14 for the Bergman c-PCR assay and the Bergmann sn-PCR assay. However, this finding is contradicted by Bergmann, et al. (2010). They found the Bergmann sn-PCR assay to be more sensitive than the adapted Gilad q-PCR, detecting CyHV-3 DNA in 100% of common carp assessed compared to the 60% of samples that tested positive by Gilad q-PCR.

While there is some discrepancy between these two comparative studies, both Monaghan et al. (2015) and Bergmann, et al. (2010) found the adapted Gilad q-PCR, Bergmann c-PCR assay and the Bergmann sn-PCR assay to be superior to any of the techniques appraised by Bercovier et al. (2005) or D Pokorova et al. (2010): Gilad c-PCR; Bergmann nPCR; Bercovier c-PCR; and CEFAS n-PCR assays.

The relative diagnostic sensitivity of PCR assays assessed by Monaghan et al. (2015) and Bergmann, M. Riechardt, et al. (2010) are graphically presented in Figure 4 and 5 respectively.

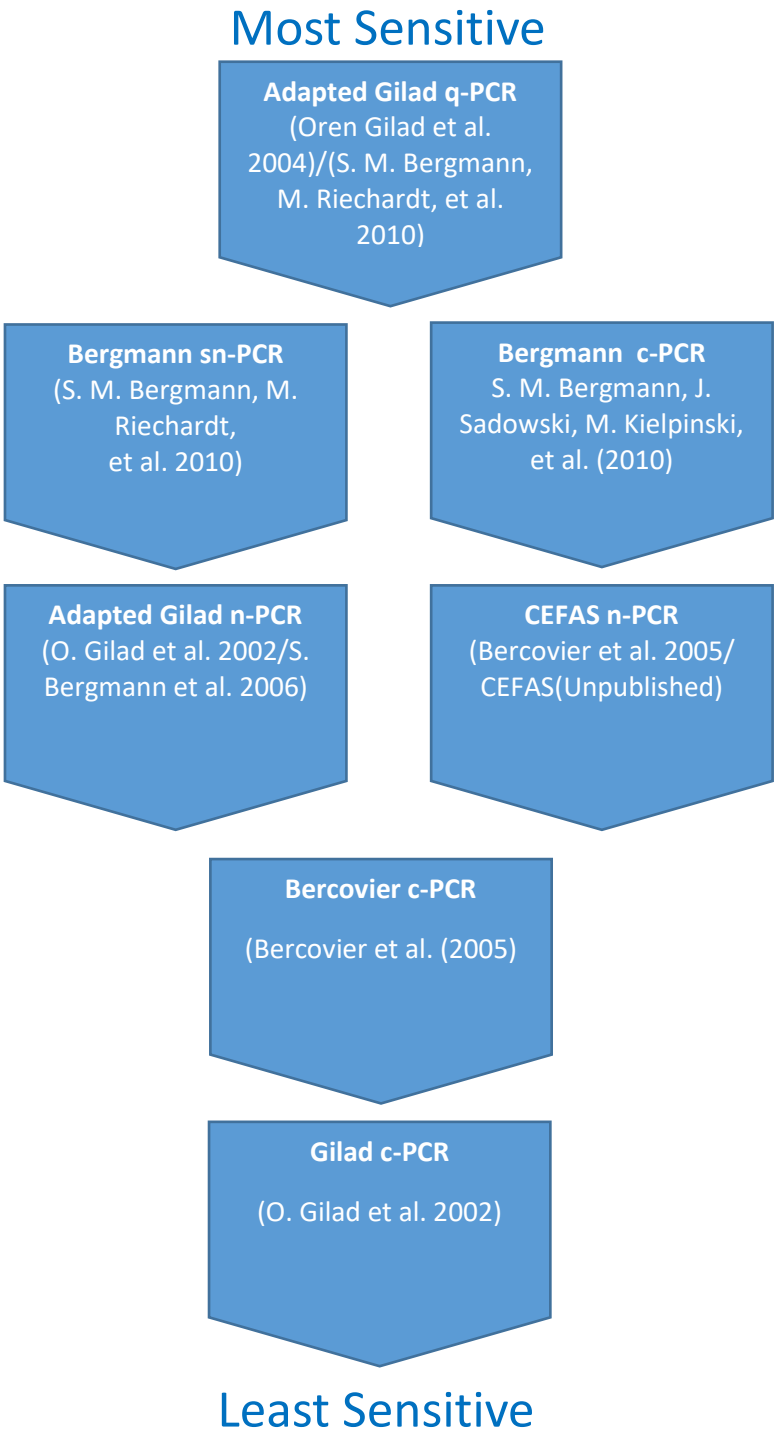

Figure 4. Relative diagnostic sensitivity of various PCR assays, as compared by S. J. Monaghan et al. (2015)

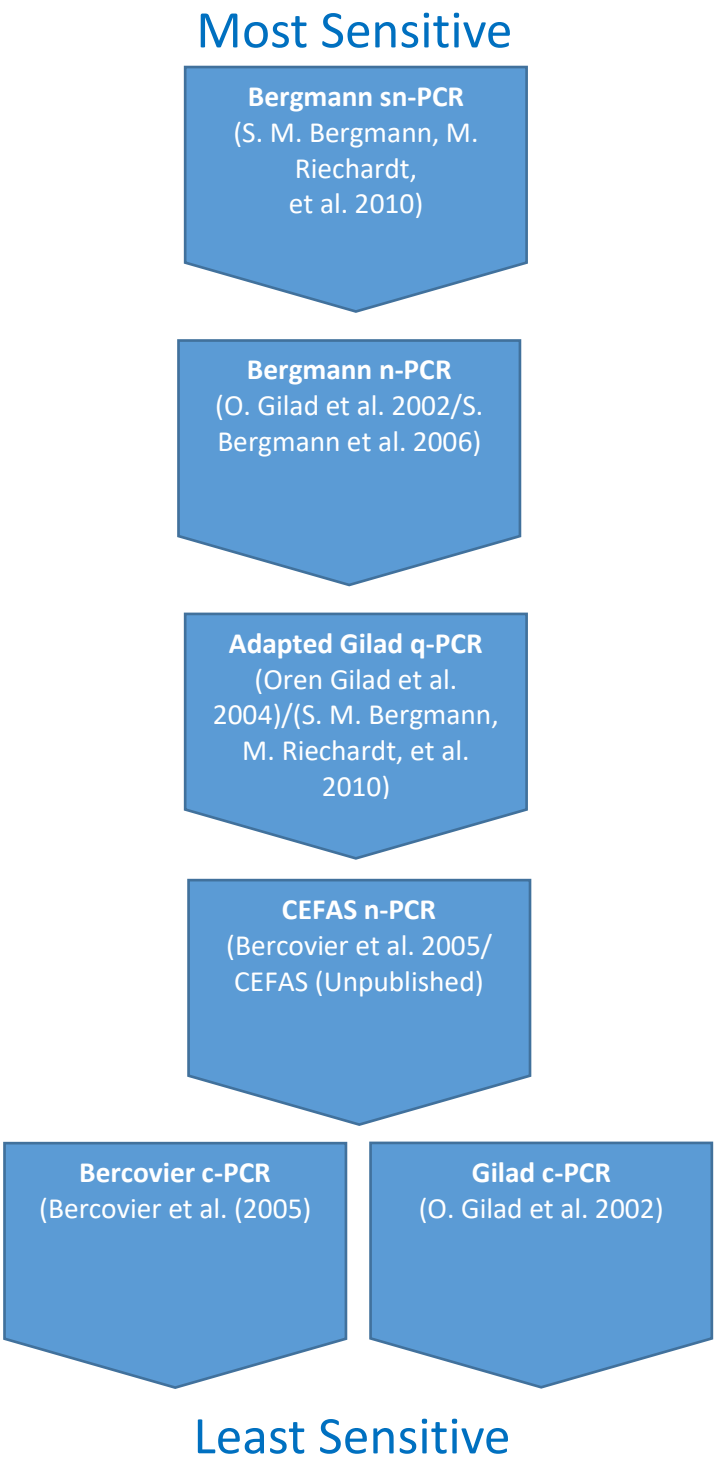

Figure 5 Relative diagnostic sensitivity of various PCR assays, as compared by S. M. Bergmann, M. Riechardt, et al. (2010).

The diagnostic sensitivity of the adapted Gilad q-PCR was also assessed by Clouthier et al. (2017) and found to be identical to the conventional Bercovier c-PCR. However, it appears that only heavily infected fish were assessed. Similarly, when Monaghan et al. (2015) assessed heavily infected fish by a range of PCR methods, little difference was observed in diagnostic sensitivity. This highlights the potential for diagnostic sensitivity to vary depending on the load of CyHV-3 in the sample being assessed.

Analytical responsiveness (i.e. the level at which template can be detected within a sample) is also a critical factor affecting the choice of diagnostic PCR assay. Analytical responsiveness is usually assessed by making sequential dilutions of a known concentration of viral DNA. These dilutions are subsequently subjected to diagnostic PCR assays to determine the lowest concentration at which the test can detect the virus.

Analytical responsiveness of a range of PCR assays was directly assessed by Pokorova et al. (2010). With the exception of the Yuasa c-PCR assay, which was less responsive, all of the PCR assays assessed: CEFAS n-PCR; Bercovier c-PCR; Bergmann n-PCR; and Gilad c-PCR had the same responsiveness, detecting as low as 12 copies of CyHV-3 DNA. Pokorova et al. (2010) did not directly assess the responsiveness of the Gilad q-PCR, however, this PCR was initially used to establish the concentration of CyHV-3 DNA in samples prior to the establishment of sequential dilutions. This is indicative of the high regard held by clinicians for the Gilad q-PCR method.

Bergmann, et al. (2010) also assessed analytical responsiveness of a range of PCR assays. The adapted Gilad q-PCR, Bergmann n-PCR and Bergmann sn-PCR assay were the most responsive, detecting as low as 1-5 copy numbers of CyHV-3 DNA . The Bercovier c-PCR, Gilad c-PCR and CEFAS n-PCR previously assessed by Pokorova et al. (2010) were less responsive, detecting CyHV-3 in samples with 100-1000 copies of CyHV-3 DNA. The analytical responsiveness of PCR assays assessed by Bergmann, et al. (2010) is graphically represented in Figure 6.

Monaghan et al. (2015) did not directly assess analytical responsiveness. However, analytical responsiveness can be implied from their investigation of diagnostic sensitivity during early infection. The ability of various PCR assays to detect CyHV-3 DNA in fish was assessed after varying time periods post challenge. If it is assumed that viral load gradually increased with increasing time post challenge, then the ability of a test to detect CyHV-3 DNA during early infection can provide an indication of the relative analytical responsiveness of the tests. In this respect, the adapted Gilad q-PCR, Bergmann c-PCR and the Bergmann sn-PCR assay were the most responsive. These tests detected CyHV-3 most often during early infection, when copy numbers of CyHV-3 DNA were likely to have been low. Again, the PCR assays previously assessed by Bercovier et al. (2005) and D. Pokorova et al. (2010); D Pokorova et al. (2010): Gilad c-PCR; Bergmann n-PCR; Bercovier c-PCR; and CEFAS n-PCR assays were found to be less responsive. The analytical responsiveness of PCR assays assessed by S. J. Monaghan et al. (2015) is graphically represented in Figure 7.

Clouthier et al. (2017) also determined that the adapted Gilad q-PCR was a highly responsive method, especially when compared to the Bercovier c-PCR. The Bercovier c-PCR limit of detection was 500 copies of CyHV-3 DNA while limit of detection for the adapted Gilad q-PCR was as low as five copies.

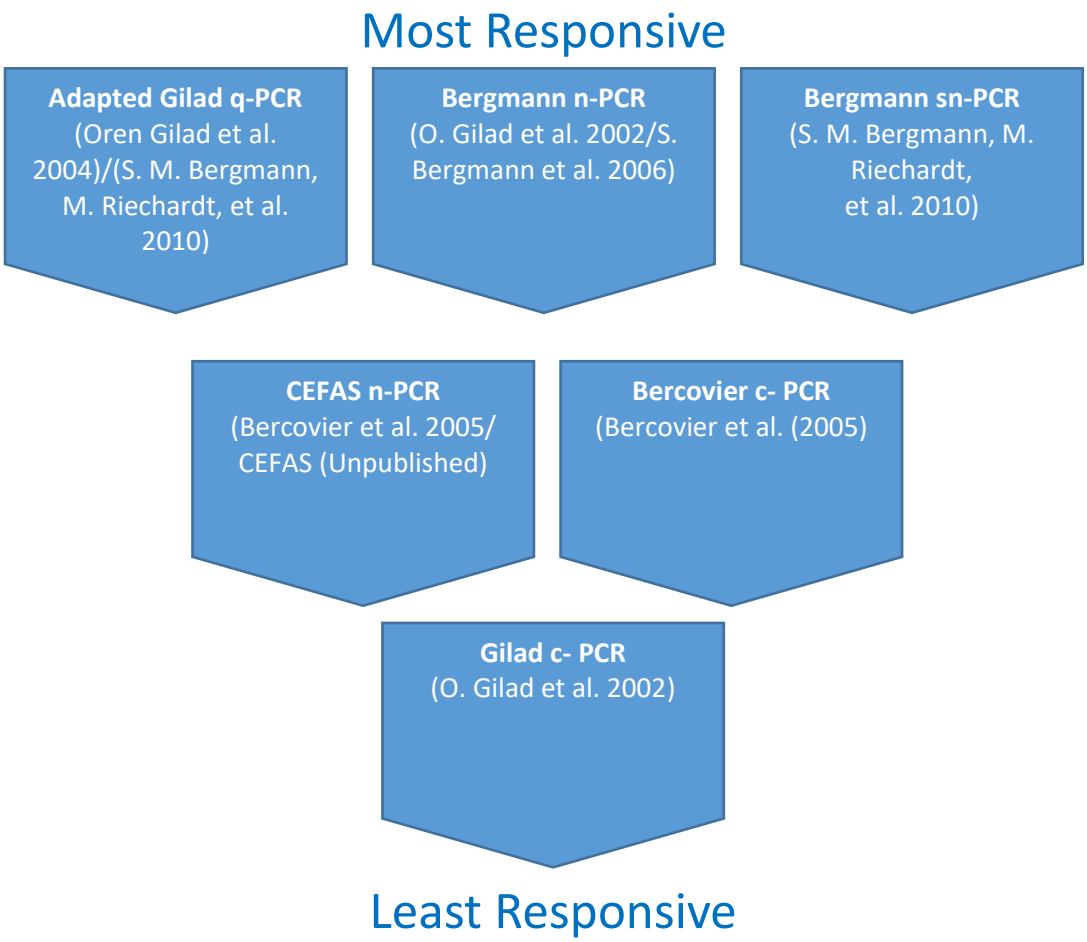

Figure 6. Relative analytical responsiveness of various PCR assays, as compared by S. M. Bergmann, M. Riechardt, et al. (2010).

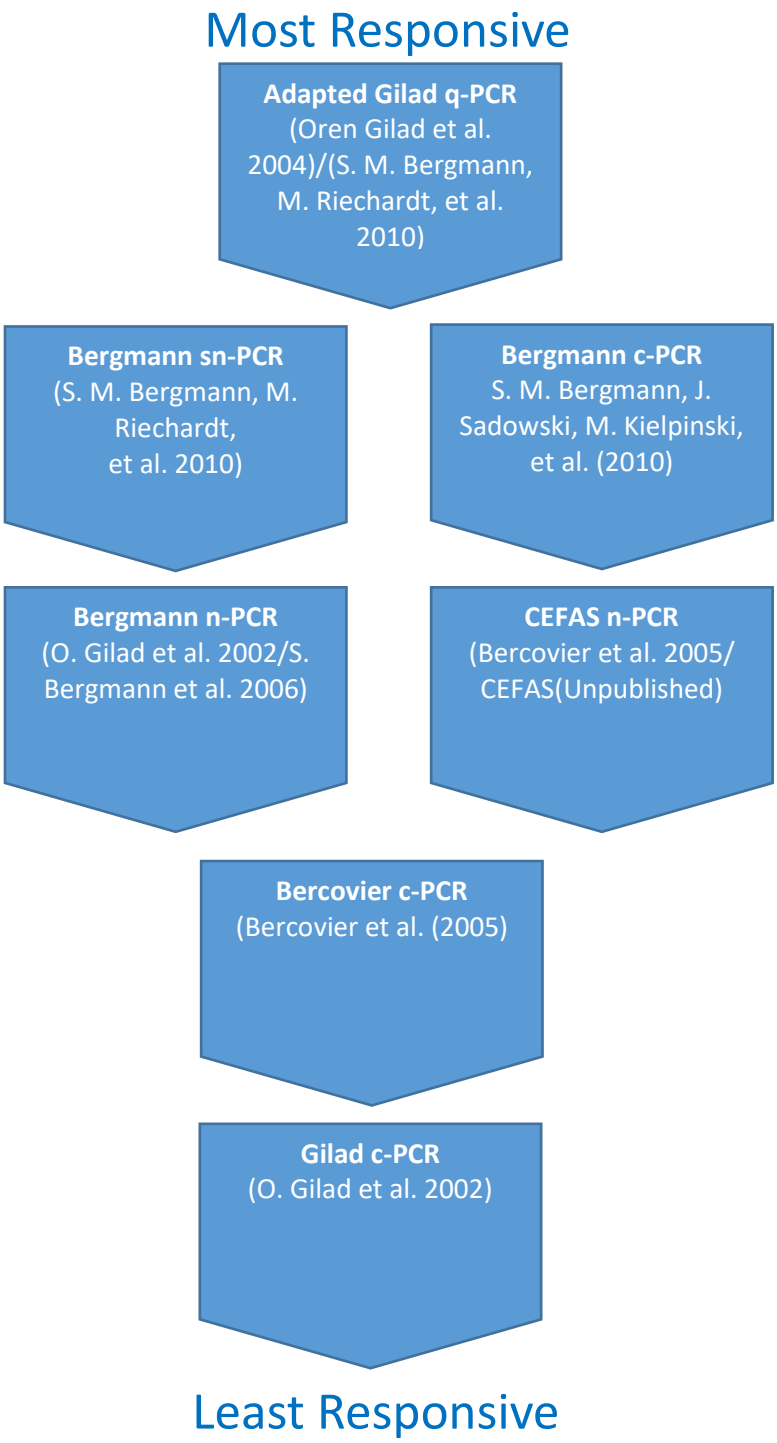

Figure 7 Implied relative analytical responsiveness of various PCR assays, as compared by S. J. Monaghan et al. (2015)

3.9.4 Conclusions on the use of molecular diagnostic methods to determine resistance of NTS to CyHV-3 infection.

In testing the resistance of NTS to CyHV-3 infection, test sensitivity and the ability to avoid false negatives is of the utmost importance. Based on the relative analytical and diagnostic sensitivity & specificity observed in these studies, the following PCR assays perform favourably:

- Adapted Gilad q-PCR – according to the method of Oren Gilad et al. (2004), modified by S. M. Bergmann, M. Riechardt, et al. (2010).

And the

- Bergmann sn-PCR assay – according to S. M. Bergmann, M. Riechardt, et al. (2010).

Both of these PCR assays have high analytical and diagnostic sensitivity & specificity and compare favourably against other PCR assays (S. M. Bergmann, M. Riechardt, et al., 2010; Clouthier et al., 2017; S. J. Monaghan et al., 2015; D Pokorova et al., 2010).

While these PCR assays can be used to provide evidence that fish have been exposed to CyHV-3 they do not necessarily imply active, replicating CyHV-3 infection in host tissues (Clouthier et al., 2017). To overcome this, an rt-PCR assay designed to amplify genes expressed during active replication, may be considered as a useful tool in a broader diagnostic protocol.

Of the rt-PCR assays available, the K. Yuasa et al. (2012) rt-PCR assay appears to be the most promising. This assay uses primers spanning the exon junction of a spliced putative terminase gene in CyHV-3. The advantage of this method is that it does not rely on the use of DNase to remove contaminating CyHV-3 DNA. As well as preventing the detection of viral DNA, this has the added advantage of preventing the unintended loss of RNA that may occur due to the use of DNase. The primer sets used by K. Yuasa et al. (2012) are advantageous as they were shown to amplify mRNA but not genomic DNA, thus eliminating the need to use DNase. However, this PCR assay has not been directly compared to other PCR assays and its sensitivity, responsiveness and specificity are uncertain.

It would be advantageous to undertake an appraisal of the method prior to its use to ensure that results observed during CyHV-3 resistance testing could be interpreted with an understanding of the level of risk of this test returning false negative results.

It is also important to understand that all PCR assays available for diagnosing CyHV-3 infection in fish have limited ability to identify the virus during latent infection. This may be overcome by applying isothermal amplification assays designed to target latently infected fish.

While the LAMP assay developed by Soliman and El-Matbouli (2010) is more responsive than alternate LAMP assays (Gunimaladevi et al., 2004; Soliman & El-Matbouli, 2005) it is only as sensitive as the single round Bercovier c-PCR assay, which is not considered to be as sensitive as the adapted Gilad q-PCR and Bergmann sn-PCR techniques discussed above. As such, LAMP is not deemed suitable as a diagnostic tool in future resistance testing of NTS to CyHV-3 infections.

However, the RPA assay developed by Prescott et al. (2016) does appear to be a useful tool for diagnosing CyHV-3 latently infected fish.. This assay is more responsive than the RPA assay developed by Soliman and El-Matbouli (2018), and more sensitive than the Gilad q-PCR in detecting

latently infected fish. As this is a relatively new technique for diagnosing CyHV-3 infection,(Prescott et al. 2016), it would be advantageous to undertake an appraisal of its performance characteristics.

3.10. Conclusions and recommendations

It is recommended that a broad-based diagnostic protocol should be developed for use in future studies that assess the resistance of NTS to CyHV-3 infection. The protocol should include clinical signs, histopathology, virus isolation in cell culture, PCR and RPA diagnostic procedures, as detailed above. The broad based approach is considered the most effective way to identify evidence of CyHV-3 infection as well as identifying any other pathogenic agents, disease, injury or distress that may be affecting the health status of subject animals. By undertaking this multifaceted approach to diagnostics, the resistance status of NTS to CyHV-3 infection can be more clearly determined.

The following molecular tests are recommended as the most suitable to be included in the broad based approach for the identification of CyHV-3 infection.

For the detection of CyHV-3 genome in fish tissues:

- Adapted Gilad q-PCR – according to the method of Gilad et al. (2004), modified by Bergmann, et al., (2010).

And

- Bergmann sn-PCR assay – according to Bergmann, et al. ,(2010).

For the detection of actively replicating CyHV-3:

- Yuasa rt-PCR according to Yuasa et al. (2012).

For the detection of latent CyHV-3 infection:

- Prescott RPA assay developed by Prescott et al. (2016).

Using all of these tools as the molecular component of a broader diagnostic protocol would increase the level of confidence that the true CyHV-3 infection status of NTS can be determined. However, it is critical that testing methodology be carefully developed to ensure that these molecular tests are performed to best-practice standards due to a range of methodological challenges associated with molecular diagnostic techniques including potential contamination, leading to false-positives, discussed in detail by Clouthier et al. (2017).

4. Occurrence of unexplained mortalities and false positives when testing the resistance of NTS to CyHV-3 infection.

Undiagnosed mortalities and false positives have the potential to confuse the findings of studies that investigate the susceptibility/resistance of a host to a specific pathogen. While the causative factors contributing to these are often unrelated, both have the potential to reduce confidence in the findings achieved. This can ultimately lead to the outcomes of the study being considered unreliable.

To ensure that the results obtained through resistance testing studies are reliable, robust and able to withstand the scrutiny of stakeholders, resistance testing protocols should be carefully considered to avoid the occurrence of undiagnosed mortalities and false positives. However, should they occur or be suspected, it is important that a strategy be devised that places them into the context of the broader diagnostic evidence available.

As highlighted in Chapter 3 the development of a diagnostic matrix that draws on results from all direct detection and diagnostic tests performed should enable the resistance status of the target species to be determined based on the overall balance of diagnostic evidence. While this may not directly reduce the occurrence of undiagnosed mortalities and false positives, it will help to place them into the context of the broader study and generate conclusions based on the balance of diagnostic evidence. Combining a matrix of diagnostics increases the overall sensitivity and specificity of any diagnostic investigation and so should be undertaken particularly where the outcomes of exposure trials are critical in management decisions for natural populations of fish.

4.1. Unexplained mortalities and false positives

An unexplained mortality is any death of a subject animal that occurs during a pathogen exposure trial for which a clear cause of death is not or cannot be provided. In exposure trials it is normal to undertake diagnostic tests on all subject animals to determine whether the target pathogen has caused infection or has caused mortalities in the exposed animals. In some instances, mortalities may occur for which specific diagnostic tests return negative results for the target pathogen. If no further diagnostic procedures are performed which can identify a cause of death then these mortalities can be referred to as unexplained mortalities.

On the face of it, these unexplained mortalities may seem somewhat inconsequential – a negative test result for the target pathogen may seem sufficient to provide evidence that it was not responsible for the death. However, as discussed in Chapter 3, the reliability of different diagnostic tests can be highly variable. As a consequence, and especially where the diagnostic sensitivity of procedures is not clearly understood, some ambiguity may remain over whether or not the test result was a true negative or in fact a false negative. And without further diagnostic evidence, it may be difficult to rule out viral infection in unexplained mortalities.

It is clear that unexplained mortalities can affect the validity of results obtained through exposure trials and a concerted effort must be made to avoid their occurrence. Where unexplained mortalities do occur, they must be placed into the broader context of all available diagnostic evidence. This will enable decisions on the resistance status of the host to be made based on all of the diagnostic evidence available, thus generating a more accurate decision making process.

False positive diagnostic results also have the potential to complicate and weaken the findings of resistance trials. False positives occur when a direct detection or diagnostic test yields a positive result when the target pathogen is actually not present.

In trials that assess the resistance status of a subject animal to a specific pathogen, direct detection and diagnostic tests are performed to determine the presence and effects of the pathogen on a subject animal. This will guide the determination of whether or not a subject animal is resistant to the pathogen. Generally speaking, diagnostic procedures are designed such that a positive test result provides evidence of pathogenic infection, thus leading to the conclusion that the subject animal is not resistant to the pathogen.

However, in some cases, positive test results may be suspected of being false positives. False positives may occur for a number of reasons. The most likely sources of false positives during resistance testing trials are: low diagnostic specificity of the test being performed (see Chapter 3 for a detailed discussion of diagnostic specificity); and procedural errors during diagnostic testing.

If false positives are suspected to have occurred due to low diagnostic specificity, then additional diagnostic procedures with high specificity may be performed to overcome the occurrence of false positives. However, for this to occur, a secondary test must be available that is known to have greater diagnostic specificity than the original test. The diagnostic sensitivity of both tests must also be known, and at least one of the tests must have high sensitivity to prevent the occurrence of false negatives.

As well as occurring due to low specificity of the diagnostic test, false positives may occur due to procedural errors. Where procedural errors are suspected to have caused false positive test results, it may be impossible to overcome these results without repeating the diagnostic procedure or repeating the exposure trial. A failure to do so will lead to confusion in the decision making process. And this has the potential to yield inaccurate decisions on the resistance status of a subject animal.

As noted in Chapter 2.41 published article were identified that assessed the susceptibility or resistance of at least one subject species to CyHV-3infection. Reviewed for information on the occurrence of unexplained mortalities and false positive test results. The results of the review are presented below.

4.2. Literature review methodology and results

38 articles identified through the systematic, quantitative review process outlined above (Chapter 2), and graphically represented in PRISMA Statement 2 (Figure 2), were reported information related to the occurrence of unexplained mortalities and false positives in CyHV-3 susceptibility/resistance testing. Where available, the full text of each research study was obtained and reviewed in detail. Of each of the studies identified, the reference lists were checked to identify additional relevant studies.

A small number of articles reported unexplained mortalities and false positives during CyHV-3 susceptibility/resistance testing. To provide a summary of the occurrence of unexplained mortalities and false positives during susceptibility testing, and to identify processes that researchers have previously used to overcome these issues, specific information from these studies is presented in Appendix 3.

To ensure that a comprehensive list of the occurrence of unexplained mortalities is presented, we have included information related to the reporting of mortalities from negative control groups

(animals that were deliberately not exposed to the virus) and any mortalities that occurred in non-common carp species. We feel that mortalities occurring in carp species other than common carp, although rare, warrant special consideration as they indicate a potential CyHV-3 infection. Mortalities of common carp exposed to CyHV-3 were not included as unexplained mortalities, even where supporting evidence of the presence of virus are not provided. For the purposes of Appendix 3, and the following discussion, mortalities of common carp exposed to CyHV-3 are deemed to have been caused by the virus and are not considered to be unexplained mortalities.

Very few of the studies reviewed refer directly to false positives during their diagnostic procedures. However, to ensure that all information related to the occurrence of false positives was captured, we extended our reporting criteria to include the occurrence of positive diagnostic results from animals known to be true negatives. This includes positive test results from naïve common carp and non-common carp species as well as negative control groups (animals that were deliberately not exposed to CyHV-3).

Summary information relating to the occurrence of unexplained mortalities and false positives in reviewed articles is presented in Appendix 3.

4.3 Occurrence of unexplained mortalities and false positives in CyHV-3 susceptibility and resistance testing.

Unexplained mortalities

Of the 39 studies reviewed and presented in Appendix 3, 32 did not report any occurrence of mortalities in negative control groups or in non-common carp species exposed to CyHV-3. Mortalities in negative control groups and non-common carp species were only reported by: St-Hilaire et al. (2005), Ito, Sano, et al. (2007), Kempter et al. (2009), Yuasa, et al. (2013), Ronsmans et al. (2014), Pospichal et al. (2016) and McColl et al. (2017). The authors of these studies applied differing approaches to dealing with these mortalities and to overcoming the potential problem of unexplained mortalities.

McColl et al..(2017) present their observations from two exposure trials that they call "*KHV Exposure 1*" and "*KHV Exposure 2*". In both studies they used groups of negative control common carp that were not exposed to CyHV-3. A small number of mortalities occurred in negative control groups from both trials. In both of these trials, the number of mortalities that occurred were exceeded in groups of common carp that were exposed to CyHV-3. Regardless of the low number of mortalities that occurred in negative control groups, moribund and dead fish were subjected to additional diagnostic procedures to establish the most likely cause of death.

In trial "*KHV Exposure 1*", negative group mortalities and moribund common carp were subjected to post-mortem examination for bacterial infection and ectoparasites and assessed by PCR for the presence of CyHV-3 DNA. No bacterial pathogens were observed and all fish were PCR negative for CyHV-3 DNA. However, the ectoparasite *Ichthyophthirius multifilliis* was observed on wet mounts of skin and gills and this protozoan parasite was put forward as the cause of death in this trial.

In trial "*KHV Exposure 2*", mortalities also occurred in negative control common carp. These were examined for clinical signs, external parasites, and by PCR. Skin ulcerations were observed on dead fish but no external parasites were observed and PCR did not detect CyHV-3 DNA. No further diagnostic evidence is provided and despite these investigative diagnostic procedures, these mortalities remain unexplained.

A small number of mortalities in negative control common carp was also observed by Ito, et al. (2007). Larvae and juveniles of two strains of common carp, that the authors called *Strain 1 and Strain 2*, were assessed for their susceptibility to CyHV-3 infection. Mortality rates and PCR results were used to compare between fish exposed to the virus and in negative control groups. No mortalities occurred in common carp of "*Strain 2*".

When larval common carp of "S*train 1*" were assessed for susceptibility to CyHV-3, 2/20 negative control fish died, while 3/40 fish exposed to the virus died. CyHV-3 DNA was not detected in any of these larval fish and no further procedures were reported to diagnose a cause of death. These mortalities remain unexplained although non-specific mortality rates in the order of 10% are not uncommon during the larval rearing of common carp (Sharma & Chakrabarti, 1999).

When juvenile common carp of "*Strain 1*" were assessed, 1/10 negative control fish died, while 20/29 fish exposed to the virus died. The negative control fish that died was negative for CyHV-3 DNA by PCR and, in the absence of additional diagnostic evidence, remains an unexplained mortality.

Kempter et al. (2009) examined 15 farmed Russian sturgeon and 14 farmed Atlantic sturgeon with a known history of exposure to CyHV-3. This was not an exposure trial, negative controls were not used and fish were not directly challenged with CyHV-3 to determine susceptibility. A significant number of mortalities were observed in Russian sturgeon. These fish were subjected to an external examinations which revealed haemorrhages at the fin bases and post mortem examination of the internal organs that revealed partial lysis of spleen and liver, ascites and swollen intestines. The gills were also pale and covered with a grainy deposit. The Atlantic sturgeon were asymptomatic, with only a single mortality observed prior to the fish being fixed in ethanol for transportation to the laboratory. PCR tests revealed the presence of CyHV-3 DNA in nine Russian sturgeon and four Atlantic sturgeon. CyHV-3 DNA was also detected in gill and kidney samples by In-situ hybridisation (ISH) and also confirmed at the protein level by immunofluorescence (IFAT).

While mortalities were observed by Kempter et al. (2009), and evidence of CyHV-3 infection was reported, the authors did not directly attribute these deaths to the virus. Despite investigating and providing a detailed report of external and internal clinical clinical signs, the cause of death for these fish is ambiguous and they remain unexplained mortalities.

Yuasa et al. (2013) also observed mortalities in a non-common carp species. In this study, three varieties of goldfish were exposed to CyHV-3 and then stressed by temperature fluctuations. Significant mortality, 40-75%, occurred in ryukin goldfish exposed to CyHV-3. Moribund fish in this group exhibited upset swimming behaviour near the water surface before dying.

The authors attributed the deaths of ryukin goldfish not to the effects of CyHV-3, but to temperature stress. The authors noted that this variety of goldfish is known to be sensitive to temperature fluctuations, with affected fish showing upset swimming behaviour consistent with that observed in the trial.

Ronsmans et al. (2014) investigated susceptibility to CyHV-3 in the early life stages of common carp. The susceptibility of embryo, larval and juvenile common carp was assessed. Embryos were classified as newly hatched fish, 3-5 days old in which the digestive tract and gills are not yet fully formed. The larval stage included fish between 3-5 days old and 2-3 weeks old. These fish had fully absorbed their yolk sack and most of their organs were formed and functional. Juvenile fish were described as being 2-3 weeks old and having similar morphology to adult common carp.

Ronsmans et al. (2014) observed low-level mortality in negative control common carp (<~10%) compared with embryo, larval and juvenile fish exposed to CyHV-3 (~50-100%). In all cases mortality rates were significantly higher in fish exposed to the virus than in negative controls ($P < 0.0001$ at 15 and 30 days post exposure). Negative controls did not show evidence of CyHV-3 infection by in vivo imaging system (IVIS). No further diagnostic evidence is provided to explain these mortalities and they remain unexplained. However, mortality of approximately 10% is not uncommon during larval rearing of common carp (Sharma & Chakrabarti, 1999).

The mortality of non-common carp species during susceptibility testing was also observed by Pospichal et al. (2016). Mortality in Stone Loach and Sterbel was observed during two rounds of experimental testing. During the first round of experimental testing mortality in non-common carp species was: stone loach 0/10 and 1/10; sterbel, 1/10 and 0/10; and in the secondary transmission trial was: Stone loach 10/10; Sterbel 0/10. PCR testing of dead fish did not reveal the presence of CyHV-3 DNA.

The high rate of mortality observed in Stone Loach in the secondary transmission trial was deemed to be due to compromised function of the biological filters that led to a higher concentration of nitrites (~1.73 mg L-1) in tank water. Due to the lack of confirmative diagnostic evidence, these mortalities remain unexplained. However, the observation and reporting of water quality parameters provides indicative evidence that this parameter played a role in causing mortality.

The work of McColl et al. (2017) reported low to high levels of mortality in 12 non-common carp species (CyHV-3 exposed and/or negative control) as well as in a single group of negative control common carp. According to the methodology presented, all dead animals were examined for clinical signs specifically consistent with CyHV-3 disease in common carp and assessed by Gilad q-PCR. In a number of non-common carp and negative control common carp mortalities, CyHV-3 was identified by Gilad q-PCR. Samples from these fish were also assessed by Yuasa rt-PCR with all animals returning negative results. Moribund animals and some healthy animals were also assessed by histopathology to identify any effects consistent with CyHV-3 infection in common carp. Diagnostic procedures designed to identify a cause of death other than CyHV-3 do not appear to have been performed on non-common carp and negative control common carp mortalities and no alternative cause of death is reported for any of these mortalities that occurred in the study. As such, all these mortalities in non-common carp species and negative controls can be considered to be unexplained mortalities.

4.3.3 False positives

Of the 39 articles reviewed during the systematic review, only K. McColl et al. (2017) reported false positives from PCR testing while a single false positive is reported by S St-Hilaire et al. (2005) during enzyme linked immunosorbent assay (ELISA) testing. This lack of reporting of false positives may have occurred for a range of reasons.

In some studies, true negative control fish were unavailable and so the reporting of post exposure false negatives is not applicable in the context of trial infections. In other cases, direct detection diagnostic procedures were only applied to mortalities, which in most cases only occurred in fish that were exposed to the virus. In these studies, known-false negatives are not applicable as direct detection tests were not applied to known-negatives. In other studies, false negatives may have occurred during the study but were not reported. We cannot comment on the frequency of this occurring, but in cases where the reporting of false negatives did not affect the conclusions drawn by the authors, it is possible that the authors failed to report the occurrence of false positive results.

It is also possible that when false positives were observed to be occurring due to a systemic procedural error, the results were considered invalid and discarded. Again, there is no evidence to support this assertion but if systemic procedural errors are observed, it would seem necessary that the trials are repeated and corrections made to diagnostic procedures.

In any case, K. McColl et al. (2017) is the only study identified by the systematic review that directly reports on the frequent occurrence of false positives during direct detection diagnostic procedures. In this study false positive Gilad q-PCR results were observed in a small number of samples from negative control groups of non-common carp species (5/353). False positive Gilad q-PCR results were also observed in a large number of negative control common carp in the first exposure trial undertaken, trial 1 (7 positive results out of 8 samples), but occurred less frequently in the remaining 8 trials that were performed (1 positive result out of 19 samples).

Positive Gilad q-PCR results were also observed for a number of viral challenged non-common carp species (104 positive results out of 921 samples). The authors argue that these were in fact false positive results, caused by inadvertent contamination of samples with viral DNA during processing of samples (rt-PCR tests on these 104 samples all returned negative results).

McColl et al. (2017) observed no false positives when Yuasa rt-PCR was applied to negative control groups.The work of McColl et al. (2016) appears to be unique, in this review, with respect to the reporting of false positive results from direct detection diagnostic procedures. A more in depth discussion of these false positives is set out in appendix 4.

4.4. Developing a diagnostic matrix to aid the interpretation of undiagnosed mortalities & false positives

While undiagnosed mortalities and false positives are the primary focus of this section, it is difficult to discuss these aspects in isolation of the broader diagnostic protocol that was applied. By undertaking a comprehensive appraisal of the diagnostic evidence presented by K. McColl et al. (2017), it is possible to develop a formalised decision making process that assists in the interpretation of undiagnosed mortalities and false positives. To achieve this, it is advantageous to include all diagnostic evidence in a matrix that optimises the interpretation of the diagnostic tests and yield a well-informed decision regarding the resistance status of NTS to CyHV-3 infection.

The absence of a gold standard diagnostic test for CyHV-3 infection in NTS makes it difficult to provide a confirmative diagnosis of infection with this virus. The power of individual diagnostic tests to confirm the absence of CyHV-3 is even more problematic as all available tests regularly return false negative results. The difficulty in diagnosing CyHV-3 infection is acknowledged by the OIE (2017b) which recommends that the diagnosis of CyHV-3 infection should not rely on a single test alone but on a combination of two or three tests with significant diagnostic advantageous afforded as test numbers increase.

To guide the development of such a matrix, a mock-matrix based on the results published by K. McColl et al. (2017) is presented in Table 2. The table . provides as much diagnostic evidence as it is possible to ascertain from the published paper. K. McColl et al. (2017) did not present PCR data for each species assessed. Data was instead presented for each trial performed, with each trial assessing multiple NTS. For the purpose of the matrix below, Gilad q-PCR results for individual species have been assumed to be equal across all NTS within each trial (we acknowledge that this is highly unlikely).

For mortality data, a species was considered to be positive to CyHV-3 when the rate of mortality in fish exposed to the virus exceeded the rate of mortality in the corresponding negative control group. Again this is only an assumption.

Animals were considered positive by pathological signs when any lesions consistent with viral infection were observed. As pathological signs may vary between species, a positive determination should not be restricted to pathological signs previously observed in common carp exposed to CyHV-3.

For Gilad q-PCR testing, a species was deemed to be positive when more than 5.3% of tests returned a positive result. This represents the percent of known false positives that occurred in negative control common carp (excluding trial 1).

Animals were considered to be positive by rt-PCR where any positive results were observed.

It is important to note that this matrix is not presented to provide evidence of the resistance status of each species assessed by K. McColl et al. (2017). However, it does provide a pertinent example of a matrix that could be used for use in future resistance testing protocols.

Table 2 Summary of diagnostic results obtained from exposure trials of Common Carp and NTS to CyHV-3 by McColl et al (2017)

	Diagnostic test applied/Result from diagnostic testing (+/-)				
Species	Mortality (excluding mortalities where diagnosis confirms an alternative cause of death)	Pathological signs (including gross signs and histology)	Gilad q-PCR	Yuasa rt-PCR	**Resistance status** (<1 +ve result = Resistant 1 +ve results = Uncertain >3 +ve result = Not Resistant)
Common carp (*C. Carpio*)	**+**	**+**	**+** (+ 90.7%)	**+** (79.6%)	**Not Resistant**
Silver perch (*B.bidyanus*)	**+**	-	**+** (+ 41.9%)	-	**Uncertain**
Murray Cod (*M. Peelii*)	-	-	**+** (+ 29.4%)	-	**Uncertain**
Golden perch	-	-	**+**	-	**Uncertain**

(*M. ambigua*)			(+ 21.1%)		
Common galaxias (*G. maculatus*)	-	-	- (+ 2.2%)	-	**Resistant**
Rainbow trout (*O. Mykiss*)	**+**	-	- (+ 2.2%)	-	**Uncertain**
Short-finned eel (*A. australis*)	-	-	**+** (+ 5.5%)	-	**Uncertain**
Salmon catfish (*N. graefei*)	-	-	**+** (+ 5.5%)	-	**Uncertain**
Crimson spotted rainbow fish (*M. duboulayi*)	-	-	**+** (5.5%)	-	**Uncertain**
Eel-tailed catfish (*T. tandanus*)	-	-	- (+ 3.2%)	-	**Resistant**
Sea mullet (*M. cephalus*)	**+**	-	- (+ 3.2%)	-	**Uncertain**
Australian smelt (*R. semoni*)	-	-	- (+ 3.2%)	-	**Resistant**
Chicken (*G. g. Domesticus*)	-	-	**+** (+ 6.1%)	-	**Uncertain**
Mouse (*M. musculus*)	-	-	**+** (+ 6.1%)	-	**Uncertain**
Carp gudgeon (*Hypseleotris sp.*)	-	-	- (+ 2.0%)	-	**Resistant**
Olive perchlet (*A. Agassizii*)	-	-	- (+ 2.0%)	-	**Resistant**
Short-headed lamprey ammocoetes (*M. mordax*)	-	-	- (+ 2.0%)	-	**Resistant**
Common Yabby (*C. destructor*)	-	-	- (+ 2.0%)	-	**Resistant**
Eastern water dragon (*I. lesuerii*)	-	-	**+** (+ 15.1%)	-	**Uncertain**
Macquarie short necked turtle (*E. macquarii*)	-	-	**+** (+ 15.1%)	-	**Uncertain**
Peron's tree frog, adult (*L. peronii*)	-	-	**+** (15.1%)	-	**Uncertain**
Peron's tree frog, tadpole (*L. peronii*)	**+**	-	**+** (15.1%)	-	**Uncertain**
Spotted Marsh frog, adult (*L. tasmaniensis*)	-	-	**+** (15.1%)	-	**Uncertain**
Bony bream (*Nematalosa erebi*)	-	-	**+** (33.3%)	-	**Uncertain**

In the example provided by Table 2 all diagnostic tests are weighted equally. Where more than two positive results are observed, the species in question is interpreted as being not resistant to CyHV-3. If all tests return a negative result then the species is determined to be resistant to CyHV-3. However, if one or two diagnostic tests yield a positive result, then the resistance status of the species is deemed to be uncertain. When this occurs, it would be recommended that resistance testing be repeated to provide additional evidence of the resistance status of the host.

Based on this matrix, only common carp would be considered to be not resistant to CyHV-3. Common galaxias, eel-tailed catfish, Australian smelt, carp gudgeon, olive perchlet, short-headed lamprey ammocoetes and common yabby would be considered to be resistant to CyHV-3. While the resistance status of the remaining 16 species would be considered to be uncertain.

The matrix could also be used to make a compelling argument that mortalities occurring in rainbow trout and sea mullet are most likely not due to CyHV-3 infection. While the resistance status of these species must remain uncertain, there is substantive diagnostic evidence against the presence of CyHV-3 infection in these species. If additional diagnostic procedures were to be added to the broader diagnostic protocol, or if an alternative diagnosis could be provided for mortalities that occurred in these species, it may be possible to assert with confidence that CyHV-3 infection was not involved in these mortalities.

However, for Peron's tree frog tadpoles and especially for silver perch, it would be more difficult to argue that the mortalities observed were not related to CyHV-3 infection. The high rate of positive results observed by Gilad q-PCR, especially for silver perch, make it hard to exclude CyHV-3 infection as a potential cause of death in these animals, despite the lack of pathological signs and positive Yuasa rt-PCR results.

The large number of species deemed by this matrix to have uncertain resistance status is predominantly due to the high proportion of positive results returned by Gilad q-PCR. In many cases, Gilad q-PCR was the only test to indicate the presence of CyHV-3. It is difficult to interpret positive Gilad q-PCR data as anything other than the presence of CyHV-3 DNA in the tissues of NTS. K. McColl et al. (2017) blame sampling protocols and the contamination of samples for yielding false positive results from Gilad q-PCR. This may have been the case, however, the large number of positive Gilad q-PCR results in some trials makes this assertion somewhat tenuous.

It is also inappropriate to use mortality, pathological signs and Yuasa rt-PCR to discredit the positive results observed by Gilad q-PCR. This is because it is possible for a NTS to become infected with CyHV-3 and yield positive Gilad q-PCR results but fail to exhibit mortality, pathological signs and positive Yuasa rt-PCR results. These tests target different aspects of CyHV-3 infection.

Mortality and pathological signs provide evidence of acute effects of the virus and Yuasa rt-PCR detects viral replication while Gilad q-PCR detects the presence of viral DNA in tissue samples. As such, mortality, pathological signs and Yuasa q-PCR cannot conclusively rule out the presence of viral DNA. This is an important consideration as the presence of CyHV-3 DNA may indicate a latent infection or subclinical infection below the threshold of detection by Yuasa rt-PCR. And while such infection may not cause acute clinical signs during short term exposure trials, it indicates spill over infections and species jumps the implication of which are unpredictable given present knowledge of CyHV-3.

The assertion that Gilad q-PCR results were false positives due to procedural errors is also a major concern that must be excluded from future NTS CyHV-3 resistance testing protocols. Such

procedural errors should be identified and rectified prior to the execution of future resistance testing protocols. Or, where such problems arise during the course of a study, trials should be repeated to avoid basing decisions on incorrect data. The reliability of diagnostic and direct detection tests is a central aspect for NTS resistance testing protocols and should not be brought into question after the completion of such trials.

4.5. Recommendations on the occurrence of unexplained mortalities and false positives when testing the resistance of NTS to CyHV-3 infection.

Unexplained mortalities and false positives have the potential to confuse the outcomes of NTS resistance testing studies. Every effort must be made to prevent their occurrence in future studies that assess the resistance status of NTS to CyHV-3 in the NCCP. In addition to the recommendations made in Chapter 3 and above for using a broad based diagnostic approach in testing resistance of NTS to CyHV-3 infections, it is recommended that:

1. A causal diagnosis should be identified for all mortalities that occur in future studies that assess the resistance status of NTS to CyHV-3 infection in the NCCP.

and

2. False positives should be avoided by ensuring that sampling procedures are designed to prevent their occurrence and if they do occur an explanation for their occurrence should be identified

and

3. A weighted decision matrix for the determination of resistance status is necessary to enable a balanced assessment of diagnostic results.

Including these critical considerations in future NTS resistance trials, will increase confidence by researchers and stakeholders in the resistance status of NTS post exposure to CyHV-3.

5. Should stressors be deliberately applied to target species when assessing the resistance of NTS to CyHV-3 infection.

With the potential for environmental stressors to affect aquatic organisms in Australian freshwater and brackish environments, it is critical that the effects of stress/immunosuppression on resistance to CyHV-3 in NTS are considered. This can be undertaken under experimental conditions maximising the potential of infections by CyHV-3 in NTS. This chapter discusses the role of stress and immunosuppression and addresses the concerns that the NTS susceptibility study undertaken by McColl et al. (2017) did not explicitly impart stress on subject animals within the testing approach.

5.1. Immunosuppression and stress

Immunosuppression refers to any act or pressure that reduces the efficacy of the immune system. Immunosuppression is well recognised in fish and aquatic organisms and can lead to increased disease incidence and mortality rates (Mateus, Power, & Canário, 2017). The aquatic environment confounds the effects of immunosuppression on aquatic organisms as this environment increases the risk of infection (Mateus et al., 2017).

One of the most common factors associated with immunosuppression in captive and wild aquatic organisms is stress. However, the level of immunosuppression that occurs is variable and can depend on a number of key factors. Different species can react differently to different stressors, while the intensity and persistence of the stimulus are also known to affect the immune system in varying ways (Mateus et al., 2017; Portz, et al., 2006).

Stress involves the disruption of homeostasis by an external influence. In response, the affected animal attempts to re-establish homeostasis through physiological and/or behavioural actions. However, this response may be inadequate, excessive or prolonged, potentially leading to immunosuppression and disease (Mateus et al., 2017).

Numerous studies have observed the immunosuppressive effects of stress on aquatic organisms (e.g. Brydges, et al., 2009; Vazzana, et al., 2002) and several excellent reviews exist on the topic (eg. Mateus et al., 2017; Portz et al., 2006). Immune system dysfunction has been observed to result from severe-acute and chronic stress causing deleterious effects on the animal (Ortuño, et al., 2001; Varsamos, et al., 2006; Vazzana et al., 2002; Yin, Lam, & Sin, 1995). External stressors can result in a reduction in: circulating leukocytes, antibody levels, cytokine expression and serum immunoglobulin M (IgM) and increased susceptibility to pathogenic challenge (Mateus et al., 2017; Portz et al., 2006).

Common stressors associated with immunosuppression in aquatic animals are sub-optimal or acute changes in water quality and handling practices (Portz et al., 2006). Specific parameters associated commonly with stress and reduced immune function in fish are temperature and dissolved oxygen levels. Handling of aquatic animals is also well established as a cause of stress and can arise from netting and crowding animals in captive systems or due to capture and handling during commercial or recreational fishing (Portz et al., 2006).

5.2. Potential Stressors in the Murray Darling Basin

As stress is known to cause immunosuppression, and susceptibility to pathogen effects if exposed, this factor may play a critical role when assessing NTS for resistance to CyHV-3 infection.. This is especially important in the context of the NCCP and the variable fresh water environments that native fish inhabit within Australia. For instancewaters in Australia regularly experience severe

fluctuations in temperature and dissolved oxygen. For example, normal annual temperature fluctuations within the Murray Darling Basin range from 8-23°C (Murry River, Albury), 9-35°C (Darling River, Wilcannia) and 11-26°C (Murray river, Lock 1), with the potential for greater fluctuations during extreme weather events. Dissolved oxygen (DO) can also fluctuate widely. While DO typically exceeds 6 mg L^{-1}, black water events, associated with inundation of the flood plain and decomposition of accumulated organic matter, can cause DO to fall below 2 mg L^{-1} (Whitworth, Baldwin, & Kerr, 2012). Such extremes are likely to cause severe-acute and chronic stress on aquatic organisms within the system. These stressful events are likely to cause immunosuppression and affect the susceptibility of aquatic animals to various pathogens.

A number of studies have been published in which a stressor was applied during CyHV-3 susceptibility/resistance testing. This literature has been reviewed and the results are presented below.

5.3. Literature review methodology

The 451 articles identified through the systematic, quantitative review process outlined above (Chapter *2.*), and graphically represented in PRISMA Statement 4 (Figure 8) were subjected to an additional screening process for this section. This additional screening was performed to identify those articles on studies that applied, assessed or discussed the effects of applying an external stressor on CyHV-3 infection dynamics. Publications were excluded where they failed to mention the potential effects of an external stressor during CyHV-3 susceptibility/resistance testing. After screening, 14 articles were identified as meeting the criteria for inclusion in this review (see PRISMA Statement 4, Figure 8). The full text of each was then obtained and reviewed in detail. Of each of the relevant articles identified, the reference lists were checked to identify additional relevant publications.

Previous studies that have applied, assessed or discussed stress in the context of CyHV-3 infection dynamics are summarised in Appendix 5. This data presents the following key information from each study: Title, Author(s), Year of Publication, Journal and Stressor Applied. These studies and their implications to assessing the resistance of NTS to CyHV-3 infection are discussed.

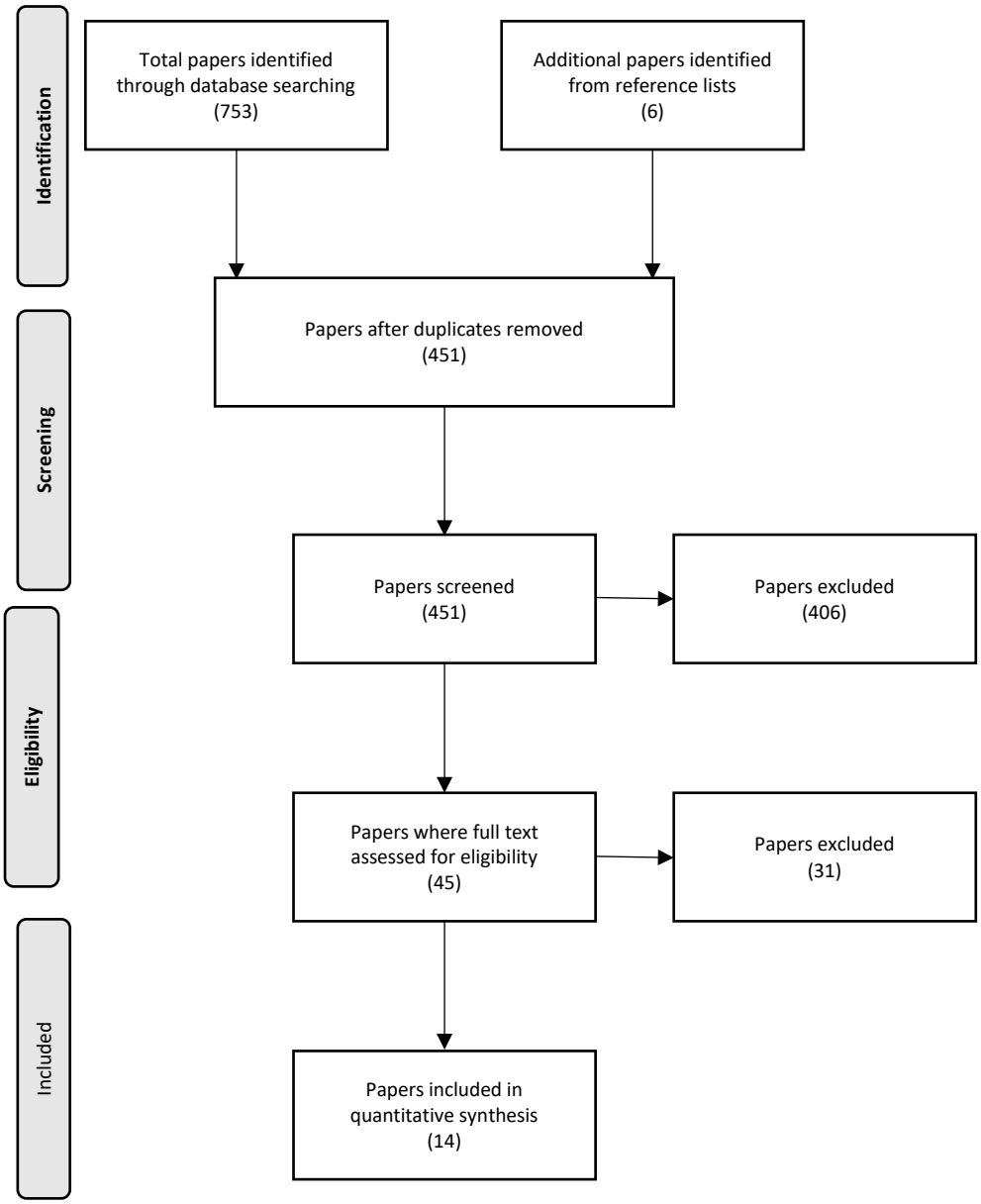

Figure 8. PRISMA statement 4. Screening process used to identify articles that applied, assessed or discussed the effects of applying an external stressor on CyHV-3 infection dynamics.

5.3. The application of stress in studies assessing susceptibility of infection to CyHV-3.

The most common stressors that have been applied to affect CyHV-3 infection dynamics are temperature fluctuations and netting/air exposure. These stressors are applied for a range of reasons: It has been shown that these stressors can: increase cortisol (a response hormone in stress) in fish plasma and holding water; decrease survivability following challenge with CyHV-3; reactivate latent or subclinical-persistent CyHV-3 infections in carp leading to clinical signs and mortality; increase the abundance of CyHV-3 in host tissues, making detection easier; and increase viral shedding and the concentration of CyHV-3 in holding water.

Temperature stress was applied in five studies that investigated susceptibility to CyHV-3 infection: Lin et al. (2017); Takahara et al. (2014); Eide et al. (2011); Mansour El-Matbouli and Soliman (2011); Yuasa et al. (2013).

Takahara et al. (2014) quantified the effects of diurnal temperature fluctuations on common carp challenged with CyHV-3. Separate groups of carp were acclimated to temperatures of 22°C and 25°C respectively. These were subsequently divided into groups that were either: 1) held at a constant temperature (22°C or 25°C), or 2) subjected to diurnal temperature fluctuations with the holding temperature fluctuating between 19 and 25°C during each 24 hour period. Common carp were exposed to CyHV-3 on the same day that temperature fluctuations commenced. These temperature fluctuations were then applied continuously for a period of 17 days. When compared to fish held at a constant temperature, daily temperature fluctuations of ±3°C decreased survivability, increased the release of CyHV-3 from fish and increased cortisol levels in holding water. This study demonstrates clear immunosuppressive effects of diurnal temperature fluctuations in fish exposed to CyHV-3.

Eide et al. (2011) presented methodology for applying temperature stress to common carp to reactivate a latent infection. The method described increased temperature from 12°C to 23°C at a rate of 1°C per day, following which the temperature was then held at 23°C for 4 days before being returned to 12°C at a rate of 1°C per day. CyHV-3 DNA was detected in common carp, by PCR, two days after the initial temperature increase and virus reactivation peaked after 8 to 12 days (coinciding with a water temperature of 21-23°C). While this procedure is described by the authors as a temperature stress, the reactivation of the virus occurred at temperatures consistent with the optimal temperature range of the virus (Oren Gilad et al., 2003). It appears likely that reactivation of the virus in this case was more likely related to the virus entering its permissive range rather than being due to immunosuppression occurring as a result of temperature stress.

Similar to Eide et al. (2011), Lin et al. (2017) applied what they refer to as temperature stress to latently infected common carp. In this study, water temperature was increased at a rate of 1°C per day from 15°C to 22°C before returning the temperature back to 15°C at a rate of 1°C per day. The increase in temperature led to reactivation of latent CyHV-3 infection. Again, this appears to be related to the virus entering its permissive range rather than a direct stress effect. While the authors also observed increases in plasma cortisol, indicating a stress response, increases in plasma cortisol were also observed in common carp held at 15°C. The increase in plasma cortisol across all groups appeared to be a consequence of handling and netting which was performed across all groups of fish. This provides indicative data, that netting and handling can causes quantifiable stress in fish which may have implications for immunosuppression and challenge by CyHV-3.

Mansour El-Matbouli and Soliman (2011) and Kei Yuasa et al. (2013) describe a more rigorous approach to imparting temperature stress. In each case, temperature was changed by 3°C per day. In the case of Mansour El-Matbouli and Soliman (2011), the temperature was increased from 14°C to 26°C, held at this temperature for five days and then returned to 14°C with this cycle repeated twice. Kei Yuasa et al. (2013) decreased the water temperature from 24 to 18°C, held this temperature for 2 days before returning the temperature to 18°C, with the cycle repeated twice.

Mansour El-Matbouli and Soliman (2011) applied stress to stimulate the reactivation and release of CyHV-3 from goldfish with a known history of exposure to infected common carp. It was shown that in the presence of a stress factor, goldfish could transmit CyHV-3 to susceptible naïve common carp by cohabitation.

In contrast to the findings of Mansour El-Matbouli and Soliman (2011), Yuasa et al. (2013) observed a limited ability for goldfish to transmit CyHV-3 to recipient common carp; even under the stressful conditions applied by temperature fluctuations. As a consequence, it was concluded that goldfish are not susceptible to CyHV-3 but may transmit the virus to common carp only under specific conditions. It was suggested that the virus may be carried on the surface of goldfish for a limited period of time following exposure to CyHV-3 in water (Yuasa et al., 2013).

Neither Mansour El-Matbouli and Soliman (2011) nor Yuasa et al. (2013) compared the effects of temperature fluctuations on goldfish with fish held in a constant-temperature environment. As a result it is difficult to determine if temperature fluctuation, in either study, induced stress and/or immunosuppression. Further research is needed to quantify the effects of temperature fluctuations on stress/immunosuppression during testing of susceptibility/resistance testing to CyHV-3 infection.

Netting/air exposure was described, as a stressor, in 6 studies that investigated susceptibility to CyHV-3 infection: S. Bergmann and J. Kempter (2011); Bergmann, et al. (2016); Fabian, et al. (2016); Fabian et al. (2013); Gaede, et al. (2017); and Pospichal et al. (2018). In each case, common carp were captured from the holding tank by netting and then subjected to at least one period of air exposure for 30 seconds to 1-2 minutes.

Bergmann and Kempter (2011) recognised difficulties in detecting CyHV-3 DNA in infected but healthy appearing common carp due to very low concentrations of virus particles in tissues. In response, a method was developed to increase the detectability of CyHV-3 DNA by PCR based methods. To achieve this, common carp were netted from their holding tank and subjected to two 30 second time periods out of the water to induce stress. While this did not lead to any clinical clinical signs associated with CyHV-3, the study was successful in enhancing the detection of CyHV-3 DNA in infected common carp. Increased copy numbers of CyHV-3 DNA were detected by real-time PCR in all common carp from days 2-3 post-netting-stress. Copy numbers of CyHV-3 DNA were then observed to decrease from 4-10 days post-netting-stress. The study also showed that applying a stressor could stimulate release of the virus from subclinically infected common carp. In stressed fish, CyHV-3 DNA was detected primarily in gill swabs, but also in some faecal samples, indicating that the stressor stimulated the release of virus particles.

Pospichal et al. (2018) applied a simulated transport event to impart stress on rainbow trout. This study appears to draw on the results of Bergmann and Kempter (2011) who showed that release of viral particles could be stimulated by applying an external stressor. In this case, rainbow trout were exposed to CyHV-3 by immersion before being placed in separate aquaria for seven days. The trout were then stressed by a simulated transport procedure that involved netting the fish, exposing the

fish to air for a period of 30 seconds, placing the fish in new aquaria for 10 minutes and then returning to the original aquaria. Carp were then cohabitated with these rainbow trout and shown to develop CyHV-3 infection. The authors did not attempt to compare infection dynamics between fish exposed to this simulated transport stressor and unstressed fish. As a result it is difficult to infer the effects of simulated transport on CyHV-3 infection dynamics from this study.

Fabian et al. (2013) also based their methodology on Bergmann and Kempter (2011), citing the potential for netting stress to reactivate latent infection and allow a more reliable detection of CyHV-3 DNA. In this instance, the authors deliberately stressed a range of wild fish species that had previously cohabitated with common carp carrying a latent CyHV-3 infection. Fish from each species were netted and held out of the water for a period of 30 seconds, allowed one minute of regeneration time and then exposed to the air for another 30 seconds. The netting procedure was repeated five times. Half of the fish from each species were killed three days after this netting procedure and assessed for the presence of CyHV-3 DNA by PCR. The remaining fish were cohabitated with naïve common carp to determine the potential for these wild fish species to act as a source of CyHV-3 and pass the infection on to the carp. CyHV-3 DNA was detected in the tissues of eight species of wild fish that had been stressed after cohabitation with latently infected common carp. The potential for five species of wild fish to pass CyHV-3 infection to common carp was also demonstrated by the presence of CyHV-3 DNA in recipient carp. Again, the authors made no comparison between stressed and unstressed fish.

Fabian et al. (2016) present another investigation of susceptibility in which the procedure of S. Bergmann and J. Kempter (2011) was applied. In this study, netting stress was applied to reactivate latent infection, induce shedding of CyHV-3 particles and to optimise the detection of the CyHV-3 DNA by molecular methods. This Short Communication presents a limited description of the methodology applied but refers to repeated netting and air exposure for about one minute, referencing Bergmann and Kempter (2011) as the source of the method. The netting stress was applied to a range of non-carp species in three separate experiments. The first was designed to determine the presence of CyHV-3 DNA in these species. Here, netting stress was applied three days prior to fish being euthanised and sampled for tissue analysis. This was applied to reactivate a potential latent or subclinical-persistent infection and optimise the potential to identify CyHV-3 in host tissues. A further two experiments were performed to determine if non-carp species could transmit the virus to naïve carp. In the first of these, a range of species were exposed to CyHV-3 by immersion, subsequently stressed by netting to induce viral shedding, and then cohabitated with common carp. The second of these experiments exposed various non-carp species to common carp exhibiting acute clinical signs of CyHV-3 infection. These non-carp species were subsequently stressed by netting to induce viral shedding prior to being cohabitated with naïve carp. The study showed only occasional and low presence of CyHV-3 DNA in non-carp species and a limited ability for these fish to transmit CyHV-3 to naïve common carp. No comparison was made between stressed and unstressed fish.

Netting stress was also applied by Gaede, et al. (2017), to reactivate potential latent CyHV-3 infection in fish, 90 days after initial exposure. This was performed on common carp, common roach (*Rutilus rutilus*), crucian carp and tench (*Tinca tinca*). While CyHV-3 DNA was detected occasionally in roach, tench and crucian carp up to 25 days post exposure, at 90 days post exposure, CyHV-3 DNA was only detected only in common carp. The effects of netting stress appear to be minimal in this study.

Pospichal et al. (2018) also applied netting stress when assessing the susceptibility of topmouth gudgeon (*Pseudorasbora parva*) to CyHV-3 infection. Netting stress was applied in two different ways. In the first instance, all fish were subjected to netting stress two days before sampling to enhance the chance of virus detection by PCR. Secondly, scaring by a net was undertaken to decrease immunity, presumably via immunosuppression. This involved scaring the fish with a net for approximately 30 minutes per day while the fish were cohabitated with known or suspected virus carriers. A further procedure was applied to enhance infection during exposure – following from the work of Raj et al. (2011a), removal of skin mucus was performed to facilitate entry of the virus through the epidermis. An additional group of fish was held without the application of an external stressor.

In Topmouth gudgeon that were not subjected to stress, CyHV-3 DNA was not detected by CEFAS n-PCR after cohabitation with infected koi carp. However, when topmouth gudgeon were subjected to scaring stress, CyHV-3 DNA was detected in two of five samples (pools of tissues from 2 fish) collected from topmouth gudgeon that had been cohabitated with infected common carp carp. In this instance Gilad q-PCR was used rather than CEFAS n-PCR. When skin mucus removal was applied, CyHV-3 was detected in 4 out of 5 samples (pools of tissues from 2 fish) collected from fish exposed to the virus. Again, Gilad q-PCR was performed on mucus-removed fish. The Gilad q-PCR was not applied to any topmouth gudgeon that had not been subjected to an external stressor.

Topmouth gudgeon from the most heavily infected group were subsequently cohabitated with ten naïve koi carp. After cohabitation, CyHV-3 was only detected in a single koi by Gilad q-PCR.

This study is of interest for a number of reasons. Firstly, it provides some evidence that a species other than common carp is susceptible to infection or at least acting as a source of CyHV-3 under the right circumstances. Secondly, it appears to demonstrate that applying a stressor during resistance testing may lead to infection of a non-common carp species by CyHV-3. Thirdly, CyHV-3 was only detected in Topmouth gudgeon when the highly sensitive and responsive Gilad q-PCR was used for diagnosis and not when the CEFAS n-PCR was applied.

The impact of applying a stressor in this study is somewhat difficult to interpret. While it is true that CyHV-3 DNA was only detected in topmouth gudgeon that had been subjected to an external stressor (scaring or mucus removal) and not in unstressed Topmouth gudgeon, different PCR methods were applied for stressed and unstressed fish. This is problematic as it precludes a like-for-like comparison from being made. Whether the differences observed were a result of the application of a stressor or due to the use of a more sensitive and responsive diagnostic technique is difficult to determine, but has important implications for the development of resistant testing protocols.

When viewed in this light, the work of Pospichal et al. (2018) can be interpreted in two ways. The study may either provide evidence that applying stress or removing skin mucus can inhibit resistance to CyHV-3, or, it may be seen to provide additional evidence that the Gilad q-PCR has superior diagnostic sensitivity compared to the CEFAS n-PCR. Despite this ambiguity, between stressed and unstressed fish, a clear comparison can be made between topmouth gudgeon that were either scared by netting or subjected to skin mucus removal.

The removal of skin mucus from Topmouth gudgeon led to CyHV-3 DNA being detected in 4four out of five tissue samples compared to two of five samples collected from fish that had been scared only. This may indicate that mucus removal enhances the entry of CyHV-3 into Topmouth gudgeon, although the low sample numbers make such an assertion somewhat tenuous.

In any case, the potential for skin mucus removal to enhance the entry of CyHV-3 into species that otherwise appear to be resistant to the virus warrants further investigation. This is a critical concern as skin lesions can occur in wild fish from a range of causes, including epizootic ulcerative syndrome which has been widely reported in a number of NTS in the Murray Darling Basin (Boys et al., 2012).

A further three studies included in the present review refer to stress when discussing the susceptibility of fish to CyHV-3 infection: S. Bergmann et al. (2010); Bergmann, et al. (2010); Kempter et al. (2012). None of these studies deliberately imparted a stressor during susceptibility testing but all commented that a lack of diagnostic evidence for CyHV-3 infection may have been related to the absence of stressful conditions. These studies recommend that a stressor be imparted when assessing fish for susceptibility to CyHV-3 infection. Their rationale for this is to: induce clinical clinical signs in fish; stimulate the release of CyHV-3 from sub-clinically infected fish; and to optimise the chance of detecting CyHV-3 in latently infected or subclinical fish.

The OIE Criteria for listing species as susceptible to infection with a specific pathogen also refers to stressors, requiring that evidence for susceptibility be classified according to the mode of transmission. For example the OIE code lists experimental procedures such as “invasive experimental procedure” and “exposure to stressors (e.g. temperature) not encountered in the host's natural or culture environment”. This highlights a general understanding that the application of a stressor can affect susceptibility/resistance to infection (OIE, 2017a).

5.4. Conclusions on imparting stress when assessing resistance to CyHV-3 infection in NTS.

Several studies have deliberately applied a stressor to affect the infection dynamics of CyHV-3 across a range of different species. Of these, (Takahara et al., 2014) and Bergmann and Kempter (2011) were the only studies to quantify the effects of applying an external stressor. Takahara et al. (2014) showed that temperature stress on common carp, as quantified by elevated cortisol levels, could decrease survival and increase the concentration of CyHV-3 in holding water. Bergmann and Kempter (2011) showed that stress could induce viral shedding and enhance the ability to detect CyHV-3 by PCR based methods. Three subsequent studies applied the methodology described by Bergmann and Kempter (2011). Lin et al. (2017) also observed that netting and handling could increase plasma cortisol in fish during susceptibility/resistance testing.

Deliberately applying stress appears to be a useful tool in studies that assess susceptibility/ resistance to CyHV-3. This is demonstrated by the relatively high percentage of studies that either applied a stressor or commented on the potential benefits of doing so. Of the 41 studies identified that assessed susceptibility/resistance of fish to CyHV-3, 34% (14 studies) applied a stressor or discussed the benefits of doing so.

Two different categories of stressors have been applied to impart stress in studies assessing susceptibility/resistance to CyHV-3: temperature fluctuation and netting stress. Based on this review, there are clear benefits of applying stress when investigating susceptibility to CyHV-3.

The first benefit applies to studies in which exposure is by cohabitation with infected fish. Applying stress to carrier fish can induce shedding of CyHV-3, thereby increasing the concentration of infectious virus in the water (Bergmann & Kempter, 2011; Takahara et al., 2014). Stress can either be applied to carrier carp or non-carp carriers. When applied to carp, stress can induce viral shedding, thus increasing the concentration of virus to which a non-carp species are exposed. Or, stress can be applied to assess the ability of a non-carp host species to infect naïve carp. In this case, the stressor

would be applied to the non-carp host, to induce viral shedding, prior to cohabitation with naïve carp.

Stress can also be applied to carp or non-carp species to optimise diagnosis of CyHV-3. As stress has been shown to increase the concentration of CyHV-3 in holding water (Takahara et al., 2014), tissues, gill swabs and faecal samples (Bergmann & Kempter, 2011), applying a stressor prior to diagnosis can enhance the potential for detection. This is especially important given the potential for CyHV-3 to develop latent or subclinical infections, during which detection of the virus can be extremely difficult (Bergmann & Kempter, 2011).

The application of temperature-fluctuation stress has also been shown to decrease survival of common carp following exposure to CyHV-3 (Takahara et al., 2014). During temperature fluctuations, increases in cortisol were observed, providing evidence of the stress effect. As temperature fluctuations increased mortality rate following exposure, applying this stressor is an important consideration in susceptibility/resistance testing as it will highlight the potential for the virus to cause disease and mortality in susceptible species.

The removal of skin mucus may also enhance the entry of CyHV-3 into carp and non-carp species (Pospichal et al., 2018; Raj et al., 2011a). Further work is required to validate the findings of Pospichal et al. (2018), that skin mucus removal facilitated the entry of CyHV-3 into topmouth gudgeon, but this factor may have important implications to the development of resistant testing protocols.

In the majority of studies discussed above, stress was applied to affect the immuno-competency of latently or sub-clinically infected fish. Of the 14 studies reviewed, only Takahara et al. (2014) and Pospichal et al. (2018) applied an external stressor to a potential host species prior to or during exposure to CyHV-3. This is somewhat surprising given that stress is well established as an immunosuppressant and known to increase susceptibility to pathogenic challenge (Mateus et al., 2017; Portz et al., 2006). For example, when sea bass were subjected to stress by temperature fluctuations, significant elevations in a number of stress parameters were observed and the fish were significantly more susceptible to nodavirus challenge (Varsamos et al., 2006).

5.5. Recommendations on the application of stress in in resistance testing of NTS to CyHV-3 infection

Based on this review, it is recommended that temperature fluctuations, as described by Takahara et al. (2014) or netting stress, according to the methods of S. Bergmann and J. Kempter (2011), be applied during studies that assess the resistance of NTS to CyHV-3 in the NCCP. It may also be applicable to use a combination of both, although this would need to be assessed prior to application. However the stress is applied it must be undertaken under an explicitly designed protocol.

Temperature fluctuation can cause stress and increases mortality in fish exposed to CyHV-3 (Takahara et al., 2014). The application of temperature fluctuations on NTS during resistance/susceptibility testing is likely to increase the chance of observing mortalities related to CyHV-3 infection. It is also reasonable to expect that clinical clinical signs associated with this virus will be exacerbated by temperature fluctuations, although this was not directly assessed by Takahara et al. (2014). The application of temperature fluctuation as a stressor is a critical consideration for future CSIRO NTS testing as it has a demonstrated potential to affect susceptibility to CyHV-3.

If carp carrying CyHV-3 infection are to be used in cohabitation trials to infect NTS, temperature fluctuations/netting stress should be performed on these carrier common carp to induce viral shedding. This has been shown to increase the concentration of CyHV-3 in holding water and optimise the potential for NTS to be infected with CyHV-3 if they are not resistant to the virus.

Temperature fluctuations and/or netting stress should also be applied to NTS prior to any attempts at virus detection. This is likely to increase the concentration of CyHV-3 in tissues, thus enhancing the efficacy of diagnostic tools used to detect this virus. This is a critical consideration given the potential for CyHV-3 to develop latent or sub-clinical persistent infections that can be extremely difficult to detect.

Removal of skin mucus according to the methods of Pospichal et al. (2018) and Raj et al. (2011b) is also tentatively advised.

6. Should all life stages of fish be evaluated in studies that investigate the resistance of NTS to CyHV-3 in the NCCP?

This literature review is designed to capture important information related to the susceptibility of different life stages of fish to a range of viral pathogens and their vulnerability to developing clinical signs and mortality. The collective data can then be used to guide the development of protocols to be used in any future NTS susceptibility/resistance testing to be performed in the NCCP.

Previous work undertaken to test the susceptibility of infection by CyHV-3 of NTS under experimental conditions (McColl et al, 2017) did not included trials specifically designed to investigate the susceptibility of all life stages of NTS to CyHV-3. For a complete assessment of vulnerability of NTS within the context of the NCCP it was deemed important that this be investigated or at least reviewed from other viral infection studies.

The empirical view is that the susceptibility of fish to many viruses is inversely related to age (e.g. Dorson & Touchy, 1981; Munday, Kwang, & Moody, 2002). For example, larval and early juvenile fish of several species are highly vulnerable to viral nervous necrosis, while the virus can have no perceptible impact on advanced juvenile or adult fish (Munday et al., 2002).

The effects of fish age on vulnerability to pathogenic viruses can exhibit complex patterns. For example, the vulnerability of common carp to the effects of CyHV-3 can be more severe in early juvenile fish, compared to advanced juvenile and adult fish (Bretzinger et al 1999; Sano et al, 2004). However, early larval common carp appear to be less susceptible to infection, and less vulnerable to the effects of CyHV-3, than later larvae and early juvenile fish (Takafumi Ito et al., 2007; Maygane Ronsmans et al., 2014). The precise mechanisms driving this relationship between life stage and susceptibility to CyHV-3 are unknown. And it is unclear if a relationship between the age of the host and its vulnerability to CyHV-3 could be expected in NTS exposed to CyHV-3 as a result of the NCCP.

6.1. Life stages of fish and immune function development

The life history of fishes is complex and can be broken into four major developmental stages: egg; larval; juvenile; and adult. In some species, a period of senescence may follow adulthood in which growth is limited and the gonads degenerate. These developmental stages are characterised by changes in morphology and physiology and by variations in immune function (Miller & Kendall, 2009; Zapata, et al., 2006).

The life history of fishes begins at fertilisation of the egg, which may occur internally within the female, or externally in the environment. The majority of fish species are oviparous, with fertilisation of the gametes and subsequent embryonic development occurring in the external environment (e.g. Miller & Kendall, 2009; Osse & Van den Boogaart, 1995; Ronsmans et al., 2014; Schilling, 2002).

The development from fertilised egg through to adult fish is usually characterised by gradual changes in morphology, although abrupt changes may occur in some species during larval development. Following fertilisation, the eggs undergo several developmental stages prior to hatching into larval fish. These larvae then undergo a series of species-specific stages, which are associated with differences in morphological and physiological characteristics. Eventually, larval fish transform into a morphological form similar to that of adult fish and are referred to as juveniles. These juveniles subsequently develop into adult fish with the onset of sexual maturity (e.g. Miller & Kendall, 2009; Osse & Van den Boogaart, 1995; Ronsmans et al., 2014; Schilling, 2002).

After fertilisation the egg passes through a series of developmental stages before hatching. This incubation period can last for varying time periods ranging from one day to several weeks (Miller & Kendall, 2009; Osse & Van den Boogaart, 1995). The development of the fertilised egg can be divided into the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching stages. (Kane, 1998; Kimmel, et al., 1995). These stages can be collectively referred to as the embryo stage although the embryonic stage may also refer to hatched larvae up to the time of first feeding (e.g. M. Ronsmans et al., 2014). During the embryonic period, nourishment is supplied by the yolk sac, which remains intact until after the larvae begin to feed (Miller & Kendall, 2009; Schilling, 2002).

At hatching, most larval fish are poorly developed and continue to rely upon the yolk sac for nourishment (Miller & Kendall, 2009). The larvae remain morphologically distinct from their adult form; the mouth may remain closed and the digestive tract may be poorly developed. Soon after hatching, most species develop good vision, the mouth opens and the larvae begin actively feeding (Miller & Kendall, 2009; Schilling, 2002). At this point larvae are often free swimming and planktonic, can maintain an upright orientation and respond to visual stimulus (Schilling, 2002). As the larvae develop, the organs and fins continue to form.

Larvae, with the presence of the yolk sac are often referred to as yolk sac larvae or yolk sac fry. First feeding often occurs prior to the complete exhaustion of the yolk sac. Regardless of the presence or absence of the yolk sac, larvae at the stage of first feeding are often referred to as first feeding larvae or first feeding fry.

The larval stage ends when the fin rays have formed, the scales start to form and pigmentation of the body begins (Miller & Kendall, 2009). This morphological change is often called metamorphosis as the larvae transform into juveniles. The timing of the morphological changes during the larval period varies widely between species. For example, the time between hatching and the mouth parts forming to allow first feeding can be as little as two days in striped jack (Masuda, 2009) and up to 44 to 59 days in Atlantic halibut (Lein & Holmefjord, 1992). As fish are poikilothermic, the rate of larval development is highly dependent on water temperature.

While morphological changes during larval development clearly affect life habit, behaviour and feeding, these changes may also affect the ability for specific viruses to infect larval fish. Levraud et al. (2007) observed that larval zebrafish were only susceptible to Spring viraemia of carp (SVCV) via bath exposure after the larvae had reached a developmental stage in which the mouth and gill slits had opened to allow respiratory function. Younger larvae could also be infected by SVCV but only via IV injection. It appears that the gills and/or the digestive tract may be an important portal of entry for SCVC in zebrafish and that natural infection cannot occur until the fish have reached this developmental stage (Levraud et al., 2007).

Juvenile fish generally look much like the adult form although in some species considerable visual differences may remain. Juvenile fish often inhabit a different ecological niche from their larval form, often transitioning from a planktonic to a pelagic or demersal habit. As the fish reach sexual maturity, they may again change habitat as they become adults (Miller & Kendall, 2009).

The adult stage begins with the first maturation of the gonads. During adulthood, most species are typified by an annual reproduction cycle, although in some species, fish may only mature once, towards the end of their life. Gonads usually mature in response to environmental stimuli and this is followed by reproduction. In some species, a period of senescence may follow adulthood during which growth may be limited and the gonads degenerate (Miller & Kendall, 2009).

During the early stages of life, the immune system of fish is not fully developed or functional (Zapata et al., 2006). At this early stage of development, the lymphomyeloid organs are not yet fully mature and as a result, the ability of larval and early juvenile fish to mount an effective adaptive immune response is restricted (Zapata et al., 2006). Adaptive immunity may not develop for several weeks after hatching and so larval and early juvenile fish must rely on maternally derived immune factors and on innate immune mechanisms (Zapata et al., 2006; Zhang, Wang, & Wang, 2013).

These maternal and innate immune factors may be activated immediately after fertilisation of the egg and be fully functional at hatching (Zapata et al., 2006; Zhang et al., 2013). The mucosal tissues of the skin, gills and digestive tract are an important mechanism of the innate immune response in larval fish. The mucus lining forms the first barrier of defence against pathogenic invasion, providing a physical and chemical barrier as well as triggering secondary innate immune responses (Vadstein et al., 2013).

As juvenile fish grow, the adaptive immune system develops. There is some evidence for the early development of lymphoid organs and lymphocytes in fish embryos and larvae, as little as three days post fertilisation (Zapata et al., 2006). However, full maturation of the adaptive immune system develops somewhat later. For example, humoral immune response has been observed six weeks post fertilisation in zebra fish and after 8 weeks in rainbow trout and common carp (summarised by Zapata et al., 2006).

The timing and development of innate and adaptive immunity may affect the susceptibility/ resistance of larval, juvenile and adult fish to pathogens. Furthermore, morphological changes that occur during larval development may also affect the time at which larval/juvenile fish become susceptible to infection. Importantly, age is known to affect the susceptibility of fish to a range of known viruses. This is an important aspect when assessing the susceptibility/resistance of specific fish species to specific viruses as life-stage and age may have a significant bearing.

To understand general and specific patterns in susceptibility/resistance of different life stages of fishes to viral pathogens, a review of the literature was undertaken.

6.2. Specific literature review methodology

This review searched for information on the effect of life stage on infection dynamics for viral diseases of fish.

The review initially focussed on viruses listed by the OIE (2018). This list was selected as it provides a suitable overview of common and globally significant viruses. The list also contains viruses from diverse taxa, facilitating a broad overview of taxonomically diverse viruses.

To provide additional information on those viruses most closely related to CyHV-3, the review subsequently searched for information specific to viruses of the family *Alloherpesviridae* that affect fish. Following this, the *alloherpesviruses* affecting cyprinids, the *cyprinivirus*es, were reviewed. Finally, the state of knowledge on infection dynamics of CyHV-3 in varying life stages of common carp was reviewed.

During the course of this review, it became apparent that there are wide variations in the ability of viruses to infect and/or affect various hosts. It was also apparent that there is no consistent terminology to differentiate between the potential of a virus to infect a host and the ability of the virus to affect the host. This creates difficulties when discussing the varying actions of the virus. To overcome this, the term “susceptible” has been used in this chapter to describe situations in which

the virus can infect the host, including in the absence of clinical signs, The term “vulnerable” is used in this chapter when the virus causes clinical signs or mortality. By using this terminology, the following discussion on the susceptibility and/or vulnerability of different life stages of fish to viral challenge will provide a better understanding of the complex interactions between viruses and hosts.

6.2.1. Viruses of fish listed by the OIE

The OIE (2018) lists a range of internationally significance viruses. Viruses on this list pose a risk to the health of cultured and/or wild aquatic animals, are well defined and are at risk of spreading internationally (OIE, 2018). The viruses on this list are regionally and internationally significant and usually the subject of extensive research. As such, they provide valuable information related to the varying susceptibility of different life stages to viral infection and disease.

Epizootic haematopoietic necrosis virus (EHNV)

EHNV is a member of the genus *Ranavirus* in the family *Iridoviridae*. EHNV is a large icosahedral virus, with a double-stranded DNA genome. A concise review of the characteristics of the virus, affected hosts and life stages is presented by Whittington, et al. (2010). The virus is endemic to south eastern Australia where it naturally infects and causes mortality in wild redfin perch (*Perca fluviatilis*) (Langdon, et al. 1986) and farmed rainbow trout (*O. mykiss*) (Whittington et al., 1999). The virus is known to affect early juvenile (fingerling), juvenile and adult fish of these species (Whittington et al., 2010). Controlled studies have also revealed a number of other species that are vulnerable to the virus. These include: Macquarie perch (*Macquaria australasica*), silver perch (*Bidyanus bidyanus*), mosquito fish (*Gambusia affinis*), mountain galaxias (*Galaxias olidus*), Murray cod (*Maccullochella peeli*), golden perch (*Macquaria ambigua*) and Australian bass (*Macquaria novemaculeata*), while Atlantic salmon (*Salmo salar*) are also susceptible to infection (Whittington et al., 2010).

The (OIE, 2018) lists all life stages of redfin perch and rainbow trout as being vulnerable to EHNV. During natural infections in areas where the virus is endemic, younger redfin perch appear to be more vulnerable to EHNV than older fish. However, it is not clear if this is a consequence of these young fish being naïve to the virus; due to younger fish inhabiting shallower, warmer water that provides a favourable environment for the virus; or due to intrinsic factors that affect the vulnerability of this age class (Whittington et al., 2010). While laboratory exposure trials and observations of natural infections have also observed the virus in adult fish, especially when the virus is first observed in a particular region, evidence of infection in larval fish does not appear to be available. It does not appear that the susceptibility or vulnerability of larval fish to EHNV has been investigated. This inhibits our ability to fully understand the effects of life-stage on the susceptibility of fish to EHNV.

Infectious hematopoietic necrosis virus (IHNV)

IHNV is the type species of the genus *Novirhabdovirus*. It is a negative-sense, single-stranded RNA virus in the family *Rhabdoviridae*. This virus occurs globally and has been observed primarily in salmonid species, although some non-salmonid species are also susceptible to infection and vulnerable to clinical signs and mortality (Bootland & Leong, 2011). Susceptible and vulnerable hosts include: rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), lake trout (*Salvelinus namaycush*), cutthroat trout (*Oncorhynchus clarkii*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), Chinook salmon (*Oncorhyncus tshawytscha*), sockeye/kokanee salmon

(*Oncorhynchus nerka*), chum salmon (*Oncorhynchus keta*), cherry salmon/masou salmon/ Yamame trout (*Oncorhynchus masou*), biwa salmon (*Oncorhynchus masou rhoduris*), amago salmon (*Oncorhynchus rhodurus*), coho salmon *Oncorhynchus kisutch*), Japanese charr (*Salvelinus leucomaenis*), Arctic charr (*Salvelinus alpinus*), Arctic grayling (*Thymallus arcticus*), mountain whitefish (*Prosopium williamsoni*), gilt-head bream (*Sparus aurata*), turbot (*Scophthalmus maximus*), seabass (*Dicentrarchus labrax*), northern pike (*Esox lucius*), pile surfperch (*Rhacochilus vacca*), European eel (*Anguilla anguilla*), herring (*Clupea pallasii*), cod (*Gadus morhua*), sturgeon (*Acipenser transmontanus*), shiner perch (*Cymatogaster aggregata*), tube-snout (*Aulorhynchus flavidus*) and Pacific lamprey (*Entosphenus tridentatus*) (Bootland & Leong, 2011).

Clinical signs and mortality are generally constrained to salmonid species with the relative impact of the disease varying between species. Age also affects vulnerability, with advanced larvae/early juvenile fish being most vulnerable (Bootland & Leong, 2011). In sockeye salmon, IHNV was observed to cause mortality rates of 80-100% in naturally spawned advanced larvae/early juvenile fish (yolk sac fry – two months post hatching). During this epizootic event, the virus was not observed to affect earlier larval stages (Traxler & Rankin, 1989). Mortality then typically reduces to below 50% in fish aged two to six months, while older fish typically experience lower mortality. However, mortality rates exceeding 45% have been observed in farmed Atlantic salmon smolts (>1 year old) (Bootland & Leong, 2011).

Infectious salmon anaemia virus (ISAV) is a member of the Genus *Isavirus* of the Family *Orthomyxoviridae.* Pathogenic and non-pathogenic variants of this virus exist – the pathogenic variant – highly polymorphic region (HPR)-deleted ISAV and the non-pathogenic variant – HPR0 (non-deleted HPR) ISAV. HPR-deleted ISAV is recognised as a lethal disease in Atlantic salmon (*Salmo salar*) (OIE, 2018). The disease may also infect brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), Atlantic herring (*Clupea harengus*), and amago trout (*Oncorhynchus masou*), while the virus has also been detected by PCR in coho salmon (*Oncorhynchus kisutch*) although active replication was not confirmed (Rimstad & Falk, 2011).

The virus has been most frequently observed in the marine stages of farmed Atlantic salmon (one year or older). However, all life stages of fish are known to be vulnerable to ISAV (OIE, 2018; Rimstad et al., 2011). Daily mortality of larger fish (≥ 1 year old) rarely exceeds 0.05-0.1%, although cumulative mortality can reach 90% in heavily stocked net pens if reactive measures are not taken. In first feeding Atlantic salmon larvae, mass mortality related to the virus can occur, with up to 80% of fish dying from the virus within several days (Nylund et al., 1999). The susceptibility and vulnerability of younger larvae is unclear. However, younger larvae housed alongside first feeding larvae experiencing a mortality event were not affected by ISAV until they reached the first feeding stage (Nylund et al., 1999). This may indicate that larval salmon only become vulnerable from the first feeding stage onwards, although this link remains somewhat tenuous.

Salmonid alphavirus (SAV)

SAV is a virus of the genus *Alphavirus,* of the family *Togaviridae.* There are several subtypes of this virus that can affect fish reared in freshwater and seawater (McLoughlin & Graham, 2007). SAV is an enveloped, spherical, single-stranded, positive-sense RNA virus that causes disease in Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta).* RNA from this virus has also been detected in common dab (*Limanda limanda*), long rough dab (*Hippoglossoides platessoides*) and plaice (*Pleuronectes platessa*) (McCleary, et al. 2014; Snow et al., 2010). However, the vulnerability of these species is unclear, with studies on these species primarily

focussing on the potential for these fish to act as carriers of the virus and vectors in its spread (McCleary et al., 2014; Snow et al., 2010).

The OIE (2018) states that all life stages of the hosts should be considered as being vulnerable to the various subtypes of SAV. When disease associated with this virus was first observed in rainbow trout reared in freshwater, it was seen to affect fish of ages ranging from fingerlings up to 1kg. However, it is not clear if larval fish are also vulnerable to the virus (Boucher & Baudin Laurencin, 1994). More recent reviews (e.g. McLoughlin & Graham, 2007) indicate that the virus can affect fish of all ages but direct reports of the virus infecting or affecting larval fish are not presented. In Atlantic salmon, the virus is usually reported in smolt aged fish (>1 year old) in their first year at sea, although McLoughlin and Graham (2007) cites 'unpublished observations' of the virus affecting salmon from all marine stages of the production cycle.

Red sea bream iridoviral disease (RSIVD)

RSIVD is caused by red sea bream iridovirus, a large, icosahedral, cytoplasmic DNA virus of the genus *Megalocytivirus* and the family *Iridoviridae* (Kurita & Nakajima, 2012; OIE, 2018; Plumb & Hanson, 2011; Sano, et al. 2011). A large number of species are known to be susceptible and/or vulnerable to the virus. These include: red sea bream (*Pagrus major*), black porgy (*Acanthopagrus schlegeli*), yellowfin sea bream (*Acanthopagrus latus*), crimson sea bream (*Evynnis japonica*), Japanese amberjack (*Seriola quinqueradiata*), greater amberjack (*Seriola dumerili*), yellowtail amberjack (*Seriola lalandi*), hybrid of yellowtail amberjack and Japanese amberjack (*S. lalandi × S. quinqueradiata*), striped jack (*Pseudocaranx dentex*), northern bluefin tuna (*Thunnus thynnus*), Japanese Spanish mackerel (*Scomberomorus niphonius*), chub mackerel (*Scomber japonicus*), Japanese jack mackerel (*Trachurus japonicus*), Japanese parrotfish (*Oplegnathus fasciatus*), spotted knifejaw (*Oplegnathus punctatus*), cobia (*Rachycentron canadum*), snubnose pompano (*Trachinotus blochii*), chicken grunt (*Parapristipoma trilineatum*), crescent sweetlips (*Plectorhinchus cinctus*), Chinese emperor (*Lethrinus haematopterus*), spangled emperor (*Lethrinus nebulosus*), largescale blackfish (*Girella punctata*), rockfish (*Sebastes schlegeli*), croceine croaker (*Pseudosciaena crocea*), Hong Kong grouper (*Epinephelus akaara*), convict grouper (*Epinephelus septemfasciatus*), Malabar grouper (*Epinephelus malabaricus*), longtooth grouper (*Epinephelus bruneus*), orange-spotted grouper (*Epinephelus coioides*), yellow grouper (*Epinephelus awoara*), greasy grouper (*Epinephelus tauvina*), brown-marbled grouper (*Epinephelus fuscoguttatus*), giant grouper (*Epinephelus lanceolatus*), Japanese sea perch (*Lateolabrax japonicas*), barramundi (*Lates calcarifer*), hybrid of striped sea bass and white bass (*Morone saxatilis × M. chrysops*), largemouth bass (*Micropterus salmoides*), bastard halibut (*Paralichthys olivaceus*), spotted halibut (*Verasper variegatus*), and torafugu (*Takifugu rubripes*) (see: OIE, 2018; Sano et al., 2011).

RSIVD predominantly affects juvenile fish, although market sized fish can also be affected (Kurita & Nakajima, 2012). Cumulative mortality of juvenile (fingerling) red sea bream exposed to RSIV can reach up to 100% of a population, while in market sized red sea bream, mortality can reach 20-60% (Kurita & Nakajima, 2012; OIE, 2018; Sano et al., 2011; Wang, et al. 2003). It has also been observed that cumulative mortality of young juvenile fish (7-10cm) can reach 50-90% during an epizootic event, while older fish (one to two years old) cultured in the same farm can experience no mortality (Wang et al., 2003). The susceptibility and vulnerability of larval fish to RSIV is less clear. A review of the literature failed to identify any studies that explored the relative susceptibility/vulnerability of larval fish to RSIV, and a number of recent reviews of the virus fail to discuss the impacts of RSBIV in larval fish (e.g. Hick, et al. 2016; Kurita & Nakajima, 2012; Sano et al., 2011).

Spring viraemia of carp (SVC)

SVC is caused by spring viraemia of carp virus (SVCV), a species in the genus *Vesiculovirus* in the virus family *Rhabdoviridae.* SVCV is a non-segmented, negative-sense, single stranded RNA virus (LaPatra, et al. 2016; OIE, 2018; M. Sano et al., 2011). Species known to be vulnerable or from which the virus has been identified via cell culture or PCR include: common carp (*Cyprinus carpio carpio*), crucian carp (*Carassius carassius*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*), grass carp (*Ctenopharyngodon idella*), goldfish (*Carassius auratus*), orfe (*Leuciscus idus*), tench (*Tinca tinca*), bream (*Abramis brama*), sheatfish (*Silurus glanis*), pike (*Esox lucius*), roach (*Rutilus rutilus*), zebra fish (*Danio rerio*), guppy (*Lebistes reticulatus*) and golden shiner (*Notemigonus crysoleucas*) (LaPatra et al., 2016; OIE, 2018; Sano et al., 2011).

It is generally accepted that younger common carp are more vulnerable to developing disease clinical signs associated with SVCV than older carp (Ahne et al., 2002; LaPatra et al., 2016). Mortality of younger carp, during spring time outbreaks, can typically reach up to 70% of the population, while in older fish, annual mortality rates are usually below 30% (Ahne et al., 2002). However, higher mortality rates of up to 90% are also reported in young fish (Ashraf et al., 2016), while yearling common carp can experience cumulative mortality of up to 50% (LaPatra et al., 2016). Younger fish (50g) are also more vulnerable than older fish (750g) at high water temperatures (Ahne, 1986; LaPatra et al., 2016). While adult fish are also affected by SVCV, the occurrence of disease clinical signs appears to be affected by age-related innate immune factors, with younger fish being more vulnerable (Ahne, 1986; Ashraf et al., 2016; LaPatra et al., 2016; OIE, 2018). Again, there is a general paucity of literature specific to the susceptibility and vulnerability of larvae of many fish species to SVCV. However, the vulnerability of larval zebrafish has been investigated, revealing an interesting pattern in the effect of age.

Levraud et al. (2007) demonstrated that early larval zebrafish (3 days post fertilisation) were not vulnerable to SVCV when exposed via immersion at 24°C overnight. However, the larvae did become vulnerable by immersion after they had reached a developmental stage in which the mouth and gill slits had opened to allow respiratory function (5.3 days post fertilisation). Younger embryos (3 days post fertilisation) could also be infected by SVCV but only via IV injection. Levraud et al. (2007) suggested that SVCV infects larval zebrafish via the gills and that natural infection cannot occur until the fish have reached this developmental stage. These findings are somewhat contradicted by López-Muñoz, et al. (2010) who observed that early larval zebrafish (3 days post fertilisation) were vulnerable to SVCV when exposed by immersion at 26°C for a period of 24 hours, although vulnerability was somewhat lower in these early larvae than in advanced larvae.

While there is some divergence between the results reported by López-Muñoz et al. (2010) and Levraud et al. (2007), these difference may be a result of differences in the period of exposure (24 hours vs overnight) or the temperature at which the larvae were reared and exposed to the virus (26°C vs 24°C). Both of these factors may have affected the developmental stage at which larvae were ultimately exposed to the virus. Regardless, both studies found that early larval zebrafish were less vulnerable to SVCV than advanced larvae. And despite some incongruence between the two studies, it appears likely that morphological ontogeny can affect the susceptibility of zebrafish to SVCV during early larval development.

Viral haemorrhagic septicaemia (VHS)

VHS is caused by viral haemorrhagic septicaemia virus (VHSV) which is classified in the genus *Novirhabdovirus*, within the family *Rhabdoviridae.* VHSV is a bullet-shaped, negative-sense, single-stranded RNA virus that can affect wild and farmed fish in marine or freshwater. VHSV has been identified in approximately 80 different species of fish from a diverse range of taxa. Families of fish from which the virus has been isolated include: *Ammodytidae, Anguillidae, Anoplopomatidae, Argentinidae, Aulorhynchidae, Carangidae, Catostomidae, Centrarchidae, Clupeidae, Cyprinidae, Embiotocidae, Esocidae, Fundulidae, Gadidae, Gasterosteidae, Gobiidae, Ictaluridae, Liparidae, Lotidae, Merlucciidae, Moronidae, Mugilidae, Ophidiidae, Osmeridae, Paralichthyidae, Percidae, Percopsidae, Petromyzontidae, Pleuronectidae, Salmonidae, Sciaenidae, Scombridae, Scophthalmidae, Scorpaenidae, Scyliorhinidae, Sebastidae, Serranidae, Soleidae, Sparidae, Stromateidae, and Trichiuridae* (OIE, 2018).

VHS is not known to infect fish eggs but may infect all other life stages of susceptible species (OIE, 2018). Younger fish appear to be more vulnerable to the virus (LaPatra et al., 2016), with up to 100% mortality being observed in rainbow trout fry, compared to mortality rates of 30-70% in older fish (Skall, et al. 2005). Smail & Snow (2011) reported that yolk sac fry, fry and fingerlings are more vulnerable to VHSV than older grower fish and that first feeding fry are also vulnerable. The underlying mechanisms driving divergent rates of mortality in these age classes are unclear but in areas in which the virus is endemic, it may be due to younger fish being naïve to the virus and not having developed humoral immunity (Skall et al., 2005). In general, vulnerability to VHS appears to be inversely related to age in fish ranging from yolk sac fry through to adult fish.

Viral encephalopathy and retinopathy (VER)

VER otherwise known as viral nervous necrosis (VNN) is a viral disease that primarily affects larval and early juvenile fish from a range of species. There are four major genotypes of VNN that are classified in the genus *Betanodavirus* within the family *Nodaviridae*. *Betanodaviruses* are non-enveloped, spherical and approximately 25 nm in diameter. The genome consists of two molecules of positive-sense ssRNA (Furusawa, et al. 2007; Munday et al., 2002; OIE, 2018).

VNN has been reported in more than 50 species of fish from a diverse range of taxa. Families known to be affected by VNN include: *Acipenseridae, Anguillidae, Chanidae, Siluridae, Gadidae, Poeciliidae, Acanthuridae, Apogonidae, Anarhichadidae, Carangidae, Centropomatidae, Cichlidae, Eleotridae, Ephippidae, Latridae, Lutjanidae, Malacanthidae, Mugilidae, Oplegnathidae, Percichthydae, Rachicentridae, Sciaenidae, Scombridae, Serranidae, Sparidae, Cichlidae, Monacantidae, Tetraodontidae, Pleuronectidae, Soleidae, Scophthalmidae, Sebastidae, Telmatherinidae, Adrianichthyidae, Anabantidae* and *Osphronemidae* (Furusawa et al., 2007; Munday et al., 2002; OIE, 2018).

VNN tends to affect larval and juvenile fish, from a broad range of species, to a greater extent than it does older fish (Munday et al., 2002). However, VNN is not restricted to the early life stages of fish, with advanced juvenile European sea bass and turbot and adult halibut also being susceptible and vulnerable to the virus (Munday et al., 2002)

The effects of VNN are usually restricted to larval and early juvenile fish in which mass mortality, of up to 100%, is often associated with the occurrence of this virus (Munday et al., 2002). However, different species appear to become vulnerable at different ages. For example, the disease has been observed as early as one day post hatching in striped jack (*Pseudocaranx dentex*), while Atlantic halibut (*Hippoglossus hippoglossus*) larvae are typically not affected by the virus until 60-70 days

post hatching (Grotmol, et al. 1999; Munday et al., 2002). It has also been demonstrated that when the virus does occur in younger Atlantic halibut larvae, fish less than 14 days old are less vulnerable to VNN when exposed by bath exposure compared to fish more than 14 days old.

It is possible that the age at which larval fish become susceptible to VNN is related to morphological ontogeny such as the timing of development of the mouth parts and digestive tract. The intestinal epithelium has been implicated as a potential portal of entry for VNN (Grotmol et al., 1999). However, the potential for the virus to access this site may be affected by morphological ontogeny of larval fish, especially the opening of the mouth and the development of the digestive tract. There is evidence that this may be the case for the differences observed between Atlantic halibut and striped jack. In these two species, the timing of development of the mouthparts, and first feeding, is highly divergent. The time of first feeding in Atlantic halibut is temperature dependent but can occur between 44 and 59 days post hatching (Lein & Holmefjord, 1992). In contrast, first feeding of striped jack can occur as little as two days post hatching (Masuda, 2009). These differences in the time of first feeding are reflected in the age at which larvae of these species typically become vulnerable to VNN. In Atlantic halibut, this usually occurs at 60-70 days post hatching while striped jack larvae are typically vulnerable from one to fourdays post hatching (Munday et al., 2002).

While differences in the onset of vulnerability to VNN are apparent during the larval stage of different species, in general, vulnerability to VNN declines from the early juvenile stage onwards (Munday et al., 2002; OIE, 2018). Mortality rates are also generally highest in larval and early juvenile fish compared to advanced juvenile and adult fish (Munday et al., 2002; OIE, 2018).

6.2.2. Alloherpesviruses of fish

CyHV-3 is a virus of the family *Alloherpesviridae*. *Alloherpesviridae* is the family of *herpesviruses* that affects fish and amphibians. There are 15 viruses in this family that affect a range of different species of fish. Concise information on these viruses can be found in *Chapter 9 Alloherpesviruses of Fish* in *Aquaculture Virology,* which provides an excellent review of the alloherpesviruses of fish (Hanson, et al. 2016). Hanson, et al. (2016) present details of taxonomy, viral structure, epidemiology, clinical signs, diagnostic tests, and susceptible/vulnerable species.

In general, herpesviruses are characterised as linear, double-stranded DNA viruses with an icosahedral nucleocapsid that is surrounded by a proteinaceous tegument layer and a host-derived envelope. Genetic sequences are not conserved between the families, but there is structural and biological conservation amongst all families of herpesviruses (Hanson, et al., 2016).

Alloherpesviruses usually affect a relatively narrow range of host species, although they may infect a number of additional species subclinically (Bergmann et al., 2016). The viruses are often detected in a broad range of species, although the evidence for active infection in these species can be inconclusive (Bergmann et al., 2016).

The alloherpesviruses are often categorised based on the species in which they were first identified or the species in which they are typically associated.

<u>Ictalurid herpesvirus</u>

There are two alloherpesviruses that occur primarily in catfish. *Ictalurid herpesviru*s 1 (IcHV-1) and *Ictalurid herpesvirus 2* (IcHV-2)

IcHV-1 primarily affects channel catfish, in which it can cause significant mortality. The vast majority of IcHV-1 cases occur in young channel catfish fingerlings and in juveniles less than 1 year old,

although adult fish can also be affected (Hanson, et al., 2016; Hedrick, et al., 1987). Vulnerability to Channel catfish virus disease (CCVD) is traditionally thought to be inversely related to age and this is generally the case for post-larval fish. However, during larval development, the opposite appears to be true with early larvae being less vulnerable than advanced larvae (Hanson, et al., 2004).

Hanson, et al. (2004) exposed channel catfish to IcHV-1 at different ages ranging from three to 60 days post hatching. Vulnerability was measured by the rate of mortality and the total cumulative mortality observed. The vulnerability of three day old larvae was very low compared to eight day old larvae and cumulative mortality continued to increase as the larvae got older, up to the oldest fish assessed, 60 days post hatching. The relative vulnerability of early larvae appeared to be related to maternal immunity, with the progeny of IcHV-1 exposed fish being less vulnerable. However, all larvae were less vulnerable during early development than in later developmental stages, indicating that factors other than maternal immunity, may also affect the vulnerability of larval channel catfish fish to CCVD (Hanson, et al., 2004).

The vulnerability of early larval channel catfish to IcHV-1 may be affected by a range of factors. Many of these are summarised by Hanson, et al., (2004), although evidence of their relative importance is not provided. Factors raised by Hanson, et al. (2004) include: potentially heightened innate immune function in younger larval fish; the presence of a more effective physical barrier to viral entry (e.g. epithelial and periodontal tissue and mucus); a lack specific virus receptors at the portal of entry; or differences in the basic kinetics of exposure that may be skewed in favour of the larvae due to surface area and water flow factors. These factors may affect the potential for the virus to gain entry into the host, they may limit the ability of the virus to replicate after infection or they may influence the progress of viral pathogenesis. Such factors may also affect viral shedding which may influence the rate of transmission to subsequent hosts (Hanson, et al., 2004).

Morphological features and ontogeny may also affect the ability of IcHV-1 to infect larval channel catfish. Nusbaum and Grizzle (1987) observed the gills to be the major portal of entry of IcHV-1 into channel catfish. The invulnerability of early larval channel catfish to IcHV-1 may be related to the incomplete development of the respiratory system in early larvae. If the mouth parts and gills are closed, it might inhibit the potential for IcHV-1 to access the gills and gain entry into the host. This is speculative but is consistent with the observation that early larval zebrafish that are less vulnerable to SVCV until after the gills have formed (Levraud et al., 2007).

IcHV-2 is the second alloherpesvirus to have been identified in *Ictalurid* fish (Borzák, et al., 2018). The virus primarily affects adult black bullhead catfish. However the virus can also affect channel catfish (Hedrick, et al.,2003). Ronald P Hedrick et al. (2003) observed that channel catfish fry with an average weight of 0.02 – 0.03 g (the age and precise developmental stage of these fish it is not specified) and juvenile channel catfish (average weight 15.8 g) were highly vulnerable to IcHV-2. Clearly, adult black bullhead catfish and early juvenile channel catfish are vulnerable and susceptible to the virus, however, the vulnerability of early larval fish to IcHV-2 is unknown.

Anguillid herpesvirus 1 (AngHV-1)

AngHV-1 is a herpesvirus of the genus *Cyprinivirus* that affects eels. AngHV-1 infection has been confirmed in Japanese (*Anguilla japonica*) and European eels (*Anguilla Anguilla*) and has been identified by PCR in the American eel (*Anguilla rostrate*) (Kempterl, et al., 2014; Sano, et al.,1990; van Beurden et al., 2012). It is generally thought that AngHV-1 affects eels of all ages (Hanson, et al.,

2016). Glass eels and juveniles eels are highly vulnerable and can experience high mortality rates (van Beurden et al., 2012). The susceptibility and vulnerability of larval eels is unclear.

A herpes-like virus was also isolated from diseased Japanese eels and tentatively designated as eel herpesvirus in Formosa (EHVF) (Ueno, et al., 1992). When carp were experimentally exposed to this virus, high mortalities were observed (Ueno et al., 1992). However, this observation has not been repeated by others and relatively little is known of this virus (Hanson, et al., 2016).

Acipenserid herpesvirus

Alloherpesviruses are also known to affect sturgeon. Two herpes virus have been identified in sturgeon: acipenserid herpesvirus 1 (AciHV-1); and acipenserid herpesvirus 2 (AciHV-2) (Hanson, et al., 2016). AciHV-1 has been observed less frequently than AciHV-2 but it has been shown to cause significant mortality in experimentally infected juvenile white sturgeons (*Acipenser transmontanus*) (Hedrick, et al.,1991). The susceptibility and vulnerability of adult and larval fish to AciHV-1 is unclear.

AciHV-2 affects a wider range of species. The virus has either been detected in or has been observed to affect white sturgeon, shortnose sturgeon (*Acipenser brevirostrum*), lake sturgeon (*Acipenser fulvescens*), Siberian sturgeon (*Acipenser baeri*) and hybrids of sterlet (*Acipenser ruthenus*) and beluga (*Huso huso*) (Hanson, et al., 2016).

AciHV-2 is more pathogenic to early juvenile (two month old) Siberian sturgeon than to 2 year old fish (Shchelkunov et al., 2009). Shchelkunov et al. (2009) observed 100% mortality when 2 month old fish were exposed to the virus compared to 36% in two year old fish. Despite this much lower rate of mortality, 90% of two year old fish became moribund after exposure to the virus. Later juvenile (five month old) white sturgeon are also highly vulnerable, and have been observed to suffer up to 80% mortality in controlled exposure trials (Watson, et al., 1995). Natural infections have also been observed in sub adult and adult fish (summarised by: Hanson, et al., 2016), although mortality rates are typically much lower in older fish compared to younger juveniles. The susceptibility and vulnerability of larval fish to AciHV-2 appears to be unexplored.

Salmonid herpesviruses

Salmonids are also affected by herpesviruses. Four phylogenetically related herpesviruses have been characterised and are labelled Salmonid herpesviruses 1 – 4 (SalHV-1 – 4).

The first of these, SalHV-1 was identified in rainbow trout in 1975 and designated as a herpesvirus by Wolf, Darlington, Taylor, Quimby, and Nagabayashi (1978). The virus has been isolated from adult rainbow trout and has been observed to cause clinical disease and mortality of fry stage rainbow trout (2.5-4 months old) (Wolf et al., 1978; Wolf & Smith, 1981). SalHV-1 is not considered to be a major threat to wild or cultured salmonids and has not been the subject to extensive study. As a consequence, there is limited knowledge on host range and the relative susceptibility and vulnerability of different life stages of fish (Hanson, et al., 2016).

SalHV-2, labelled *Oncorhynchus masou* virus by the (OIE, 2018) is the aetiological agent responsible for *Oncorhynchus masou* virus disease. Species known to be susceptible to SalHV-2 include: masu salmon (*O. masou*), coho salmon (*Oncorhynchus kisutch*), kokanee salmon (*Oncorhynchus nerka*), rainbow trout (*O. mykiss*), and Chum salmon (*Oncorhynchus keta*) (Hanson, et al., 2016).

While adult fish can be infected with SalHV-2, larval and juvenile fish are more vulnerable to clinical signs and mortality (Kimura, et al.,1983). Kimura et al. (1983) investigated the vulnerability of kokanee salmon, chum salmon and coho salmon when exposed to SalHV-2 immediately after hatching, and at one month old. Using mortality as a measure, it was shown that one month old alevins of different species of salmonid had varying levels of vulnerability to SalHV-2. The most vulnerable species was kokanee salmon, which experienced 100% cumulative mortality within ~27 days post exposure, while 83% of chum salmon and 39% of coho salmon succumbed to the virus during 110 days post exposure. Cumulative mortality of newly hatched larvae was greatly reduced in kokanee salmon (~59%), chum salmon (35%) and coho salmon (~12%). In all cases, newly hatched larvae were far less vulnerable to the virus and suffered much reduced levels of mortality compared to 1 month old fish.

The vulnerability of chum salmon and masu salmon was also investigated when fish were exposed to the SalHV-2 at ages of up to seven months old (Kimura et al., 1983). When newly hatched chum salmon were exposed to the virus, cumulative mortality reached 35%. This increased to 83 % in fish exposed to the virus at one month old and to 98% when fish were exposed to the virus at three months of age. As the age at which fish were exposed to the virus increased beyond this point, cumulative mortality then declined, such that six month old fish only experienced seven percent mortality and only two percent of fish died when exposed to virus at seven months of age.

In masu salmon, cumulative mortality was highest when fish were exposed to the virus at 1 month old with 87% of fish dying after exposure. Cumulative mortality then decreased when masu salmon were exposed to the virus at three and five months of age, with 65% and 24% of fish dying in these groups respectively (Kimura et al., 1983).

Differences in the degree of vulnerability to SalHV-2 have been observed: in different species of susceptible fish; in different age groups of fish of a single species; and in different age groups across different species. This highlights the potential for widely divergent patterns of susceptibility and vulnerability to a single virus that can occur across different species of fish.

SalHV-3 is a herpes virus that causes serious disease in lake trout and has also been detected in cisco (*Coregonus artedi*) (Glenney, Barbash, & Coll, 2016). In lake trout, SalHV-3 has only been documented in aquaculture facilities (L. Hanson, Doszpoly, van Beurden, de Oliveira Viadanna, et al., 2016). SalHV-3 primarily affects lake trout up to one year old and can establish a carrier state in adult fish, where it can be detected by PCR (Bradley, et al.,1989; Glenney et al., 2016; Kurobe, et al.,2009). Using mortality as a measure of vulnerability, Bradley et al. (1989) observed that the fry (age unknown) of lake trout were more vulnerable to SalHV-3 than juvenile fish (10-13cm in length, age unknown). Mortality of fry could reach nearly 100%, while in juveniles, mortality rates were 27% to 30.5 %.

SalHV-4 is a typically benign herpesvirus of Atlantic salmon that has occasionally been implicated in significant disease and mortality events, primarily in cultured fish (Bylund, et al., 1980; Doszpoly,et al., 2013; Hanson, et al., 2016). The virus typically affects one and two year old Atlantic salmon, especially during smoltification, although it has also been observed to effect Atlantic salmon in the freshwater stages of culture (<1 year old) (Bylund et al., 1980; Doszpoly et al., 2013). The susceptibility and vulnerability of larval fish to SalHV-4 is unclear.

An additional herpesvirus has recently been identified in salmonids and is tentatively designated as salmonid herpesvirus-5 SalHV-5 (Glenney et al., 2016), although little is currently known of this virus, its host range, or the susceptibility and vulnerability of different aged of fish.

6.2.3. Cyprinivirus

Alloherpesviruses have also been observed in cyprinids, the family of fish that contains common carp. There are three herpesviruses that primarily affect cyprinids – Cyprinid herpesvirus 1, 2 and 3 (CyHV-1, 2 and 3). CyHV-1 and 2 are closely related to CyHV-3 in terms of sequence and genome structure, as is the eel herpesvirus AngHV-1 (Davison et al., 2013; Waltzek et al., 2005). The C*yprinivirus*es, as they have been labelled, typically only affect a narrow range of hosts, although the viruses may be detected in a broader range of host species by various direct detection methods (Bergmann et al., 2016; Hanson, et al., 2016). A brief review of the ability of CyHV-1, 2 and 3 to infect and affect different life stages of fish will be presented below.

CyHV-1, previously designated as *Herpesvirus cyprini,* and also known as carp pox, is a disease that causes mortality and papillomas in common carp. The disease has been observed in common carp for centuries but the virus was first identified and described during the mid 1980s (Sano, et al.,1985). TSano, et al., (1991) investigated the vulnerability of common carp, grass carp, crucian carp and willow shiner (*Gttathopogon elongatus caertdescens*) to CyHV-1. Clinical signs and mortality were only observed in common carp. Furthermore, the virus was reisolated from common carp but not from any of the other fish species assessed. Direct detection methods were not available at the time of the study.

Sano et al. (1991) assessed the vulnerability of larval and early juvenile common carp to CyHV-1. When mortality was used as a measure of vulnerability, 2 week old fish were more vulnerable to the virus than four or eight week old fish. In two week old fish, cumulative mortality reached 86-97% compared to 20% in four week old fish and 0% in eight week old fish. Fish younger than two weeks old were not assessed. It appears that vulnerability to CyHV-1 is inversely related to age, at least for advanced larval and juvenile common carp.

Unlike CyHV-1 that appears restricted to common carp, CyHV-2 is known to affect at least two species of cyprinids – gold fish and Prussian carp – and has been detected by TEM in diseased crucian carp (Fichi et al., 2013; Hedrick, et al.,2006). Because of this, it has been suggested that CyHV-2 should be considered to be a multi-host pathogen (Becker et al., 2014). Hybrids of common carp and goldfish have also been assessed for susceptibility and vulnerability to CyHV-2. When these fish were deliberately exposed to CyHV-2 no fish died, but viral DNA was detectable in fish 25 days after exposure.

Jung and Miyazaki (1995) provide the first description of CyHV-2, which was observed in goldfish up to one year old (5-15 g). The fish appeared listless and epizootics had been previously reported in which mortality had reached nearly 100% (Jung & Miyazaki, 1995). Since then, CyHV-2 has also been reported in adult Prussian carp during an epizootic that killed the vast majority of Prussian carp in a lake in the upper Elbe Basin in the Czech Republic (Danek et al., 2012).

Typically, CyHV-2 is reported in juvenile or adult goldfish greater than two months old. It is not clear if age affects vulnerability to the virus. It has been observed that mortality in affected populations of goldfish can reach 50-100% in 2 month old fish (Groff, et al.,1998). However, in a population of older goldfish, weighing ~5 g, exposed to the virus, only 1 of 14 goldfish died (Stephens, et al., 2004). Jeffery et al. (2007) examined an incidence of CyHV-2 in first year goldfish ranging from 10 – 20g in

weight. Although a detailed age effect is not presented, the authors report that mortalities were first reported in smaller fish (70 mm) and were later observed in fish of all sizes (70-100 mm). Cumulative mortality rates were observed to be highest in smaller fish (Jeffery et al., 2007). The susceptibility and vulnerability of early larval fish to CyHV-2 is currently unclear.

6.2.4. Current knowledge of CyHV-3 infection dynamics in larval, juvenile and adult carp

Common carp are highly susceptible and vulnerable to CyHV-3 (e.g. Boutier et al., 2015; R. P. Hedrick et al., 2000; OIE, 2017b). While the virus has been detected in a range of other species, and mild clinical affects have been observed in goldfish (Bergmann et al., 2010), no other species appear to be seriously affected by CyHV-3. However, hybrids of common carp × goldfish (*Carassius auratus*) and common carp × crucian carp (*Carassius carassius*) are also susceptible to CyHV-3 infection and vulnerable to the clinical effects of the virus (Bergmann, et al., 2010; R. P. Hedrick et al., 2006).

It also appears that a number of other fish species are not resistant to CyHV-3 infection, albeit subclinically. The presence of CyHV-3 has been detected in a range of non-common carp species including: goldfish (*C. auratus*); grass carp (*Ctenopharyngodon idella*); ide (*Leuciscus idus*); ornamental catfish (*Ancistrus sp.*); Russian sturgeon (*Acipenser gueldenstaedtii*); Atlantic sturgeon (*A. oxyrinchus*); stone loach (*Barbatula barbatula*); hybrids between sterlet (*Acipenser ruthenus*) and beluga (*Huso huso*); and topmouth gudgeon (*Pseudorasbora parva*) (Bergmann et al., 2009; Ilouze et al., 2011; Kempter et al., 2009; Pospichal et al., 2016; Pospichal et al., 2018). And although the authors raise the possibility of sample contamination, CyHV-3 DNA was also detected in a range of endemic and introduced species in Australian waters (McColl et al., 2017). In a study undertaken by McColl et al. (2017), CyHV-3 DNA was most frequently identified in trials that assessed silver perch (*Bidyanus bidyanus*), Murray cod (*Maccullochella peeli*), golden perch (*Macquaria ambigua)* and bony bream (*Nematalosa erebi*). In all of these species, no clinical signs consistent with a viral infection were observed, although mortality of silver perch exposed to the virus exceeded that observed in negative controls (McColl et al., 2017).

The vulnerability of various cell lines in cell culture to CyHV-3 has also been assessed (e.g. Davidovich, et al.,2007; Wang et al., 2018). Davidovich et al. (2007) observed that cell lines derived from species other than common carp were able to replicate CyHV-3 and that CPE could be elucidated. Cell lines derived from silver carp and goldfish were vulnerable to CyHV-3, indicating the presence of viral receptors in these species, at least at a cellular level (Davidovich et al., 2007).

It also appears that several species of fish (e.g. goldfish, tench, vimba, common bream, common roach, European perch, ruffe, gudgeon, rudd, northern pike, Prussian carp, silver carp, ide, and grass carp) can act as a reservoir of CyHV-3 and transmit the virus to naïve common carp (Bergmann, et al., 2010; Mansour El-Matbouli & Soliman, 2011; Fabian et al., 2016; Fabian et al., 2013; Kempter et al., 2012; Radosavljević et al., 2012).

The virus also appears to be capable of persisting in some of these species for an extended period of time. In goldfish, the virus appears to be able to persist for up to 60 days, after which the virus can be transmitted to naïve common carp, and it may potentially persist for up to one year, although the potential for transmission after this time is unknown (Bergmann et al., 2010; Haenen & Hedrick, 2006). This suggests that the virus can replicate in the host, as was demonstrated by Mansour El-Matbouli and Soliman (2011). Mansour El-Matbouli and Soliman (2011) observed positive RT-PCR results from goldfish exposed to CyHV-3, although these results have subsequently been disputed as the RT-PCR applied by Mansour El-Matbouli and Soliman (2011) could potentially have detected

genomic DNA rather than mRNA associated with viral replication (McColl et al., 2017; Yuasa et al., 2012).

CyHV-3 has been detected in a wide range of cyprinid and non-cyprinid species of fish. However, it only appears to seriously affect common carp (e.g. Boutier et al., 2015; Hedrick et al., 2000; OIE, 2017b), with mild clinical signs also reported in goldfish, crucian carp and common roach (Bergmann et al., 2010; Sadler et al, 2007; Gotesman et al, 2013;). The virus clearly infects and affects juvenile and adult common carp. Smaller common carp are generally more vulnerable to clinical clinical signs associated with CyHV-3 although under certain conditions, larger fish can also be significantly affected (Perelberg et al., 2003; Sano et al., 2004).

Early larval common carp are less vulnerable to CyHV-3 than advanced larvae (Ito et al., 2007; Ronsmans et al., 2014). Takafumi Ito et al. (2007) observed that one, three and four day old larval carp were not vulnerable to CyHV-3 when exposed by immersion. Very low levels of mortality were observed in these larval fish and CyHV-3 was not detected in mortalities or surviving fish by Gilad c-PCR. However, when 13 day, 18 day and two month old fish were exposed to CyHV-3 by immersion, mortality of up to 100% was observed. By contrast, Ronsmans et al. (2014) observed that larval carp are susceptible to infection with CyHV-3, and the virus can replicate in the host at ages ranging from 0 – 35 days post hatching. However, vulnerability, as measured by mortality, was much lower in early larval fish (0 – 10 days post hatch) than in early (21 – 28 days post hatching) and late juvenile fish (35 days post hatching).

There are a number of possible explanations for the lower susceptibility and vulnerability of larval common carp to CyHV-3: larval fish may be protected by transient maternal antibodies; essential host factors for infection (such as cell surface receptors or aspects of the internal cellular environment) may be absent in larval carp, as postulated by Ito et al. (2007); morphological development may prevent the virus from accessing the portal of entry; or, innate immune mechanisms, such as those found in epidermal mucus, may be advantageously expressed in larval but not juvenile or adult fish.

Maternal immunity may also provide a protective effect to CyHV-3 for larval common carp. Maternal immunity can be passively conferred to larval fish through the transfer of maternal antibodies (). However, this does not appear to have played a role in the low susceptibility and vulnerability of larval common carp to CyHV-3 observed by Ronsmans et al. (2014). The broodstock used by Ronsmans et al. (2014) were proved to be free from CyHV-3 prior to being used in the study. Thus, maternal immunity does not appear to have played a role in reduced susceptibility and vulnerability of larval carp to CyHV-3 observed in this study.

The absence of essential host factors for infection also doesn't appear to be the causative for reduced susceptibility and vulnerability of larval common carp to CyHV-3. Based on the observation of Ronsmans et al. (2014), it appears unlikely that larval common carp lacked viral receptors for CyHV-3, at least at the cellular level. Or that the internal environment of the cells was unsuitable for larval replication. When larval and juvenile fish were exposed to the virus via IP injection, the virus was able to infect and replicate in nearly all fish, independent of the age at which they were inoculated. Furthermore, when the epidermal mucus was removed, there was an increase in the rate of infection. Thus, the reduced susceptibility and vulnerability of larval carp to CyHV-3 does not appear to be due to the absence of viral receptors at a cellular level.

Morphological development may play a role in the reduced susceptibility and vulnerability of larval common carp to CyHV-3. Ito et al. (2007) observed that when early larval common carp (one, three and four days post hatching) were exposed to CyHV-3, mortality was extremely low and the virus could not be detected by Gilad c-PCR in the small number of mortalities that occurred. However, when the same fish were exposed to the virus as juveniles (13 days, 18 days and two months post hatching) almost all of the fish died and the virus was detected by Gilad c-PCR in the majority of dead fish.

The low susceptibility and vulnerability of the early larval fish assessed by Ito et al. (2007) (one to four days post hatching) may be related to the morphology of these early larval fish. In early larvae (0 – 5 days post hatching), delayed development of the digestive tract and respiratory system may inhibit viral entry. The periodontal mucosa (Fournier et al., 2012) and the skin (Costes et al., 2009; Raj et al., 2011b) have been implicated as potential portals of entry for CyHV-3. The mouth parts are not well developed in early larval carp (≤5 days post hatching) (Huttenhuis et al., 2006). As a result, CyHV-3 may be unable to infect early larval carp as the periodontal is not accessible to facilitate viral entry. A similar hypothesis has been postulated for zebrafish exposed to SVCV (Levraud et al., 2007). The impact of larval morphology on susceptibility and vulnerability to CyHV-3 is speculative but it appears likely that a complex range of interconnected factors affect the varying susceptibility and vulnerability of different life stages of common carp to CyHV-3.

While Ito et al. (2007) observed very low mortality of early larval carp after exposure to CyHV-3, Maygane Ronsmans et al. (2014) observed moderate to high rates of mortality when carp were exposed to the virus in the early stages of development (0-10 days post hatching). It is unclear why these results contradict those of Ito et al. (2007). However, it may be that CyHV-3 is able to take advantage of multiple portals of entry including the pharyngeal periodontal mucosa, the epidermal mucosa and the gills. It is plausible that those fish assessed by Takafumi Ito et al. (2007) may have achieved a greater level of protection from the epidermal mucosa (for unknown reasons) than those assessed by Ronsmans et al. (2014). In this scenario, the larvae assessed by Ito et al. (2007) may have resisted infection through the epidermal mucosa while the pharyngeal periodontal mucosa and gills were unable to be accessed by the virus as they had not yet developed. The contradictory findings presented by Takafumi Ito et al. (2007) and Ronsmans et al. (2014) highlight the limitations of our current knowledge on the infection dynamics of CyHV-3 in fish of varying ages.

Regardless of the causative factors for observed differences in the susceptibility and vulnerability of larval common carp to CyHV-3, there is strong evidence that innate immune mechanisms play a role in limiting the susceptibility and vulnerability of larval common carp to CyHV-3. Innate immune factors in the epidermal mucus appear to be a major inhibitor of CyHV-3 entry into larval, as well as juvenile and adult carp (Raj et al., 2011b; Ronsmans et al., 2014). The epidermal mucus appears to act as an innate immune barrier against CyHV-3 entry into common carp (Raj et al., 2011b; Ronsmans et al., 2014). Ronsmans et al. (2014) observed that seven day old carp are more likely to be infected if the skin mucus is removed prior to exposure by immersion. Likewise, when the epidermal mucus is intact, carp larvae appear to be more susceptible and vulnerable when exposed to the virus by IP injection than by immersion (Ronsmans et al., 2014). The importance of epidermal mucus in inhibiting the entry of CyHV-3 has also been observed in topmouth gudgeon (Pospichal et al., 2018).

However, in the earlier developmental stages of common carp, the protection provided by the epidermal mucus appears to be much more effective than in juvenile or adult fish (Raj et al., 2011b; Ronsmans et al., 2014). Ronsmans et al. (2014) put forward two hypotheses for this greater

protection. The first hypothesis is that the epidermal mucus layer of larval carp forms a more uniform mechanical barrier due to the composition of the mucus, the hydrodynamic properties of the fish, or due to reduced physical interactions between the larval fish and their environment. The second hypothesis is that the mucus produced by larval carp contains a greater level of antiviral agents capable of disabling CyHV-3. No evidence is provided to support either of these hypotheses and the practical difficulties of exploring such hypotheses are acknowledged.

As the knowledge of CyHV-3 grows, a complex pattern in the susceptibility and vulnerability of common carp to CyHV-3 appears to be emerging. In general, it can be said that all stages of common carp are susceptible to infection and vulnerable to the clinical effects of CyHV-3 (OIE, 2017b; Perelberg et al., 2003; Ronsmans et al., 2014; Sano et al., 2004). However, infection and clinical effects are reduced in larval fish, with the epidermal mucosa appearing to play a critical role in preventing viral entry (Ito et al., 2007; Ronsmans et al., 2014). Morphological ontogeny may also reduce the susceptibility of larval fish. Clinical signs and mortality are usually most severe in advanced larvae and young juvenile fish, less pronounced in later juvenile and adult fish and much reduced in early larval common carp.

While the effect of life stage on susceptibility and vulnerability to CyHV-3 has been investigated in common carp, the susceptibility and vulnerability of other species to CyHV-3 infection has only been investigated in advanced juvenile and adult fish. This is unsurprising, given that the vast majority of susceptibility/resistance studies identified in this review have been performed on juvenile and adult fish. However, as CyHV-3 DNA has been detected in a wide range of host organisms, it would be interesting to undertake additional research to determine if a pattern of susceptibility similar to that observed in common carp also exists in other fish species. In the context of the NCCP, this is a critical consideration as it is currently unclear if NTS are resistant to CyHV-3 infection, or the effects of this virus, at all life stages.

6.2.5. General patterns in the effect of life stage on vulnerability of fish to viruses

It is clear that life stage can affect the vulnerability of fish to viruses. Different viruses can affect fish of different ages to varying degrees. While there is great variability in the vulnerability of fish of different ages to different viral pathogens, some general patterns do appear to exist.

Of the 20 viruses discussed above, the majority appear to have the greatest impact on advanced larval or early juvenile fish. Vulnerability then declines as the age of fish exposed to the virus increases. This appears to be the case for:

- EHNV – Although all life stages of redfin perch are vulnerable to EHNV, in areas where the virus is endemic, it appears to affect younger redfin perch more than it does older fish, although the mechanism behind this difference is unclear (Whittington et al., 2010).
- IHNV – Advanced larval and early juvenile fish are more vulnerable to IHNV than older fish. In salmonids, IHNV can cause mortality rates of 80-100% in advanced larvae/early juvenile fish (yolk sac fry – two months post hatching) (Traxler & Rankin, 1989), with mortality rates reducing to below 50% in fish aged two to six months, while older fish typically experience lower mortality (Bootland & Leong, 2011).
- ISAV – All life stages of susceptible fish are known to be vulnerable to ISAV (OIE, 2018; Rimstad et al., 2011). However, younger fish are more vulnerable, with cumulative mortality of up to 80% caused by the virus, within a matter of days, in first feeding Atlantic salmon larvae (Nylund et al., 1999), while daily mortality of larger fish (≥ 1 year old) rarely exceeds 0.05-0.1% of the population

- RSIV – Cumulative mortality of juvenile (fingerling) red sea bream exposed to RSIV can reach up to 100% of a population, while in market sized red sea bream, mortality generally doesn't exceed 20-60% (Kurita & Nakajima, 2012; OIE, 2018; Sano et al., 2011; Wang et al., 2003). In farmed fish in Taiwan, it was also observed that cumulative mortality of young juvenile fish (7-10cm) reached up to 90% of the population, while older fish (one to twoyears old) cultured in the same farm remained free from mortality (Wang et al., 2003).
- SVCV – Mortality of younger carp exposed to SVCV during spring time outbreaks can typically reach up to 70% of the population, while in older fish, annual mortality rates are usually below 30% (W Ahne et al., 2002; LaPatra et al., 2016).
- VHSV – Younger fish appear to be more vulnerable to VHSV (LaPatra et al., 2016), with up to 100% mortality being observed in rainbow trout fry, compared to mortality rates of 30-70% in older fish (Skall et al., 2005). Smail and Snow (2011) report that yolk sac fry, first feeding fry and fingerlings are more vulnerable to VHSV than older grower fish.
- VNN – VNN tends to affect larval and juvenile fish, from a broad range of species, to a greater extent than it does older fish (Munday et al., 2002). However, VNN is not restricted to the early life stages of fish, with advanced juvenile European sea bass and turbot and adult halibut also being susceptible and vulnerable to the virus (Munday et al., 2002).
- IcHV-1 – The vast majority of IcHV-1 cases occur in young channel catfish fingerlings and in juveniles less than 1 year old (Hanson, et al., 2016; Hedrick et al., 1987). Vulnerability to CCVD is considered to be inversely related to age, although the reverse may be true in early larval fish (Hanson, et al., 2004).
- AciHV-1 – The relative vulnerability of different life stages of fish to AciHV-1 is poorly explored. However, AciHV-1 has been shown to cause significant mortality in experimentally infected juvenile white sturgeons (*Acipenser transmontanus*) (R. Hedrick et al., 1991).
- AciHV-2 – Early juvenile sturgeon are more vulnerable to AciHV-2 than older fish (Shchelkunov et al., 2009). Mortality rates of up to 100% have been observed in two month old Siberian sturgeon; up to 80% in 5 month old white sturgeon; and up to 36% in two year old Siberian sturgeon (Shchelkunov et al., 2009; Watson et al., 1995).
- SalHV-1 – SalHV-1 is not considered to be a major threat to wild or cultured salmonids and there is limited knowledge on host range and the relative susceptibility and vulnerability of different life stages of fish (Hanson, et al., 2016). However, SalHV-1 has been observed to cause more severe clinical disease and mortality in fry stage rainbow trout (2.5 – 4 months old) compared to adult rainbow trout (Wolf et al., 1978; Wolf & Smith, 1981).
- SalHV-2 – While adult fish can be infected with SalHV-2, larval and juvenile fish are more vulnerable to clinical signs and mortality. Newly hatched larvae of kokanee, chum and coho salmon are less vulnerable than one month old fish. For chum salmon, vulnerability is highest when fish are exposed at three months of age, and in masu salmon, when fish are exposed at one month old. The vulnerability of chum and masu salmon then declines when older fish are exposed to the virus (Kimura et al., 1983).
- SalHV-3 – SalHV-3 primarily affects lake trout up to one year old and can establish a carrier state in adult fish, where it can be detected by PCR (Bradley et al., 1989; Glenney et al., 2016; Kurobe et al., 2009). Using mortality as a measure of vulnerability, Bradley et al. (1989) observed that the fry (age unknown) of lake trout were more vulnerable to SalHV-3 than juvenile fish (10-13cm in length, age unknown). Mortality of fry could reach nearly 100%, while in juveniles, observed mortality rates were 27% to 30.5 %.

- CyHV-1 – Vulnerability of common carp to CyHV-1 decreases with increasing age. Larval common carp (two weeks old) are more vulnerable to CyHV-1 than later larvae/juvenile fish (four or eight week old). In two week old fish, cumulative mortality can reach 86-97% compared to 20% in four week old fish and 0% in eight week old fish (Sano et al., 1991).
- CyHV-3 – All life stages of common carp are susceptible to infection with CyHV-3 (OIE, 2017b; Perelberg et al., 2003; Ronsmans et al., 2014; Sano et al., 2004). However, clinical signs and mortality are usually most severe in young juvenile fish, less pronounced in later juvenile and adult fish and much reduced in early larvae of common carp. In early larval fish, the epidermal mucosa appears to play a critical role in preventing viral entry (Takafumi Ito et al., 2007; Maygane Ronsmans et al., 2014), while morphological ontogeny may also reduce the susceptibility of early larval fish.

For only three of the 20 viruses discussed in this review, are juvenile and adult fish considered to have equivalent vulnerability to the effects of the virus: SAV (McLoughlin & Graham, 2007), IcHV-2 (Borzák et al., 2018; Hedrick et al., 2003) and SalHV-4 (Bylund et al., 1980; Doszpoly et al., 2013).

For AngHV-1 and CyHV-2, there is limited information on which to base a presumption of the effect of fish age on the vulnerability of the host. However, for both of these viruses, there is some limited evidence that younger fish are more vulnerable (Groff et al., 1998; Jeffery et al., 2007; van Beurden et al., 2012).

The vulnerability of early larval fish is less clear. For ten of the viruses reviewed, there is insufficient information available to determine the relative vulnerability of larval fish. It is unclear if this relates to the limited capacity of these viruses to infect/affect larval fish and/or a deficiency of readily observed natural outbreaks of the viruses in larval fish.

Published information on the vulnerability of larval fish is available for the remaining ten viruses reviewed. A summary of this information is presented in Table 3.

For seven of the ten viruses presented in Table 3. experimental or observational studies have revealed that there is a limited capacity for the virus to affect the very early larval stages of fish. The weight of evidence for this affect is variable between the different viruses. However, in all cases, there is evidence for a period of reduced vulnerability during the very early larval period. This relationship is somewhat surprising, given the empirical view that the susceptibility of fish to viruses is inversely related to age (e.g. Dorson & Touchy, 1981; Munday et al., 2002) and a view which should be adopted in the context of exposure testing within the NCCP.

It is also interesting to note that in those cases highlighted in Table 3, the period of constrained vulnerability, during early larval development, was always followed immediately by a period of severe vulnerability. Thus, it appears that although early larval fish may, in some cases, be protected from the effects of the virus, when these protective effects cease, the vulnerability of advanced larvae/early juvenile fish can be at its maximum.

It is apparent that in some cases, early larval fish can have a heightened resistance to viral infection. And that the age at which vulnerability peaks varies in different host species. There are a range of factors that may be affecting the vulnerability of early larval fish to various pathogenic viruses. The most common factors that have been suggested include: potentially heightened innate immune function in younger larval fish; the presence of a more effective physical barrier to viral entry (e.g. epithelial and periodontal tissue and mucus); a lack of specific virus receptors at the portal of entry; differences in the basic kinetics of exposure that may be skewed in favour of the larvae due to

surface area and water flow factors; or differences in morphological and immunological ontogeny (. Hanson, et al., 2004; Levraud et al., 2007; Ronsmans et al., 2014).

However, much of the hypothesising around the causative factors for differences in the vulnerability of different life stages and in different species is speculative. This prevents us from making well-informed assumptions for the vulnerability of different life stages to different viruses. Clearly, more experimental work is required, not only to understand the underlying factors that affect the vulnerability of different life stages to specific viruses, but also to demonstrate, conclusively, which life stages are susceptible to infection and vulnerable to the effects of specific viruses.

Table 3 Summary of the viruses for which the vulnerability of larval fish has been investigated

Virus	Effects of the virus on larval fish	Life stage of fish known to have greatest vulnerability	Reference/s
IHNV	Naturally spawned first feeding yolk sac fry of sockeye salmon are more vulnerable than older fish and can experience mortality reaching nearly 100% of the population. Younger alevins cohabitating with diseased fish were not affected.	Advanced larvae (First feeding yolk sac fry)	(Bootland & Leong, 2011; Traxler & Rankin, 1989)
ISAV	Mass mortality was observed in first feeding hatchery reared Atlantic salmon larvae but not in earlier larval stages housed alongside the infected fish.	Advanced larvae (First feeding larvae)	(Nylund et al., 1999; Rimstad et al., 2011)
SVCV	*The vulnerability of larval carp is unclear.* Early larval zebrafish are less vulnerable than advanced larvae.	Early juvenile common carp Advanced larval zebrafish (First feeding larvae)	(Ahne et al., 2002; Levraud et al., 2007; López-Muñoz et al., 2010)
VHS	Yolk sac fry, first feeding fry and fingerlings are more susceptible than later juvenile fish. *The vulnerability of earlier larvae is not reported.*	Advanced larvae (Yolk sac fry, fry and fingerlings – specific age not reported)	(Skall et al., 2005; Smail & Snow, 2011)
VNN	Larvae of many species of fish are susceptible to VNN. However, susceptibility may be delayed until after first feeding in some species. The link between vulnerability and morphological ontogeny is tenuous for this virus and requires further investigation. However, the earliest age at which VNN has been observed in a range of fish species is highly variable.	Early to advanced larvae and early juveniles (Variable between host species. Vulnerability possibly greatest after first feeding.)	(Grotmol et al., 1999; Munday et al., 2002)
IcHV-1	Advanced larvae from 8 to 60 days old are more vulnerable than early larvae, less than 3 days old.	Advanced larvae and early juveniles	(Hanson, A. et al., 2004; L. Hanson, et al., 2016; Hedrick et al., 1987)
SalHV-2	Newly hatched kokanee, chum salmon and coho salmon are less vulnerable than one month old larvae. For chum salmon, vulnerability is greatest in 3 month old fish. In masu salmon vulnerability is	Advanced larvae (1-3 months old depending on species)	(Kimura et al., 1983)

	greatest in 1 month old fish. Vulnerability then decreases as the fish grow older.		
SalHV-3	When lake trout fry (unspecified age) are exposed to the virus, cumulative mortality can reach nearly 100%. When lake trout are exposed to the virus as juveniles (10-13 cm long) cumulative mortality is reduced to ~30%. *The vulnerability of earlier larvae is not reported.*	Advanced larvae (Fry, age unknown)	(Bradley et al., 1989)
CyHV-1	Mortality rates were observed to be highest in 2 week old common carp compared to 4 or 8 week old fish. *The vulnerability of earlier larvae is not reported.*	Advanced larvae (2 months old)	(Sano et al., 1991)
CyHV-3	Early larval fish (1 – 10 days old) are less vulnerable to CyHV-3 than older fish (> 13 days old). Mortality of up to 100% can occur when fish are exposed to the virus at 13 or 21 days post hatching.	Advanced larvae and early juveniles	(Ito et al., 2007; Ronsmans et al., 2014)

Two general comments can be made based on the collective information presented in this review.

1. Where the vulnerability of larval fish has been investigated or observed, the very early larval stages are often less vulnerable to pathogenic viruses.
2. Vulnerability usually peaks in advanced larvae/early juvenile stages before declining in later juvenile and adult fish.

Most of the viruses reviewed in this report affect advanced larvae and early juvenile fish more profoundly than they do late juvenile or adult fish. The clinical effects of one virus, VNN, is almost entirely restricted to larval and early juvenile fish of the majority of species that it affects. Based on this information, it could easily be assumed that the vulnerability of fish to viral diseases is inversely related to age. However, during the early larval period, the reverse appears to apply, at least for certain viruses. For a range of viruses (ISAV, SVCV, VNN, IcHV-1, SalHV-2, CyHV-1 and CyHV-3), early larval fish are less vulnerable than advanced larvae or early juveniles. However, even for these viruses, vulnerability of the host is highest in advanced larvae and early juvenile fish.

Difficulties remain in making broad, generalised statements as to the effect of fish age on vulnerability to viral pathogens. Different patterns of age-related vulnerability are reported for different viruses and different host species.

However, for the majority of viruses reviewed in this report, the most vulnerable life stage is advanced larvae and early juvenile fish, at a time when morphological ontogeny allows viral entry but innate and humeral immune factors are unable to successfully control the virus. The effects of the virus on the host then usually decline as the fish grow older..

6.3. Conclusions on the evaluation of all stages of fish when assessing resistance of NTS to CyHV-3 infection.

The effects of fish age on vulnerability to pathogenic viruses can exhibit complex patterns. For example, the vulnerability of common carp to the effects of CyHV-3 can be more severe in early juvenile fish, compared to advanced juvenile and adult fish. However, early larval common carp appear to be less susceptible to infection, and less vulnerable to the effects of CyHV-3, than later larvae and early juvenile fish (Ito et al., 2007; Ronsmans et al., 2014). The precise mechanisms driving this relationship between life stage and susceptibility to CyHV-3 infection are unknown. And it is unclear if a relationship between the age of the host and its vulnerability to CyHV-3 could be expected in NTS exposed to CyHV-3 as a result of the NCCP.

This review has revealed that the life stage of fish can greatly affect susceptibility and vulnerability to pathogenic viruses. Across a range of viruses, generalised patterns in the relative susceptibility and vulnerability of fish of different life stages do appear to exist, with advanced larvae and early juvenile fish usually being the most susceptible and vulnerable stages. However, these patterns are not universal and exceptions to the rule do exist.

With advanced larval and early juvenile fish the most susceptible and vulnerable developmental stages of fish to viral challenge In the majority of cases,, it would be prudent to include these age classes in all trials that seek to assess the resistance of NTS of fish to CyHV-3 infection.

As older fish are generally less vulnerable to pathogenic viruses, only testing such fish may provide misleading results as to the true resistance status of NTS of fish to CyHV-3 infection. If older fish only are assessed, it may be difficult to induce clinical signs (including histological changes) or mortality during exposure trials. Direct detection methods such as PCR may also be less effective on older, less vulnerable fish due to potentially lower viral loads in these fish. If the viral load is lower in such fish, then direct detection methods may lack the analytical responsiveness required to successfully identify the presence of the virus.

For CyHV-3, all life stages of common carp are susceptible to infection and vulnerable to the clinical effects of CyHV-3 (OIE, 2017b; Perelberg et al., 2003; Ronsmans et al., 2014; Sano et al., 2004). However, infection and clinical effects are reduced in larval common carp, with the epidermal mucosa appearing to play a critical role in preventing viral entry (Ito et al., 2007; Ronsmans et al., 2014). Morphological ontogeny may also reduce the susceptibility of larval common carp to infection. Clinical signs and mortality are usually most severe in advanced common carp larvae and young juvenile fish, less pronounced in later juvenile and adult fish and much reduced in early larval common carp.

Based on a range of viruses, this review has shown that this general pattern may not hold for other fish species if they are susceptible to CyHV-3 infection. A failure to assess all life stages in CyHV-3 resistance testing may mean that the true resistance status of NTS of fish to CyHV-3 infection is uncertain, as it does not appear to be possible to make concrete assumptions as to the relative susceptibility and vulnerability of different life stages of fish to CyHV-3 infection.

In their study, McColl et al. (2017) only tested the susceptibility of juvenile and mature NTS of fish to CyHV-3 infection. With no testing of larval NTS of fish, uncertainty on the resistance of NTS of fish to CyHV-3 infection remains.

6.4. Recommendations on the evaluation of all life stages of fish when assessing resistance of NTS to CyHV-3 infection

It is recommended that larvae, juveniles and adults of NTS of fish be assessed for their resistance to CyHV-3 infection.

7. What is the potential for NTS beyond those previously investigated to become infected by CyHV-3? Should future CyHV-3 resistance testing include a wider range of NTS?

Host specificity is perhaps the most important issue in relation to the use of a pathogenic virus as a biocontrol measure. This chapter addresses the question of which NTS of fish should be tested for resistance to CyHV-3 infection and addresses the concern that the NTS of fish susceptibility testing undertaken by McColl et al. (2017) did not include all species that may be exposed to CyHV-3 if it were to be released into Australian waters.

7.1. Introduction

While 21 NTS were assessed by McColl et al. (2017), the criteria for each species' inclusion in susceptibility testing was not clearly articulated, and concerns were raised that the range of species assessed was not sufficiently extensive.

McColl et al. (2017) selected the 21 NTS they tested on the basis of them being a representative range of animal taxa that could conceivably come in contact with CyHV-3 if it were to be released into Australian waters. The range of NTS tested represented teleost fish, invertebrates, lampreys, amphibians, reptiles, birds and mammals, any of which could potentially be exposed to CyHV-3 by drinking water, or coming into contact with water, containing the virus.

McColl et al. (2017) also noted that because there are no native cyprinids in Australia, it was important to test native fish from the Order Siluriformes, the Australian teleost taxon most closely related to the cyprinids. Salmon catfish (Neoarius graeffei) and eel-tailed catfish (Tandanus tandanus) were included for this reason.

As set out in Chapter 3, the NCCP is being developed on the basis that CyHV-3 cannot infect NTS. Working under this definition, it is essential to have the highest possible level of confidence that all NTS that may be exposed to CyHV-3 if it were to be released into Australian waters are truly resistant to infection.

To determine which NTS of fish are likely to be exposed to CyHV-3, it is first necessary to consider the potential geographic spread of the virus if it were to be released. Following this, consideration needs to be given to which NTS of fish need to be tested for resistance to give confidence that all NTS of fish likely to be exposed are resistant to CyHV-3 infection.

Given that it is not possible to test all NTS of fish for resistance, reviewing published literature on studies investigating the resistance status of fish to CyHV-3, and whether taxonomy plays a role in susceptibility and resistance to infection, may provide insight into which Australian NTS of fish should be tested. If CyHV-3 is released into the MDB as part of the NCCP, a large number of NTS from a diverse range of habitats will be exposed to the virus. When this occurs, it is imperative that these NTS are not negatively affected by the virus.

7.2. Potential geographic spread of CyHV-3 in Australia

7.2.1. Spread of CyHV-3 in other parts of the world

In determining which fish species are likely to be exposed to CyHV-3 and hence potentially infected by the virus, if it were to be released into Australian waters, the potential geographic spread of the virus needs to be considered.

It is likely that the virus would disseminate widely across Australian freshwater ecosystems as it is spread by abiotic vectors such as water flow); and biotic vectors such as the movement of infected carp and other aquatic carriers of the virus The downstream flow of infected carp or water may also lead to marine species of fish inhabiting nearshore watersbeing exposed to the virus.

The potential for CyHV-3 to infect fish in such habitats may in part be determined by the ability of the virus to persist in saline water. Marine species that transiently inhabit freshwater environments or low-salinity river discharge water, may be exposed to the virus as they pass through these habitats.

If the virus is unable to persist in saline waters, marine species may never the less be exposed to the virus via the consumption of infected carp, or their tissues, that wash into the marine environment. This is of particular concern given that CyHV-3 is known to infect common carp via the consumption of infectious material, which facilitates viral entry through the pharyngeal periodontal mucosa (Fournier et al., 2012).

It is difficult to precisely determine the potential spread of CyHV-3 throughout Australia. On one hand, the discontinuous and fragmented abundance of common carp in waters outside of the Murray Darling Basin may be considered to be an obstacle to the widespread dissemination of the virus across the Australian continent. However, this assumes that common carp are the only potential carrier of the virus in Australian waters. This is currently uncertain. On the other hand, the rapid and expansive spread of CyHV-3 throughout North America, Europe and Asia indicates that, in the presence of suitable host species, the virus can readily translocate over vast distances via a range of vectors and pathways.

Since the first known occurrences of CyHV-3 in the United Kingdom in 1996, Germany in 1997 and Israel and the USA in 1998 (Bretzinger, et al.,1999; Perelberg et al., 2003), CyHV-3 rapidly spread throughout much of the world. It has now been observed in the United Kingdom (Haenen, et al.,2004), throughout the European continent (Bergmann, et al., 2006; Haenen et al., 2004; Novotny et al., 2010), across Asia (Haenen et al., 2004; Ilouze et al., 2011; Pikulkaew, et al., 2009; Sano et al., 2004), in north America (Garver et al., 2010; Hedrick et al., 2000) and in South Africa (Haenen et al., 2004).

There is clearly a great potential for CyHV-3 to spread rapidly over vast distances. In North America, the virus was first observed in wild fish in New York and South Carolina in north eastern USA, during 2004. In 2005, the virus had spread into Wisconsin in northern central USA and by 2006 the virus was detected in Texas in the south of the USA. By 2007, the virus was causing mortality in wild common carp in the Kawartha Lakes region of eastern Ontario, Canada and in 2008 had spread to an additional 15 bodies of water in central Ontario. By 2009 the virus was also detected in wild fish in Arizona, in the south west of the USA (Garver et al., 2010).

A similar pattern of rapid spread has been observed in the United Kingdom and Japan. In the UK, CyHV-3 was implicated in fish kills in angling waters in 2003. Further detections of CyHV-3 occurred in 2004 and 2005 and by 2006, the virus had been reported, and confirmed, at 23 separate sites in southern England.

In Japan, CyHV-3 was first observed in 2003, when it was implicated in fish kills of farmed common carp in Ibaraki Prefecture of eastern Japan. Within a year, outbreaks of the virus were being reported in all prefectures across Japan andby 2008, CyHV-3 DNA was detected in 93 Japanese rivers

of the 103 assessed, with mortality of common carp reported in approximately half of these rivers (Becker, Ward, & Hick, 2018; Aoki et al, 2007)

7.2.2. Factors assisting the spread of CyHV-3 and suitability of environmental conditions in Australia

The rapid spread of CyHV-3, both within continents and across continental borders, has been assisted by a range of abiotic and biotic vectors.

Water appears to be one of the primary vector of transmission, although it is possible that animate vectors such as other fish species, parasitic invertebrates and piscivorous birds and mammals are also involved in transmission of the virus (OIE, 2017b). The trade and movement of infected ornamental common carp (koi) is thought to be a major vector in the spread of CyHV-3 (OIE, 2017b; Rathore et al, 2012). However, there is also growing evidence that a range of other potential carrier species have the capacity to transmit CyHV-3 (Radosavljevic et al, 2012) and may contribute to its spread.There is also some evidence that CyHV-3 may be able to persist in plankton and sediments (Oidtmann et all, 2018; Minomoto et al, 2011) and in invertebrate species such as molluscs and crustaceans which may be incidentally or deliberately transported to geographically distant regions (for example, by the transportation of water in boats or the deliberate movement of invertebrate species used as fishing bait).

CyHV-3 can persist in water for at least four hours at 23-25°C, after which it can still infect common carp and cause clinical disease (Perelberg et al., 2003). However, Perelberg et al. (2003) also found that carp did not develop clinical signs when they were exposed to virus that had been maintained in water for 21 hours at 23-25°C.

The survivability of CyHV-3 in environmental water and sediment has also been assessed by exposing cell cultures to virus that had been held in these media for varying lengths of time. It was found that the virus remained infective for up to three days in environmental water or sediment at 15°C or 20°C, but this could be extended to seven days if the water was sterilised by filtering or autoclaving. Bacteria with anti-CyHV-3 properties were observed in water samples and appear to play a role in reducing the survivability of CyHV-3 in water and sediment (Shimizu, Yoshida, Kasai, & Yoshimizu, 2006). It appears that the survivability of CyHV-3 in water can be prolonged, and is somewhat dependent on the abundance of bacteria with anti-CyHV-3 activity.

CyHV-3 also appears to be able to persist in ecosystems with much wider thermal extremes. In Lake Biwa, Shiga Prefecture Japan, CyHV-3 DNA was routinely detected in water samples throughout 2007 and 2008 (Minamoto, et al.,2009). During this period, water temperatures ranged from 5.0°C to 36.1°C. Although the virus was not detected in water samples below ~10°C, when the water temperature increased the virus was again detected in environmental water (Minamoto et al., 2009). This demonstrates the potential for the virus to reactivate after periods of unfavourable thermal conditions.

CyHV-3 has been recorded in farmed and wild fish from latitudinally wide-spread locations. The virus has been recorded from equatorial and tropical locations in Indonesia, Singapore and Malaysia, through subtropical locations in South Africa, southern USA and Asia and across numerous temperate locations in North America, Europe and Asia. Clearly, the virus has the potential to infect fish and persist in geographically and thermally disparate locations.

Environmental conditions in Australian aquatic ecosystems appear to be favourable to the persistence and spread of CyHV-3. Given that fish are poikilothermic, water temperature is perhaps

the most important factor affecting the persistence of this piscivorous virus. CyHV-3 has a wide thermal tolerance, infecting common carp at temperatures between 13°C and 28°C (Becker et al. 2014)

In Australia, water temperatures suitable for CyHV-3 infection (13°C – 28°C) and persistence (5°C – 36.1°C) are likely to occur in aquatic systems throughout the entire country. Specific water temperature data are collected by the Australian Bureau of Meteorology from a range of freshwater ecosystems in Australia. Over the past ten years, water temperatures have ranged between: ~5°C and ~22.5°C at Meadowbank (central Tasmania) and Kennedys Creek (southern Victoria); ~7°C and 31°C in the Kent River (southern Western Australia); ~10°C and 32.5°C in Cooper Creek (central South Australia); 23°C and 34°C at Kalpower Crossing (far northern Queensland). At all of these geographically disparate locations, water temperature would be seasonally favourable for CyHV-3 infection and for the establishment of long-term CyHV-3 persistence. As a consequence, all Australian freshwater ecosystems should be considered to be suitable for the persistence of CyHV-3.

In certain locations, heavy rainfall and river runoff can also cause nearshore locations to experience periods of very low salinity. This is the case for numerous river mouths around the Australian coastline. These locations are likely to provide environmental conditions that are periodically suitable for CyHV-3 to persist in near-shore habitats.

Across the Australian continent, nearly all freshwater and many nearshore environments should be considered to provide conditions suitable for the persistence of CyHV-3 in the aquatic environment and its infection of fish tissues.

7.3. Fish species potentially exposed to CyHV-3 if it were to be released into Australian waters

As discussed above, the potential for CyHV-3 to spread rapidly across geographically wide areas and the suitability of freshwater environments and many nearshore environments across Australia, it should be assumed that if released into Australian waters, CyHV-3 will ultimately spread to waters across much of the continent.

Accordingly, there is potential for all NTS of fish inhabiting freshwater ecosystems and nearshore environments across Australia to be exposed to the virus. Based on this, the pertinent question is whether all these NTS of fish are resistant to CyHV-3 infection?

Prima facie, this question can be answered comprehensively only through experimental procedures that provide robust data on the resistance status of all these NTS of fish. This is not possible given the very large number of fish species potentially involved.

In the absence of such data, review of published literature on the resistance status of fish species that have previously been investigated may yield information on factors that affect resistance to CyHV-3 infection. With such knowledge, it may be possible to select NTS of fish that are most likely to be potentially susceptible to infection if CyHV-3 were to be released into Australian waters.

One factor that may affect the resistance of fish species to CyHV-3 is taxonomy. If taxonomy does affect the resistance status of fish to CyHV-3, this may enable some inferences to be made as to the resistance status of many, as yet unexamined, non-common carp species, and it may be possible to select NTS of fish for resistance testing based on these inferences.

In general, the susceptibility of fish to Alloherpesviruses tends to occur amongst taxonomically related species (Bergmann et al., 2016; Hanson, et al., 2016). CyHV-3 is generally considered to be host specific due to the general observation that serious clinical signs are seemingly restricted to common carp (Bergmann et al., 2016; Hanson, et al., 2016). However, a concise review of the literature, examining the published knowledge on resistance to CyHV-3, does not appear to exist. This constrains our ability to make well informed assertions as to the host-specificity of CyHV-3.

The published scientific literature has been reviewed to look for insights on the resistance status of fishes to CyHV-3 infection and the influence of taxonomy in this regard. The results of this review are presented below.

7.4. Literature review methodology

The 41 articles identified through the systematic, quantitative review process outlined in Chapter 2, and graphically represented in PRISMA Statement 2 (Figure 2), were subjected to an additional screening process. This was performed to identify those articles that assessed the resistance status of non-common carp species to CyHV-3 infection. Studies were excluded where they only investigated the resistance of common carp to CyHV-3 infection. After screening, 18 articles were identified as meeting the criteria for inclusion in the review.. The full text of each article was then obtained and reviewed in detail. Of each of the relevant articles identified, the reference lists were checked to identify additional relevant articles.

This process is graphically represented in PRISMA Statement 5 (Figure 9).

Summary information from these 18 articles is presented in Appendix 6.

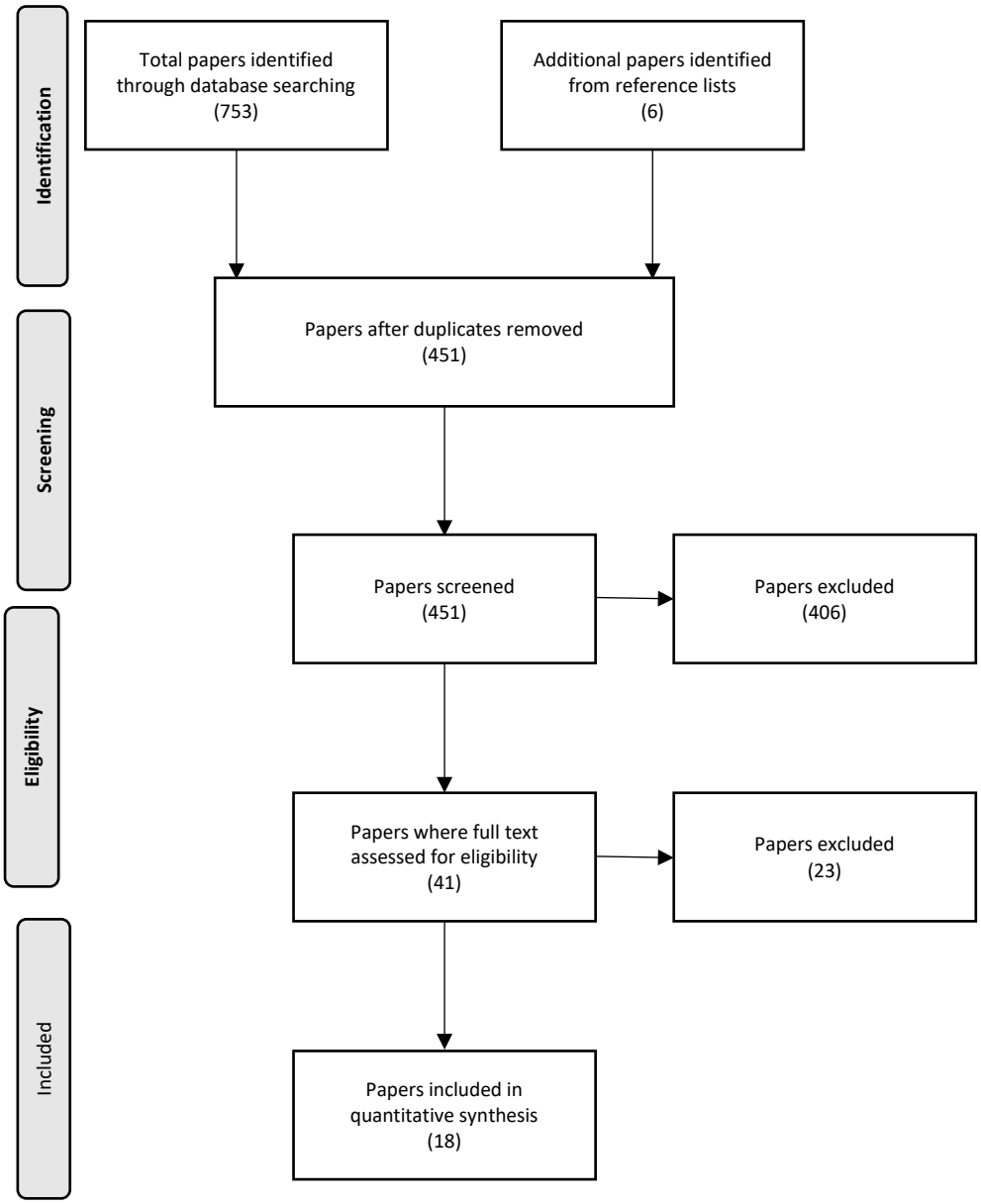

Figure 9. PRISMA Statement 5. Screening process used to identify articles reporting studies that assessed the resistance status of non-common carp species to CyHV-3.

7.5. Resistance status of fishes to CyHV-3 infection and the role of taxonomy

7.5.1. Global occurrence of CyHV-3 – Species known to be resistant/not-resistant to CyHV-3

Through this systematic quantitative literature review, a total of 18 published articles were identified that have previously assessed the resistance status of non-common carp species to CyHV-3 infection. These articles present data related to the resistance status of a total of 23 different species of fish, representing 8eight taxonomic orders and ten families (Appendix 6).

In addition to these species, studies performed by Bergmann et al. (2009), Bergmann, et al., (2007), El-Matbouli, et al. (2007), Fabian et al. (2013) Kempter & Bergmann (2007), Kempter et al. (2009); Kempter. et al. (2008) and Sadler et al. (2008), used various direct detection methods (PCR, ISH and IFAT) to investigate a range of species for the presence of CyHV-3 DNA in their tissues. These species will be discussed separately from those identified in the systematic quantitative review as they do not represent the findings of structured resistance testing procedures (i.e.formal procedures). As a consequence, the exposure status of many of the fish investigated in this work is uncertain (Appendix 7)

Additionally, McColl et al. (2017) investigated the susceptibility of 21 species of endemic Australian species to CyHV-3 infection. These species represent 15 taxonomic orders and 20 families. These findings will also be discussed separately as the results presented make it difficult to determine the resistance status of the species assessed. While the authors assert that no species assessed were susceptible to the virus, the occurrence of unexplained mortalities in animals exposed to the virus and the frequent occurrence of positive results when exposed fish were assessed by Gilad q-PCR, makes it difficult to determine if these animals were truly resistant to infection by CyHV-3. Furthermore, Gilad q-PCR data are not reported for individual species, making it difficult to interpret the results.

Excluding species assessed by McColl et al. (2017), those species for which resistance has been assessed through experimental procedures are presented in Appendix 6. Each species that has been assessed for resistance is listed, with pertinent information presented from each individual study in which they were assessed. The information presented includes: taxonomic details; the resistance status of each species as determined in each individual study; the weight of evidence reported in each study; the occurrence of clinical signs; and the reference for the published paper.

In Appendix 6 species are listed as “resistant”, “not-resistant”, “detected” or “uncertain”. Species are listed as “resistant” where exposure to the virus is known but there is no evidence of the presence of the virus in the host. Species are listed as “not-resistant” where there is compelling evidence that the virus can infect the host and establish an active, replicating infection. Such evidence may include the detection of the virus in host tissues through various direct and indirect detection methods coupled with either: the occurrence of clinical signs; or a demonstrated ability for the virus to be transmitted from this intermediate host species to recipient naïve common carp. This does not imply that the virus can cause immediate clinical effects in that species. However, if the virus is able to be transmitted from a novel host to naïve recipient common carp, this is highly indicative of the potential for the virus to replicate in these intermediate host species, and, for the purposes of this review, they are considered to be not-resistant to CyHV-3 infection.

Of the 23 non-common carp species listed in Appendix 6, published evidence is available to indicate that 19 species are not-resistant to CyHV-3 infection. Of these species, mild clinical signs were only

observed in three species: goldfish; crucian carp; and common roach. For the remaining species, classification as non-resistant is is based on the detection of the virus in host tissues by direct detection methods (predominantly PCR), coupled with the observation that the virus can be subsequently transmitted to naïve common carp. The use of PCR data alone as evidence for infection must be taken with some caution. PCR tests targeting viral DNA can only provide evidence of the presence of a segment of viral genome and cannot demonstrate active viral replication. As such, where viral DNA is detected by PCR, and in the absences of other corollary data, the resistance status of the host is considered to be uncertain.

Directly comparing the resistance status of different species is often constrained by the use of varying methodologies applied in different studies and because of the variable reporting of specific results. Contradictory results are also reported between different studies that have assessed the resistance status of the same species.

Of the 23 fish species listed in Appendix 6, the most represented taxonomic group is the cypriniformes, from which 14 species have been investigated. The majority of these species represent the taxonomic family cyprinidae with a single species from the family nemacheilidae having also been assessed. Most of these species can be considered to be not-resistant to CyHV-3. The reasons for this interpretation are detailed below.

The resistance status of stone loach, a cypriniforme of the family nemacheilidae was investigated by Pospichal et al. (2016). When stone loach were cohabitated with CyHV-3-infected common carp, CyHV-3 DNA was detected in their tissue by CEFAS n-PCR. Interestingly, CyHV-3 DNA was not detected in any stone loach sampled after 15 days of exposure, but was detected in seven of nine fish sampled 30 days after cohabitation with CyHV-3 infected common carp. When these stone loach were subsequently cohabitated with naïve common carp, all stone loach and carp died. Despite this high mortality, CyHV-3 DNA was not detected in any of these stone loach or common carp by CEFAS n-PCR (the authors provided evidence that mortalities were related to a malfunction of the biofilters and/or bacterial infection and not due to infection with CyHV-3). As it could not be shown that stone loach could transmit the virus to naïve common carp, the resistance status of this species to CyHV-3 is considered to be uncertain.

Goldfish (*Carassius auratus*) are the cyprinid species that has been investigated most often, having been tested for resistance in eight separate studies. In three of these studies, goldfish were considered to be resistant to the CyHV-3. The evidence presented for this finding is variable.

Perelberg et al. (2003) presented the first investigation into the resistance status of goldfish. Goldfish were exposed to common carp known to be infected with the virus. After this, no clinical signs or mortality were observed in the exposed goldfish. These goldfish were subsequently cohabitated with naive common carp. Following this, no clinical signs or mortality were observed in the naïve common carp. Other direct or indirect detection methods were not applied to the common carp or goldfish. Hedrick, et al.(2006) exposed goldfish to CyHV-3 via intraperitoneal (IP) injection. Following this, no mortality of goldfish was observed and the virus could not be detected by Gilad c-PCR. Yuasa et al. (2013) also determined that goldfish were resistant to CyHV-3. In their study, goldfish were exposed to CyHV-3 and later cohabitated with naïve common carp. No clinical signs or mortality were observed in these goldfish or common carp and the virus could not be detected in either species by Bercovier c-PCR.

In contrast to the findings of Perelberg et al. (2003), Hedrick et al. (2006) and Yuasa et al. (2013), five studies have presented compelling evidence that goldfish are not-resistant to CyHV-3. In the report of the Koi Herpes Virus workshop prepared by Haenen and Hedrick (2006), a presentation by S. Bergmann is said to have reported on the occurrence of CyHV-3 in goldfish. Results from rt-PCR (modified after Gilad c-PCR) and from an unspecified n-PCR are referred to. It is stated that CyHV-3 was identified in goldfish 7, 14 45, 60 and 365 days after the fish were injected with the virus. It is also eluded to that specific proteins related to CyHV-3 were found in goldfish and that CyHV-3 was isolated after cohabitation with infected common carp. As this is only the report of a workshop presentation, it is difficult to assess the reliability of the information presented by Haenen and Hedrick (2006), however, it is included here as it is considered valid and pertinent to the resistance status of goldfish. These findings are also supported by the studies of S. Bergmann et al. (2010), Mansour El-Matbouli and Soliman (2011), Radosavljević et al. (2012) and Fabian et al. (2016). In each of these studies, when goldfish were exposed to CyHV-3 by immersion, IP injection or cohabitation with infected common carp, the virus could subsequently be detected in these fish by various PCR assays, IFAT, ISH and/or the occurrence of mild clinical signs (leucopoenia). In each case, infected goldfish could also transmit the virus to naïve common carp by cohabitation.

In summary based on the findings of eight studies that have investigated the resistance status of goldfish to CyHV-3, goldfish are considered to be not-resistant to CyHV-3 infection. While three studies have appeared to confirm that goldfish are resistant, it appears that this is most likely related to the analytical responsiveness of the detection methods used to detect the virus in these studies, rather than the inability of the virus to infect goldfish.

Crucian carp (*Carassius carassius*) also appear not-resistant to CyHV-3. As with goldfish, the presentation of S. Bergmann published in the report of the Koi Herpesvirus workshop prepared by Haenen and Hedrick (2006) implicates crucian carp as being not-resistant to the virus. While the methodology and results presented in this report are somewhat lacking in detail, crucian carp were also found to be not-resistant to CyHV-3 by Gaede, Steinbrück, et al. (2017). Gaede, Steinbrück, et al. (2017) detected CyHV-3 by Gilad q-PCR and ISH in the tissues of crucian carp that had been exposed to the virus by immersion. Mild clinical signs – mild mucoid plaques on the head and fins – were also observed in crucian carp after the first week of exposure. Based on this evidence, crucian carp should be considered to be not-resistant to CyHV-3 infection.

Prussian carp (*Carassius gibelio*) can also be considered to be not-resistant to CyHV-3. While Fabian et al. (2013) failed to identify CyHV-3 in Prussian carp or to transmit the virus from Prussian carp to naïve common carp, the exposure history of the Prussian carp investigated was unknown as these were wild fish collected carp farms. However, when Prussian carp were intentionally exposed to CyHV-3 under controlled conditions (cohabitation with infected common carp), the virus could be subsequently detected in their tissues by Bercovier c-PCR (Radosavljević et al., 2012). When these Prussian carp were cohabitated post initial exposure (14 days and 21 days) with common carp, the virus was transmitted to these common carp, confirmed by the detection of viral DNA by Bercovier c-PCR.

There is also strong evidence that grass carp (*Ctenopharyngodon idella*) and silver carp (*Hypophthalmichthys molitrix*) are not-resistant to CyHV-3. In the first investigation of grass carp and silver carp by Perelberg et al. (2003) clinical signs or mortality were not observed in either species after cohabitation with infected common carp. When these two species were subsequently cohabitated with common carp the recipient common carp did not become sick or die. However, more recent studies (J Kempter et al., 2012; Radosavljević et al., 2012) have shown that CyHV-3 can

be detected in the tissues of grass carp and silver carp, by various PCR methods, after exposure by cohabitation with infected common carp. And that both of these species can transmit the virus to common carp, where it can be detected by various PCR methods.

CyHV-3 has also been detected by Gilad q-PCR in wild gudgeon (*Gobio gobio*) inhabiting carp farms in Germany (Fabian et al., 2013). When naïve common carp were cohabitated with these gudgeon, the virus was transmitted to these common carp, where it was detected by Gilad q-PCR. Gudgeon have also been infected with CyHV-3 when exposed by cohabitation with infected common carp under controlled conditions. However, when these gudgeon were cohabitated with common carp, the virus did not appear to be transmitted to these recipient common carp and it was not detected by Gilad q-PCR (Fabian et al., 2016). Despite some contradictory findings, the presence of viral DNA in gudgeon and the ability of the virus to be transmitted to naïve common carp, observed by Fabian et al. (2013) suggests that gudgeon are not-resistant to CyHV-3 infection.

The resistance status of ide (*Leuciscus idus*) has been investigated by a single study in which the fish were exposed to CyHV-3 by immersion (Fabian et al., 2016). These fish were subsequently cohabitated with common carp. After this process, CyHV-3 was detected in a single ide and a single common carp by Gilad q-PCR. In a separate experiment Fabian et al. (2016) exposed ide to CyHV-3 by cohabitation with infected common carp. The virus was subsequently detected in these ide by Gilad q-PCR. However, it could not be demonstrated that these ide could transmit the virus to common carp with these common carp testing negative by Gilad q-PCR. Again, the results for ide are somewhat contradictory. Despite this, the presence of CyHV-3 DNA in ide after exposure through immersion or cohabitation with infected carp, and the observation that CyHV-3 can be transmitted from ide to common carp indicates that they are not-resistant to CyHV-3 infection.

CyHV-3 has also been detected in wild common dace (*Leuciscus leuciscus*) by Gilad q-PCR (Fabian et al., 2013). However, when these fish were cohabitated with common carp, the virus could not be detected in these common carp. While viral DNA was detected in common dace, the inability to demonstrate that the virus could be transmitted to common carp leads us to consider the resistance status of common dace to CyHV-3 to be uncertain.

Topmouth gudgeon (*Pseudorasbora parva*) also appear to be not-resistant to becoming infected with CyHV-3. When Pospichal et al. (2018) removed skin mucus from topmouth gudgeon and cohabitated them with infected common carp, the virus was detected in these topmouth gudgeon by Gilad q-PCR. When these topmouth gudgeon were subsequently cohabitated with naïve common carp, the virus was detected in a single common carp by Gilad q-PCR. This study not only indicates that topmouth gudgeon are not-resistant to CyHV-3 infection, but that removing skin mucus increases the likelihood of topmouth gudgeon becoming infected. It is unclear if removal of skin mucus would increase the likelihood of other species of fish becoming infected with CyHV-3. This finding may have significant implications to future studies that assess the resistance status of fish to CyHV-3.

Three separate studies have detected the presence of CyHV-3 DNA in the tissues of common roach. Using Gilad q-PCR, Gaede, et al. (2017) detected CyHV-3 in the tissues of common roach that had been exposed to the virus by immersion. Slight abrasions were also observed on the fins of these fish in the first days post infection. Kempter et al. (2012) and Fabian et al. (2013) also detected CyHV-3 DNA in the tissues of wild common roach by various nested and quantitative PCR techniques. Fabian et al. (2013) was unable to demonstrate that infected common roach could transmit the virus to naïve common carp, however, when Kempter et al. (2012) exposed naïve common carp to infected

roach, the virus could be detected in common carp using Bergmann n-PCR or CEFAS n-PCR. This combined data provides evidence that common roach can be a source for CyHV-3 infection of common carp meaning they are a source of live virus.

Some contradictory findings have also been observed for rudd. Fabian et al. (2016) exposed rudd to CyHV-3 by immersion and then cohabitated these fish with naïve common carp. Following this, CyHV-3 could not be detected in rudd or common carp by Gilad q-PCR. In contrast to this, Fabian et al. (2013) detected CyHV-3 DNA in wild rudd and demonstrated that the virus could be transmitted to common carp by cohabitation, as detected by Gilad q-PCR. Although there is some discrepancy between these two studies, the presence of viral DNA in wild fish, and the ability of these fish to transmit the virus to naïve common carp indicates that rudd are not-resistant to CyHV-3.

Tench also appear to be non-resistant to infection with CyHV-3. CyHV-3 DNA has been detected in wild tench (Fabian et al., 2013; Kempter et al., 2012) by a range of quantitative and nested PCR techniques. And these tench could transmit the virus to naïve common carp, where it could be detected by various PCR assays. Viral DNA has also been detected in tench after exposure by immersion (Fabian et al., 2016; Gaede, et al., 2017) and cohabitation (Fabian et al., 2016; Radosavljević et al., 2012). And it has been demonstrated that the virus could be transmitted to naïve common carp by cohabitation, with the virus being detected in recipient carp by Bercovier c-PCR (Radosavljević et al., 2012). This provides compelling evidence that tench are not-resistant to CyHV-3.

The majority of cyrpiniformes that have been investigated for resistance to CyHV-3 appear to be not-resistant to CyHV-3. Under the definition of resistance used in this review, and based on the published literature, goldfish, crucian carp, Prussian carp, grass carp, silver carp, gudgeon, ide, topmouth gudgeon, common roach, rudd and tench appear to be not-resistant to CyHV-3 infection, while the resistance status of stone loach and common dace must remain uncertain. Only for a single species of cypriniformes that has been assessed by experimental methodology is there insufficient evidence to suggest that they are not-resistant to CyHV-3. Fabian et al. (2016) exposed common nase to CyHV-3 by immersion and were subsequently unable to identify the virus in their tissues by Gilad q-PCR.

Despite compelling evidence that CyHV-3 can infect a range of non-common carp cyprinid species, clinical signs in these species have rarely been observed. And when clinical signs have occurred, they have only been very minor. For example, mild leucopoenia was observed in goldfish, mild mucoid plaques were observed in crucian carp and slight fin abrasions were apparent on common roach exposed to the virus.

The next most investigated taxonomic order are the perciformes. Four species of perciformes, from the sciaenidae and percidae families have been investigated for resistance to CyHV-3. These are American silver perch (*Bairdiella chrysoura*) of the sciaenidae family and ruffe (*Gymnocephalus cernua*), European perch (*Perca fluviatilis*) and pike perch (*Sander lucioperca*), from the percidae family. Only for American silver perch is there insufficient information to indicate that the species is not-resistant to the virus. For the remaining three species, there is evidence to suggest that these species are not-resistant to CyHV-3 infection.

American silver perch were assessed by cohabitating them with common carp known to be infected with the virus. This caused no clinical signs or mortality to develop in American silver perch following exposure. When these American silver perch were subsequently cohabitated with naïve common

carp, no clinical signs or mortality developed in these carp. No direct or indirect detection methods were used to detect the virus in fish tissues (Ayana Perelberg et al., 2003).

The resistance status of ruffe has been investigated in two published studies. Fabian et al. (2013) failed to detect CyHV-3, by Gilad q-PCR, in the tissues of ruffe collected from carp farms with a history of CyHV-3. However, CyHV-3 was detected in common carp that were cohabitated with these ruffe. This finding is difficult to interpret. It could possible indicate that CyHV-3 was present in ruffe at a level below the analytical responsiveness of the Gilad q-PCR assay but that when the virus infected common carp, it replicated to a level that could be detected. This is speculative but the ability of CyHV-3 to persist in ruffe is supported by the findings of Kempter et al. (2012) who identified CyHV-3 DNA in the tissues of wild ruffe by Bercovier c-PCR. When these ruffe were cohabitated with common carp, the virus could be detected in these carp by Bercovier c-PCR. This indicates that ruffe are not-resistant to being infected by CyHV-3.

European perch also appear to be not-resistant to CyHV-3. DNA of the virus was detected in wild fish collected from carp farms with a known history of exposure to CyHV-3 (Fabian et al., 2013; J Kempter et al., 2012) and in captive fish deliberately exposed to the virus by immersion (Fabian et al., 2016) using a variety of PCR assays. It has also been observed that the virus can be transmitted to naïve common carp by cohabitation, where it can be detected by Bergmann n-PCR and CEFAS n-PCR (Kempter et al., 2012).

A single species from the orders salmoniformes, siluriformes, acipenseriformes, gasterosteiformes, cichliformes and esociformes have also been investigated for their resistance to CyHV-3.

There is strong evidence that rainbow trout (*Oncorhynchus mykiss*) of the family salmonidae and the order salmoniformes are not-resistant to CyHV-3. When exposed to the virus by immersion, CyHV-3 could be detected in exposed fish by Gilad q-PCR, Bergmann sn-PCR, ELISA and SNT. The virus could also be transmitted from these rainbow trout to common carp, where it caused clinical signs and mortality and could be detected by Gilad q-PCR, Bergmann sn-PCR, ELISA and SNT. While the virus does not appear to cause any clinical signs in rainbow trout, it is clear that the virus can persist in this species and that active virus can then be transmitted to naïve common carp (Sven M Bergmann et al., 2016).

From the order siluriiformes, brown bullhead (*Ameiurus nebulosus*) of the siluridae family has been assessed for resistance to CyHV-3. CyHV-3 was detected in Brown bullhead collected from carp farms with a history of CyHV-3 by Gilad q-PCR (Fabian et al., 2013). However, it could not be demonstrated that the virus could be transmitted to common carp by cohabitation. Because of this, the resistance status of brown bullhead to CyHV-3 should be considered to be uncertain.

One species from the order Acipenseriformes and the family Acipenseridae has been assessed for resistance to CyHV-3. When sterbel, a hybrid of sterlet (*Acipenser ruthenus*) and beluga (*Huso huso*) were exposed to CyHV-3 via cohabitation with infected common carp, CyHV-3 DNA was identified in their tissues by CEFAS n-PCR (Pospichal et al., 2016). Interestingly, CyHV-3 was not identified at 15 days post exposure to infected carp, but was identified in 2 out of 9 sterbel assessed 30 days after exposure. The ability of these sterbel to transmit the virus was assessed by cohabitating these sterbel with naïve common carp. While significant mortality of common carp occurred during cohabitation with these sterbel, CyHV-3 could not be identified in these mortalities by CEFAS n-PCR. As a consequence, the resistance status of sterbel to CyHV-3 should be considered to be uncertain.

The resistance status of three-spined stickleback (*Gasterosteus aculeatus*), of the family Gasterosteidae and the order Gasterosteiformes, to CyHV-3 can also be considered to be uncertain. CyHV-3 has been detected in three-spined stickleback collected from carp farms with a history of CyHV-3 (Fabian et al., 2013) and in fish intentionally exposed to the virus by immersion (Fabian et al., 2016). As was observed for sterbel, three-spined stickleback were not observed to transmit the virus to naïve common carp.

The resistance status of tilapia (*Oreochromis niloticus*) of the order cichliformes and the family cichlidae has only been investigated by one published study. Perelberg et al. (2003) found that tilapia were not vulnerable to clinical effects or mortality when exposed to the virus by cohabitation with infected common carp. Furthermore, when tilapia that had been exposed to the virus were subsequently cohabitated with naïve common carp, the virus did not appear to be transmitted to these carp. However, exposed tilapia have not been assessed by any direct or indirect detection methods. As a consequence, tilapia can be tentatively assumed to be resistant to CyHV-3, although this should be confirmed through resistance studies that incorporate advanced direct detection methods.

Finally, northern pike, a member of the esocidae family of the order esociformes has also been investigated. CyHV-3 could not be detected in wild northern pike collected from a carp farm with a history of CyHV-3. However, when these pike were cohabitated with naïve common carp, the virus could be detected in these carp by Gilad q-PCR. As with ruffe, this finding is difficult to interpret. Again, it is possible that CyHV-3 was present in northern pike at a level below the analytical responsiveness of the Gilad q-PCR assay but that when the virus infected common carp, it replicated to a level that could be detected. This is speculative and further work is required to better understand the resistance status of northern pike to CyHV-3. The resistance status of northern pike is considered to be uncertain.

Based on the data presented in the published scientific literature, of the 23 species that have been assessed, three are tentatively considered to be resistant to CyHV-3. These are: common nase, which tested negative by Gilad q-PCR after being exposed to the virus by immersion (Fabian et al., 2016); and American silver perch and tilapia which did not develop clinical signs after cohabitation with common carp, and could not induce clinical signs in common carp when cohabitated with naïve fish (Perelberg et al., 2003).

A further six species are considered to have uncertain resistance status. These include: stone loach, common dace, brown bullhead, sterbel, three-spined stickleback and northern pike. With the exception of northern pike, the DNA of CyHV-3 has been detected in all of these species. However, in all cases, it has not been demonstrated that the virus can be transmitted from these hosts to naïve common carp. The virus has not been detected in northern pike, but when northern pike collected from a carp farm with a history of CyHV-3 were cohabitated with common carp, the virus was detected in these recipient carp.

The majority of fish that have been investigated for resistance to CyHV-3, should be considered to be not-resistant to infection, as resistance is defined in this review. Of the 23 species that have been assessed, 14 can be considered to be not-resistant to CyHV-3 infection. These are: goldfish, crucian carp, Prussian carp, grass carp, silver carp, gudgeon, ide, top mouth gudgeon, common roach, rudd, tench, ruffe, European perch and rainbow trout. For all of these species there is compelling evidence that the virus can enter the host, where it can be identified by various direct detection methods, and indicative evidence that it can establish a replicating infection. This was usually demonstrated by the

ability of these non-common carp species to transmit the virus to naïve common carp through cohabitation. In most cases, clinical signs could not be induced in recipient carp but the virus was detected by various direct detection methods, predominantly PCR.

The lack of clinical signs in recipient common carp is somewhat puzzling given that common carp are known to be highly vulnerable to the virus. It is plausible that the viral DNA detected in recipient carp was not indicative of active infection in these carp, or in the intermediate species. Inactive virions of CyHV-3 or fragments of viral DNA may have been passed from the intermediate host to common carp, where it was detected by PCR. It is impossible to rule out this scenario. However, in ruffe and in northern pike, viral DNA was detected only in recipient carp and not in these intermediate species. This may be a result of low viral load in ruffe and northern pike, due to reduced viral replication in these species but increased viral replication in the recipient carp, enabling detection by PCR. It is also true that clinical signs and low-level mortality could be induced in common carp cohabitated with CyHV-3-infected rainbow trout, providing strong evidence that active virus could be transmitted from rainbow trout to recipient common carp.

It is also possible, that the lack of clinical signs in recipient common was due to a very low viral load being released by intermediate hosts. It is possible, although somewhat speculative, that non-common carp species release relatively low concentrations of the virus, which can infect common carp subclinically but are insufficient to induce clinical signs. It has previously been observed that common carp exposed to low viral titres can survive exposure to the virus with an absence of clinical signs. Further investigation is required to understand why common carp have rarely developed clinical signs after being infected with the virus through cohabitation with non-common carp carrier species.

In addition to the 23 different species that have been assessed for resistance to CyHV-3 through formal experiments, wild fish of an additional 31 species have been investigated for the presence of CyHV-3 in their tissues. The studies presented in Appendix 7 were performed to identify CyHV-3 DNA in wild fish. This process was undertaken to determine the potential for these species to carry the virus and act as a vector in the transmission and spread of the virus.

The studies presented in Appendix 7 did not investigate the potential for CyHV-3 to cause clinical signs in non-common carp species, but were performed to determine the potential for such species to carry the virus asymptomatically. These studies used direct detection methods, typically PCR targeting genomic DNA, to identify the presence of the virus in tissues of wild animals that were likely to have been exposed to the virus through cohabitation with infected common carp. However, the precise exposure history of the animals is often uncertain, given that exposure was uncontrolled.

Because the exposure history of the fish is unknown, it is difficult to make well informed conclusions as to the resistance status of the species presented in Appendix 7. None the less, the detection of viral DNA in such species is important information as it provides an indication that the virus can enter these fish, and persist in their tissues.

The use of PCR to identify the presence of the virus in fish tissues also raises important considerations. The PCR tests used to detect CyHV-3 in the species presented in Appendix 7 have the power to identify a fragment of DNA specific to CyHV-3. However, as the PCR test does not detect viable virus, it is plausible that fragments of DNA or unviable virus could be present in host tissues, without the presence of viable virus. Because of this, Yuasa rt-PCR is sometimes used to detect replicating CyHV-3. However, the analytical responsiveness of this assay is unknown, and it may not

detect the virus when there is a very low concentration of replicating virus. Furthermore, the Yuasa rt-PCR cannot detect latent, or non-replicating infections. As a consequence, conventional PCR assays, targeting viral DNA are the most commonly used detection methods.

Because a positive PCR test (targeting genomic DNA) can only confirm the presence of a specific segment of DNA, it cannot confirm that a species is not-resistant to the virus. To ensure clarity in the information presented, Appendix 7 provides a summary of those species that have been assessed for the presence of CyHV-3, with findings presented as "detected" or "not-detected".

Of the 31 species for which wild fish have been subjected to direct detection methods targeting CyHV-3 DNA, 23 species, from six taxonomic families and 6 orders have returned positive results. Those species that have tested positive for CyHV-3 are: Russian sturgeon (*Acipenser gueldenstaedtii*), Atlantic sturgeon (*Acipenser oxyrinchus*), Common bream (*Abramis brama*), Common barbell (*Barbus barbus*), Goldfish (*Carassius auratus*), Crucian carp (*Carassius carassius*), Common nase (*Chondrostoma nasus*), Spined loach (*Cobitis taenia*), Grass carp (*Ctenopharyngodon idella*), Gudgeon (*Gobio gobio*), Belica (*Leucaspius delineates*), Blue back ide (*Leuciscus idus*), Common dace (*Leuciscus leuciscus*), Common roach (*Rutilus rutilus*), European chub (*Squalius cephalus*), Tench (*Tinca tinca*), Vimba bream (*Vimba vimba*), Northern pike (*Esox lucius*), European perch (*Perca fluviatilis*), Zander (*Sander lucioperca*), European bullhead (*Cottus gobio*), Sheatfish (*Ictalurus melas*), Bristlenose catfish (*Ancistrus sp.*) (Bergmann et al., 2009; Bergmann, et al., 2007; El-Matbouli, Saleh, et al., 2007; Fabian et al., 2013; Kempter & Bergmann, 2007; Kempter et al., 2009; Kempter. et al., 2008; Sadler et al., 2008).

While the resistance status of these 23 species must remain uncertain, when coupled with the information presented in Appendix 6, the detection of CyHV-3 in wild fish from a high number of species, representing diverse taxa, is indicative of a wider trend. That is, that the virus appears to be able to enter into and possibly persist in a diverse range of fish species, and that in many cases, the virus can subsequently be transmitted to naïve common carp. This is most interesting, given that the virus has not been implicated as the causative of severe clinical signs in any species other than common carp, while minor clinical signs have only been reported in goldfish, crucian carp and common roach. Thus, although the virus appears to be capable of entering into and persisting in a wide range of species, from diverse taxa, it appears unlikely that the virus causes severe disease or mortality in any species other than common carp.

McColl et al. (2017) also investigated the resistance of a range of endemic Australian species to CyHV-3. The results presented by McColl et al. (2017) are somewhat difficult to interpret as Gilad q-PCR and Yuasa rt-PCR data is not presented for individual fish species, but for each discrete trial, in which numerous fish species were assessed. McColl et al. (2017) instead, appears to focus on mortality data and the absence of clinical signs in non-common carp species exposed to the virus. The evidence presented by McColl et al. (2017) indicates that the virus does not affect any of the NTS assessed. However, because of the way the results are presented, it is difficult to make a clear assessment as to whether the NTS were infected by the virus subclinically.

High rates of positive Gilad q-PCR results were observed in trials that investigated Murray cod, golden perch and silver perch (McColl et al., 2017). The number of positive results from each species is unclear. However, Gilad q-PCR data is presented for a single trial in which only bony bream were assessed. For this species, ten out of 30 fish exposed to the virus tested positive to the virus by Gilad q-PCR. While the study was affected by the frequent occurrence of false positives in Gilad q-PCR testing, the authors state that these were reduced in the later trials of the study as experimental

protocols were improved. It appears that bony bream were assessed in the last trial performed and that false positives should be less likely in this trial. If this is indeed the case, it appears likely that CyHV-3 DNA was present in a large number of bony bream. This is insufficient to indicate that bony bream are not-resistant to infection, but further trials should be performed to determine if the presence of viral DNA in bony bream is indicative of a multiplying or otherwise developing or latent viral infection in this non-common carp species.

McColl et al. (2017) applied Yuasa rt-PCR to determine if viral replication was occurring in bony bream and other non-common carp species. Non-common carp species were always negative by Yuasa rt-PCR. However, as previously discussed, the analytical responsiveness of the Yuasa rt-PCR is uncertain and it is possible that this PCR may return false negatives when the virus is present at low concentrations. It is also possible that the virus can establish a latent, or temporarily non-replicating infection in bony bream and other non-common carp species, which would not be detected by Yuasa rt-PCR.

Unfortunately, McColl et al. (2017) did not apply a prescribed repeatable external stressor to optimise direct detection methods (Chapter 5), use the highly accurate Adapted Gilad q-PCR and Bergmann sn-PCR assays, apply the Prescott RPA assay to detect latent CyHV-3, or appraise viral isolation in cell culture using a range of different cell lines (including the newly developed KB cell line (Wang et al., 2018)) (as discussed in Chapter 3). The potential for exposed fish to infect naïve common carp was also not assessed. This makes it somewhat difficult to assert with confidence that all fish assessed by were indeed resistant to CyHV-3, as the authors assert. As such, the resistance status of those fish assessed by McColl et al. (2017) must remain ambiguous.

Collectively, it appears highly likely that CyHV-3 can enter a range of host species, from diverse taxa, and that the virus can persist in these species before being transmitted to naïve common carp. This observation has led some authors to question if there is any species specificity in infections with CyHV-3 (Bergmann et al., 2016). There is strong evidence that CyHV-3 can enter rainbow trout, establish an active replicating infecting and be transmitted from these rainbow trout to naïve common carp where it can cause clinical signs and mortality (Bergmann et al., 2016).

The suggestion of viral persistence in rainbow trout, and other non-common carp species, is important as this may be a critical predetermining factor that could theoretically favour spill over infections or species jumps to occur (Flanagan et al., 2012; McColl et al., 2016).

Based on the findings of this review, it is clear that CyHV-3 has been detected in a wide range of fish species, from diverse taxa, and it is highly likely that in many cases the virus can be transmitted from non-common carp species to recipient carp through cohabitation. This is indicative that the virus can establish a multiplying or otherwise developing or latent viral infection in these species.

There are no clear effects of taxonomy on the potential for CyHV-3 to infect fish. The virus has been detected in 33 species of fish from a diverse range of taxonomic families. However, clinical signs have only been observed in cyprinids. Common carp are highly vulnerable to the effects of the virus, with mortality reaching up to 100%. Mild clinical signs are also reported in goldfish, crucian carp and common roach, all members of the cyprinidae family. By contrast, no clinical signs have been reported as being observed in fish from any other taxonomic family.

This may indicate that cyprinids are the only family of fish that are vulnerable to CyHV-3. However, without additional experimental work, it is impossible to rule out clinical effects in taxonomic

families that have not yet been assessed. It may be that family-specific factors affect the vulnerability of non-cyprinid fish to CyHV-3, but this is currently unknown.

In general, it appears that CyHV-3 may have the potential to infect a number of species and can be transmitted from these fish to common carp. This is indicative, although not confirmative, of a multiplying or otherwise developing or latent viral infection. Further research is required to confirm that the virus can establish such an infection in non-common carp species and be transmitted to recipient common carp. This may be achieved through the application of stressors in non-common carp carrier species to increase the rate of viral replication and shedding, and through the application of stressors in recipient common carp to increase viral replication and increase the likelihood of clinical signs developing.

Further research is required to better understand the potential for CyHV-3 to establish viral infection in non-common carp species. However, based on the available evidence in the scientific literature, it appears likely that CyHV-3 can be detected and may infect a range of species from diverse taxa. More specific diagnostic test sensitivity and specificity is required during exposure testing to determine the nature of the viral host interaction in NTS. Presence of viable virus within NTS in the context of the NCCP, may also increase the chances of spill over infections and species jumps to non-common carp species. This factor should be carefully considered prior to additional resistance testing of NTS in the NCCP.

7.2. Conclusions and recommendations

It appears likely that a range of geographically disparate locations, including almost all freshwater and some nearshore ecosystems in Australia would be exposed to CyHV-3 post any strategic freshwater release. The rapid and wide-reaching spread of CyHV-3 around much of the globe demonstrates the ability of the virus to spread across vast distances in relatively short periods of time. It is likely that this rapid spread is promoted by the translocation of infected common carp, other carrier species of fish, aquatic invertebrates and water and plankton.

Based on the detection of CyHV-3, or evidence of the presence of CyHV-3, in a wide range of fish species from diverse taxa, and the ability for the virus to be transmitted from a range of non-common carp species to naïve common carp, it is possible that some endemic Australian NTS may be similar in there interactions with CyHV-3 post exposure. This does not suggest that these species will be vulnerable to the effects of the virus during short-term clinical trials. However, it does raise some concerns around the potential for longer-term clinical effects and may favour the occurrence of spillover infections or species jumps. The likelihood of this occurring is impossible to predict and therefore further NTS exposure testing is indicated.

Based on the available evidence, severe clinical signs are restricted to common carp. And while the virus can cause minor clinical signs in goldfish, crucian carp and common roach, severe clinical signs and mortality have not been observed in non-common carp species. Based on this observation, it appears unlikely that endemic Australian species will experience severe clinical signs or mortality due to infection with CyHV-3. It must be stated, however, that this assessment is based solely on previously published scientific studies that have failed to show evidence of clinical signs in any species other than common carp, goldfish, crucian carp and common roach. We also reiterate that the only way to comprehensively assess the resistance status of NTS is to undertake further rigorous experimental work.

If further NTS resistance testing is to be carried out by the FRDC, a process must be developed that selects NTS based on a logical set of criteria. These criteria must facilitate strong inferences to be made as to the resistance status of all other endemic Australian NTS.

Based on this review, it appears unlikely that taxonomy effects the potential for CyHV-3 to infect fish. However, taxonomy does appear to influence the likelihood of fish experiencing clinical signs associated with CyHV-3 infection. The only species other than common carp in which clinical signs have been associated with CyHV-3 infection were all members of the cyprinidae family. This may imply that clinical signs are restricted to members of this family, however, it is unclear what is driving this relationship.

The ability of CyHV-3 to cause clinical signs in cyprinid fish may be due to the presence of virus receptors, or it may be due to morphological or physiological features that permit infection and/or the development of clinical signs. Immunological factors may also play an important role in the development of clinical signs. These factors may be related to taxonomy, however, until the factors associated with resistance and vulnerability are known, it is impossible to determine the effects of taxonomy. Thus, although the only fish known to be affected by CyHV-3 are cyprinids, it is impossible to state with confidence that other taxonomic families or orders are not vulnerable to the effects of the virus.

As a consequence, it appears to be important that future NTS assessed in the NCCP, should include representative species from all taxonomic families likely to be exposed to the virus in Australian

waters. This will provide additional evidence that the virus cannot cause clinical signs in endemic Australian fish populations.

Other factors may also play an important role in selection of species for testing. In addition to taxonomy, environmental, social and economic factors may contribute to the selection of species to be assessed in resistance testing.

It may be considered particularly important to assess the potential for CyHV-3 to infect species of environmental significance. This may include species that are considered threatened or vulnerable by the International Union for Conservation of Nature and Natural Resources (IUCN) as well as species that are considered to play a crucial role in maintaining ecosystem balance.

Socially important species may also warrant special consideration as the infection of such species may have significant social impacts. This may include species that are considered to be important by recreational fishers or other users of natural aquatic systems.

It may also be critical that economically important species are assessed. If commercially harvested species of fish are vulnerable to CyHV-3, this may affect their abundance and thereby the commercial capture of these species. Aquaculture species may also be significantly affected if they are vulnerable to the virus. However, the implications of the virus to commercially important species are not restricted to the ability of CyHV-3 to cause disease in these species. Even if such fish are infected subclinically, this may have critical implications to such species gaining access to important market sectors. Currently CyHV-3 is listed by the OIE, and the international trade of fish carrying the virus may be subjected to rigorous quarantine restrictions. Thus, the detection of CyHV-3 in commercially harvested or cultivated species may affect the ability of Australian fisheries and aquaculture industries to export such species.

Environmental, social and economic factors may not directly influence the likelihood of a species becoming infected by CyHV-3. However, it may have a considerable bearing on the severity of implications, should they become infected from a planned release of the virus. Addressing the resistance status of such species may play a critical role, not only in ensuring that the NCCP has minimal impacts on ecological communities, but also in ensuring stakeholder engagement and support of the project.

Based on this review at least some endemic Australian non-common carp species are yet to be determined definitively resistant to CyHV-3 infection. It is therefore recommended that these species previously tested with equivocal results of resistance, be re-tested and in addition other species should be assessed in future NTS resistance testing performed as part of the NCCP. Species representing all taxonomic families present in Australian freshwater and nearshore ecosystems should be assessed for resistance to CyHV-3. Additionally, environmentally, socially and economically important species inhabiting these ecosystems should be assessed to determine if the virus can enter these species and establish a multiplying or otherwise developing or latent viral infection.

8. Sectional Recommendations

Chapter 3 What is the most suitable diagnostic approach to determine the resistance status of NTS to CyHV-3 infection?

It is recommended that a broad-based diagnostic protocol should be developed for use in future studies that assess the resistance of NTS to CyHV-3 infection.

The protocol should include clinical signs, histopathology, virus isolation in cell culture, PCR and RPA diagnostic procedures, as detailed above.

The following molecular tests are recommended as the most suitable to be included in the broad based approach for the identification of CyHV-3 infection.

For the detection of **CyHV-3 genome** in fish tissues:

- Adapted Gilad q-PCR – according to the method of Gilad et al. (2004), modified by Bergmann, et al., (2010).

And

- Bergmann sn-PCR assay – according to Bergmann, et al. ,(2010).

For the detection of actively replicating CyHV-3:

- Yuasa rt-PCR according to Yuasa et al. (2012). (with further Fit for Purpose validation)

For the detection of latent CyHV-3 infection:

- Prescott RPA assay developed by Prescott et al. (2016).

Chapter 4 Occurrence fo unexplained mortalities and false positives when testing the resistance of NTS to CyHV-3 infection.

Every effort must be made to prevent the occurrence of unexplained mortalities and false positives and in studies that assess the resistance status of NTS to CyHV-3 in the NCCP. This is done by:

Identifying a causal diagnosis for all mortalities (or at least a statistically significant number of the affected cohort) that occur during the course of any exposure testing to assess the resistance status of NTS to CyHV-3 infection in the NCCP.

and

False positives should be avoided by ensuring that sampling procedures are designed to prevent their occurrence and if they do occur an explanation for their occurrence should be identified

and

A weighted decision matrix for the determination of resistance status is necessary to enable a balanced assessment of diagnostic results.

Chapter 5 Application of stressors to resistance testing of NTS to CyHV-3

It is recommended that **temperature fluctuations**, as described by Takahara et al. (2014) and/or **netting stress**, according to the methods of Bergmann and Kempter (2011), be applied during studies that assess the resistance of NTS to CyHV-3 in the NCCP **AND** the stress is applied it must be undertaken under an explicitly designed protocol.

Additionally removal of skin mucus according to the methods of Pospichal et al. (2018) and Raj et al. (2011b) is also tentatively advised.

Chapter 6 Should all life stages of fish be evaluated in studies that investigate the resistance of NTS to CyHV-3 infection?

It is recommended that larvae, juveniles and adults of NTS of fish be assessed for their resistance to CyHV-3 infection.

Chapter 7 Should future CyHV-3 resistance testing include a wider range of NTS?

It is recommended that in addition to those species previously assessed for resistance to CyHV-3, additional species should be assessed in future NTS resistance testing performed as part of the NCCP. Species representing all **taxonomic families** present in Australian freshwater and nearshore ecosystems should be assessed for resistance to CyHV-3.

Additionally, **environmentally, socially and economically important species** inhabiting these ecosystems should be specifically assessed to determine if the virus can enter these species and establish a multiplying or otherwise developing or latent viral infection.

The scope of species selection will be determined by risk based assessment by the NCCP governed by practicality.

9. References

Ahne, W. (1986). The influence of environmental temperature and infection route on the immune response of carp (Cyprinus carpio) to spring viremia of carp virus (SVCV). *Veterinary Immunology and Immunopathology, 12*(1), 383-386. doi: https://doi.org/10.1016/0165-2427(86)90144-3

Ahne, W., Bjorklund, H., Essbauer, S., Fijan, N., Kurath, G., & Winton, J. (2002). Spring viremia of carp (SVC). *Diseases of aquatic organisms, 52*(3), 261-272.

Aoki, I. Hirono, K. Kurokawa, H. Fukuda, R. Nahary, A. Eldar, A.J. Davison, T.B. Waltzek, H. Bercovier, R.P. Hedrick (2007) Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide J. Virol., 81, pp. 5058-5065

Ashraf, U., Lu, Y., Lin, L., Yuan, J., Wang, M., & Liu, X. (2016). Spring viraemia of carp virus: recent advances. *Journal of General Virology, 97*(5), 1037-1051. doi: doi:10.1099/jgv.0.000436

Becker, J. A., Tweedie, A., Rimmer, A., Landos, M., Lintermans, M., & Whittington, R. J. (2014). Incursions of Cyprinid herpesvirus 2 in goldfish populations in Australia despite quarantine practices. *Aquaculture, 432*, 53-59. doi: https://doi.org/10.1016/j.aquaculture.2014.04.020

Becker, J. A., Ward, M. P., & Hick, P. M. An epidemiologic model of koi herpesvirus (KHV) biocontrol for carp in Australia. *Australian Zoologist, 0*(0), null. doi: 10.7882/az.2018.038

Bercovier, H., Fishman, Y., Nahary, R., Sinai, S., Zlotkin, A., Eyngor, M., . . . Hedrick, R. P. (2005). Cloning of the koi herpesvirus (KHV) gene encoding thymidine kinase and its use for a highly sensitive PCR based diagnosis. *Bmc Microbiology, 5*. doi: 10.1186/1471-2180-5-13

Bergmann, S., & Kempter, J. (2011). Detection of koi herpesvirus (KHV) after re-activation in persistently infected common carp (Cyprinus carpio L.) using non-lethal sampling methods. *Bull Eur Assoc Fish Pathol, 31*, 92-100.

Bergmann, S., Kempter, J., Sadowski, J., & Fichtner, D. (2006). First detection, confirmation and isolation of koi herpesvirus (KHV) in cultured common carp (Cyprinus carpio L.) in Poland. *BULLETIN-EUROPEAN ASSOCIATION OF FISH PATHOLOGISTS, 26*(2), 97.

Bergmann, S., Lutze, P., Schütze, H., Fischer, U., Dauber, M., Fichtner, D., & Kempter, J. (2010). Goldfish (Carassius auratus auratus) is a susceptible species for koi herpesvirus (KHV) but not for KHV disease (KHVD). *Bulletin of the European Association of Fish Pathologists, 30*(2), 74-84.

Bergmann, S., Schütze, H., Fischer, U., Fichtner, D., Riechardt, M., Meyer, K., . . . Kempter, J. (2009). Detection of koi herpes virus (KHV) genome in apparently healthy fish. *Bulletin of the European Association of Fish Pathologists, 29*(5), 145-152.

Bergmann, S. M., Cieslak, M., Fichtner, D., Dabels, J., & Monaghan, S. (2016). Is There Any Species Specificity in Infections with Aquatic Animal Herpesviruses. *The Koi Herpesvirus (KHV): An Alloherpesvirus Model. Fish Aquac J, 7*(169), 2.

Bergmann, S. M., & Kempter, J. (2011). Detection of koi herpesvirus (KHV) after re-activation in persistently infected common carp (Cyprinus carpio L.) using non-lethal sampling methods. *Bulletin of the European Association of Fish Pathologists, 31*(3), 92-100.

Bergmann, S. M., Kempter, J., Sadowski, J., & Fichtner, D. (2006). First detection, confirmation and isolation of koi herpesvirus (KHV) in cultured common carp (Cyprinus carpio L.) in Poland. *Bulletin of the European Association of Fish Pathologists, 26*(2), 97-104.

Bergmann, S. M., Lutze, P., Schütze, H., Fischer, U., Dauber, M., Fichtner, D., & Kempter, J. (2010). Goldfish (Carassius auratus auratus) is a susceptible species for koi herpesvirus (KHV) but not for KHV disease (KHVD). *Bulletin of the European Association of Fish Pathologists, 30*(2), 74-84.

Bergmann, S. M., Riechardt, M., Fichtner, D., Lee, P., & Kempter, J. (2010). Investigation on the diagnostic sensitivity of molecular tools used for detection of koi herpesvirus. *Journal of Virological Methods, 163*(2), 229-233. doi: 10.1016/j.jviromet.2009.09.025

Bergmann, S. M., Sadowski, J., Kielpinski, M., Bartlomiejczyk, M., Fichtner, D., Riebe, R., . . . Kempter, J. (2010). Susceptibility of koi x crucian carp and koi x goldfish hybrids to koi herpesvirus (KHV) and the development of KHV disease (KHVD). *Journal of Fish Diseases, 33*(3), 267-272. doi: 10.1111/j.1365-2761.2009.01127.x

Bergmann, S. M., Sadowski, J., Kiełpiński, M., Bartłomiejczyk, M., Fichtner, D., Riebe, R., . . . Kempter, J. (2010). Susceptibility of koi × crucian carp and koi × goldfish hybrids to koi herpesvirus (KHV) and the development of KHV disease (KHVD). *Journal of Fish Diseases, 33*(3), 267-272. doi: 10.1111/j.1365-2761.2009.01127.x

Bergmann, S. M., Stumpf, P., Schutze, H., Fichtner, D., Sadowski, J., & Kempter, J. (2007). Similarities and heterogenicity of koi herpes virus (KHV) genome detected in ornamental fish without clinical signs. *Aquaculture, 272*, S245-S245. doi: 10.1016/j.aquaculture.2007.07.034

Bergmann, S. M., Stumpf, P., Schütze, H., Fichtner, D., Sadowski, J., & Kempter, J. (2007). Similarities and heterogenicity of koi herpes virus (KHV) genome detected in ornamental fish without clinical signs. *Aquaculture, 272*(Supplement 1), S245. doi: https://doi.org/10.1016/j.aquaculture.2007.07.034

Bootland, L. M., & Leong, J. A. C. (2011). Chapter 2 Infectious haematopoietic necrosis virus. In P. T. K. Woo & D. W. Bruno (Eds.), *Fish diseases and disorders. Volume 3: viral, bacterial and fungal infections.* (Vol. 3): CABI.

Borzák, R., Haluk, T., Bartha, D., & Doszpoly, A. (2018). Complete genome sequence and analysis of ictalurid herpesvirus 2. *Archives of Virology, 163*(4), 1083-1085. doi: 10.1007/s00705-017-3683-8

Boucher, P., & Baudin Laurencin, F. (1994). Sleeping disease (SD) of salmonids. *Bulletin of the European Association of Fish Pathologists (United Kingdom)*.

Boutier, M., Ronsmans, M., Rakus, K., Jazowiecka-Rakus, J., Vancsok, C., Morvan, L., . . . Vanderplasschen, A. (2015). Chapter Three - Cyprinid Herpesvirus 3: An Archetype of Fish Alloherpesviruses. In M. Kielian, K. Maramorosch, & T. C. Mettenleiter (Eds.), *Advances in Virus Research* (Vol. 93, pp. 161-256): Academic Press.

Boys, C. A., Rowland, S. J., Gabor, M., Gabor, L., Marsh, I. B., Hum, S., & Callinan, R. B. (2012). Emergence of Epizootic Ulcerative Syndrome in Native Fish of the Murray-Darling River System, Australia: Hosts, Distribution and Possible Vectors. *PLOS ONE, 7*(4), e35568. doi: 10.1371/journal.pone.0035568

Bradley, T. M., Medina, D., Chang, P., & McClain, J. (1989). Epizootic Epitheliotropic Disease of lake trout (Salvelinus namaycush): history and viral etiology. *Diseases of aquatic organisms, 7*, 195-201. doi: 10.3354/dao007195

Bretzinger, A., Fischer-Scherl, T., Oumouna, M., Hoffmann, R., & Truyen, U. (1999). Mass mortalities in Koi carp, Cyprinus carpio, associated with gill and skin disease. *Bulletin of the European Association of Fish Pathologists, 19*(5), 182-185.

Brydges, N. M., Boulcott, P., Ellis, T., & Braithwaite, V. A. (2009). Quantifying stress responses induced by different handling methods in three species of fish. *Applied Animal Behaviour Science, 116*(2), 295-301. doi: https://doi.org/10.1016/j.applanim.2008.09.003

Bylund, G., Valtonen, E. T., & Niemela, E. (1980). Observations on epidermal papillomata in wild and cultured Atlantic salmon Salmo salar L. in Finland. *Journal of Fish Diseases, 3*(6), 525-528. doi: doi:10.1111/j.1365-2761.1980.tb00438.x

CEFAS. (Unpublished).

Clouthier, S. C., McClure, C., Schroeder, T., Desai, M., Hawley, L., Khatkar, S., . . . Anderson, E. D. (2017). Diagnostic validation of three test methods for detection of cyprinid herpesvirus 3 (CyHV-3). *Diseases of aquatic organisms, 123*(2), 101-122. doi: 10.3354/dao03093

Costes, B., Raj, V. S., Michel, B., Fournier, G., Thirion, M., Gillet, L., . . . Vanderplasschen, A. (2009). The Major Portal of Entry of Koi Herpesvirus in Cyprinus carpio Is the Skin. *Journal of Virology, 83*(7), 2819-2830. doi: 10.1128/jvi.02305-08

Danek, T., Kalous, L., Vesely, T., Krasova, E., Reschova, S., Rylkova, K., . . . Knytl, M. (2012). Massive mortality of Prussian carp Carassius gibelio in the upper Elbe basin associated with herpesviral hematopoietic necrosis (CyHV-2). *Diseases of aquatic organisms, 102*(2), 87-95. doi: 10.3354/dao02535

Davidovich, M., Dishon, A., Ilouze, M., & Kotler, M. (2007). Susceptibility of cyprinid cultured cells to cyprinid herpesvirus 3. *Archives of Virology, 152*(8), 1541-1546. doi: 10.1007/s00705-007-0975-4

Davison, A. J., Kurobe, T., Gatherer, D., Cunningham, C., Korf, I., Fukuda, H., . . . Waltzek, T. B. (2013). Comparative Genomics of Carp Herpesviruses. *Journal of Virology, 87*(5), 2908-2922. doi: 10.1128/jvi.03206-12

Dixon, P. F., Joiner, C. L., Way, K., Reese, R. A., Jeney, G., & Jeney, Z. (2009). Comparison of the resistance of selected families of common carp, Cyprinus carpio L., to koi herpesvirus: Preliminary study. *Journal of Fish Diseases, 32*(12), 1035-1039. doi: 10.1111/j.1365-2761.2009.01081.x

Dong, C., Weng, S., Li, W., Li, X., Yi, Y., Liang, Q., & He, J. (2011). Characterization of a new cell line from caudal fin of koi, Cyprinus carpio koi, and first isolation of cyprinid herpesvirus 3 in China. *Virus Research, 161*(2), 140-149. doi: 10.1016/j.virusres.2011.07.016

Dorson, M., & Touchy, C. (1981). The influence of fish age and water temperature on mortalities of rainbow trout, Salmo gairdneri Richardson, caused by a European strain of infectious pancreatic necrosis virus. *Journal of Fish Diseases, 4*(3), 213-221. doi: doi:10.1111/j.1365-2761.1981.tb01128.x

Doszpoly, A., Karaseva, T. A., Waltzek, T. B., Kalabekov, I. M., & Shchelkunov, I. S. (2013). Atlantic salmon papillomatosis in Russia and molecular characterization of the associated herpesvirus. *Diseases of aquatic organisms, 107*(2), 121-127.

Eide, K. E., Miller-Morgan, T., Heidel, J. R., Kent, M. L., Bildfell, R. J., LaPatra, S., . . . Jin, L. (2011). Investigation of koi herpesvirus latency in koi. *Journal of Virology, 85*(10), 4954-4962.

El-Matbouli, M., Rucker, U., & Soliman, H. (2007). Detection of Cyprinid herpesvirus-3 (CyHV-3) DNA in infected fish tissues by nested polymerase chain reaction. *Diseases of aquatic organisms, 78*(1), 23-28. doi: 10.3354/dao01858

El-Matbouli, M., Saleh, M., & Soliman, H. (2007). Detection of cyprinid herpesvirus type 3 in goldfish cohabiting with cyhv-3-infected koi carp (Cyprinus carpio koi). *Veterinary Record, 161*(23), 792-793. doi: 10.1136/vr.161.23.792

El-Matbouli, M., & Soliman, H. (2011). Transmission of Cyprinid herpesvirus-3 (CyHV-3) from goldfish to naïve common carp by cohabitation. *Research in Veterinary Science, 90*(3), 536-539. doi: https://doi.org/10.1016/j.rvsc.2010.07.008

Fabian, M., Baumer, A., Adamek, M., & Steinhagen, D. (2016). Transmission of Cyprinid herpesvirus 3 by wild fish species – results from infection experiments. *Journal of Fish Diseases, 39*(5), 625-628. doi: 10.1111/jfd.12399

Fabian, M., Baumer, A., & Steinhagen, D. (2013). Do wild fish species contribute to the transmission of koi herpesvirus to carp in hatchery ponds? *Journal of Fish Diseases, 36*(5), 505-514. doi: 10.1111/jfd.12016

Fichi, G., Cardeti, G., Cocumelli, C., Vendramin, N., Toffan, A., Eleni, C., . . . Susini, F. (2013). Detection of Cyprinid herpesvirus 2 in association with an Aeromonas sobria infection of Carassius carassius (L.), in Italy. *Journal of Fish Diseases, 36*(10), 823-830. doi: doi:10.1111/jfd.12048

Flanagan, M. L., Parrish, C. R., Cobey, S., Glass, G. E., Bush, R. M., & Leighton, T. J. (2012). Anticipating the Species Jump: Surveillance for Emerging Viral Threats. *Zoonoses and Public Health, 59*(3), 155-163. doi: doi:10.1111/j.1863-2378.2011.01439.x

Fournier, G., Boutier, M., Raj, V. S., Mast, J., Parmentier, E., Vanderwalle, P., . . . Vanderplasschen, A. (2012). Feeding Cyprinus carpio with infectious materials mediates cyprinid herpesvirus 3 entry through infection of pharyngeal periodontal mucosa. *Veterinary Research, 43*. doi: 10.1186/1297-9716-43-6

Furusawa, R., Okinaka, Y., Uematsu, K., & Nakai, T. (2007). Screening of freshwater fish species for their susceptibility to a betanodavirus. *Diseases of aquatic organisms, 77*(2), 119-125.

Gaede, L., Steinbruck, J., Bergmann, S. M., Jager, K., Grafe, H., Schoon, H. A., . . . Truyen, U. (2017). Koi herpesvirus infection in experimentally infected common carp Cyprinus carpio (Linnaeus, 1758) and three potential carrier fish species Carassius carassius (Linnaeus, 1758); Rutilus rutilus (Linnaeus, 1758); and Tinca tinca (Linnaeus, 1758) by quantitative real-time PCR and in-situ hybridization. *Journal of Applied Ichthyology, 33*(4), 776-784. doi: 10.1111/jai.13368

Gaede, L., Steinbrück, J., Bergmann, S. M., Jäger, K., Gräfe, H., Schoon, H. A., . . . Truyen, U. (2017). Koi herpesvirus infection in experimentally infected common carp Cyprinus carpio (Linnaeus, 1758) and three potential carrier fish species Carassius carassius (Linnaeus, 1758); Rutilus rutilus (Linnaeus, 1758); and Tinca tinca (Linnaeus, 1758) by quantitative real-time PCR and in-situ hybridization. *Journal of Applied Ichthyology, 33*(4), 776-784. doi: 10.1111/jai.13368

Garver, K. A., Ai-Hussinee, L., Hawley, L. M., Schroeder, T., Edes, S., LePage, V., . . . Lumsden, J. S. (2010). Mass mortality associated with koi herpesvirus in wild common carp in Canada. *Journal of Wildlife Diseases, 46*(4), 1242-1251. doi: 10.7589/0090-3558-46.4.1242

Gilad, O., Yun, S., Adkison, M. A., Way, K., Willits, N. H., Bercovier, H., & Hedrick, R. P. (2003). Molecular comparison of isolates of an emerging fish pathogen, koi herpesvirus, and the effect of water temperature on mortality of experimentally infected koi. *Journal of General Virology, 84*(10), 2661-2667. doi: doi:10.1099/vir.0.19323-0

Gilad, O., Yun, S., Andree, K. B., Adkison, M. A., Zlotkin, A., Bercovier, H., . . . Hedrick, R. P. (2002). Initial characteristics of koi herpesvirus and development of a polymerase chain reaction assay to detect the virus in koi, Cyprinus carpio koi. *Diseases of aquatic organisms, 48*(2), 101-108. doi: 10.3354/dao048101

Gilad, O., Yun, S., Zagmutt-Vergara, F. J., Leutenegger, C. M., Bercovier, H., & Hedrick, R. P. (2004). Concentrations of a Koi herpesvirus (KHV) in tissues of experimentally-infected Cyprinus carpio koi as assessed by real-time TaqMan PCR. *Diseases of aquatic organisms, 60*(3), 179-187.

Gilad, O., Yun, S., Zagmutt-Vergara, F. J., Leutenegger, C. M., Bercovier, H., & Hedrick, R. P. (2004). Concentrations of a Koi herpesvirus (KHV) in tissues of experimentally infected Cyprinus carpio koi as assessed by real-time TaqMan PCR. *Diseases of aquatic organisms, 60*(3), 179-187. doi: 10.3354/dao060179

Glenney, G. W., Barbash, P. A., & Coll, J. A. (2016). A Quantitative Polymerase Chain Reaction Assay for the Detection and Quantification of Epizootic Epitheliotropic Disease Virus (EEDV; Salmonid Herpesvirus 3). *Journal of Aquatic Animal Health, 28*(1), 56-67. doi: doi:10.1080/08997659.2015.1121935

Gotesman, M., Abd-Elfattah, A., Kattlun, J., Soliman, H., & El-Matbouli, M. (2014). Investigating the interactions of Cyprinid herpesvirus-3 with host proteins in goldfish Carassius auratus. *Journal of Fish Diseases, 37*(9), 835-841. doi: 10.1111/jfd.12172

Gray, W. L., Mullis, L., LaPatra, S. E., Groff, J. M., & Goodwin, A. (2002). Detection of koi herpesvirus DNA in tissues of infected fish. *Journal of Fish Diseases, 25*(3), 171-178. doi: 10.1046/j.1365-2761.2002.00355.x

Groff, J. M., LaPatra, S. E., Munn, R. J., & Zinkl, J. G. (1998). A viral epizootic in cultured populations of juvenile goldfish due to a putative herpesvirus etiology. *Journal of Veterinary Diagnostic Investigation, 10*(4), 375-378.

Grotmol, S., Bergh, Ø., & Totland, G. K. (1999). Transmission of viral encephalopathy and retinopathy (VER) to yolk-sac larvae of the Atlantic halibut Hippoglossus hippoglossus: occurrence of nodavirus in various organs and a possible route of infection. *36*(2). doi: 10.3354/dao036095

Gunimaladevi, I., Kono, T., Venugopal, M., & Sakai, M. (2004). Detection of koi herpesvirus in common carp, Cyprinus carpio L., by loop-mediated isothermal amplification. *Journal of Fish Diseases, 27*(10), 583-589.

Haenen, O., & Hedrick, R. (2006). Koi herpesvirus workshop. *Bulletin of the European Association of Fish Pathologists, 26*(1), 26-37.

Haenen, O., Way, K., Bergmann, S., & Ariel, E. (2004). The emergence of koi herpesvirus and its significance to European aquaculture. *Bulletin of the European Association of Fish Pathologists, 24*(6), 293-307.

Haenen, O. L. M., Way, K., Bergmann, S. M., & Ariel, E. (2004). The emergence of koi herpesvirus and its significance to European aquaculture. *Bulletin of the European Association of Fish Pathologists, 24*(6), 293-307.

Hanson, L., A. , Rudis, M., R. , & Petrie-Hanson, L. (2004). Susceptibility of channel catfish fry to Channel Catfish Virus (CCV) challenge increases with age. *Diseases of aquatic organisms, 62*(1-2), 27-34.

Hanson, L., Doszpoly, A., van Beurden, S. J., de Oliveira Viadanna, P. H., & Waltzek, T. (2016). Chapter 9 - Alloherpesviruses of Fish. In F. S. B. Kibenge & M. G. Godoy (Eds.), *Aquaculture Virology* (pp. 153-172). San Diego: Academic Press.

Hanson, L., Doszpoly, A., van Beurden, S. J., Viadanna, P. H. O., & Waltzek, T. (2016). Alloherpesviruses of Fish *Aquaculture Virology* (pp. 153-172).

Hedrick, R., Gilad, O., Yun, S., Spangenberg, J., Marty, G., Nordhausen, R., . . . Eldar, A. (2000). A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. *Journal of Aquatic Animal Health, 12*(1), 44-57.

Hedrick, R., McDowell, T., Groff, J., Yun, S., & Wingfield, W. (1991). Isolation of an epitheliotropic herpesvirus from white sturgeon Acipenser transmontanus. *Diseases of aquatic organisms, 11*, 49-56. doi: 10.3354/dao011049

Hedrick, R. P., Gilad, O., Yun, S., Spangenberg, J. V., Marty, G. D., Nordhausen, R. W., . . . Eldar, A. (2000). A Herpesvirus Associated with Mass Mortality of Juvenile and Adult Koi, a Strain of Common Carp. *Journal of Aquatic Animal Health, 12*(1), 44-57. doi: 10.1577/1548-8667(2000)012<0044:AHAWMM>2.0.CO;2

Hedrick, R. P., Groff, J. M., & McDowell, T. (1987). Response of Adult Channel Catfish to Waterborne Exposures of Channel Catfish Virus. *The Progressive Fish-Culturist, 49*(3), 181-187. doi: 10.1577/1548-8640(1987)49<181:ROACCT>2.0.CO;2

Hedrick, R. P., McDowell, T. S., Gilad, O., Adkison, M., & Bovo, G. (2003). Systemic herpes-like virus in catfish Ictalurus melas (Italy) differs from Ictalurid herpesvirus 1 (North America). *Diseases of aquatic organisms, 55*(2), 85-92.

Hedrick, R. P., Waltzek, T. B., & McDowell, T. S. (2006). Susceptibility of Koi Carp, Common Carp, Goldfish, and Goldfish × Common Carp Hybrids to Cyprinid Herpesvirus-2 and Herpesvirus-3. *Journal of Aquatic Animal Health, 18*(1), 26-34. doi: 10.1577/H05-028.1

Hedrick, R. P., Waltzek, T. B., & McDowell, T. S. (2006). Susceptibility of koi carp, common carp, goldfish, and goldfish x common carp hybrids to cyprinid herpesvirus-2 and herpesvirus-3. *Journal of Aquatic Animal Health, 18*(1), 26-34. doi: 10.1577/H05-028.1

Hick, P., Becker, J., & Whittington, R. (2016). Chapter 8 - Iridoviruses of Fish. In F. S. B. Kibenge & M. G. Godoy (Eds.), *Aquaculture Virology* (pp. 127-152). San Diego: Academic Press.

Honjo, M.N., Minamoto, T., Kawabata, Z., (2011) Reservoirs of Cyprinid herpesvirus 3 (CyHV-3) DNA in sediments of natural lakes and ponds

VETERINARY MICROBIOLOGY Volume 155, Issue 2-4: 183-190

Huttenhuis, H. B. T., Taverne-Thiele, A. J., Grou, C. P. O., Bergsma, J., Saeij, J. P. J., Nakayasu, C., & Rombout, J. H. W. M. (2006). Ontogeny of the common carp (Cyprinus carpio L.) innate immune system. *Developmental & Comparative Immunology, 30*(6), 557-574. doi: https://doi.org/10.1016/j.dci.2005.08.001

Ilouze, M., Davidovich, M., Diamant, A., Kotler, M., & Dishon, A. (2011). The outbreak of carp disease caused by CyHV-3 as a model for new emerging viral diseases in aquaculture: a review. *Ecological Research, 26*(5), 885-892. doi: 10.1007/s11284-010-0694-2

Ishioka, T., Yoshizumi, M., Izumi, S., Suzuki, K., Suzuki, H., Kozawa, K., . . . Kimura, H. (2005). Detection and sequence analysis of DNA polymerase and major envelope protein genes in koi herpesviruses derived from Cyprinus carpio in Gunma prefecture, Japan. *Veterinary Microbiology, 110*(1-2), 27-33. doi: 10.1016/j.vetmic.2005.07.002

Ito, T., Kurita, J., Sano, M., & Iida, T. (2007). Difference in the susceptibility of strains and developmental stages of carp to koi herpesvirus. *Fish Pathology, 42*(4), 233.

Ito, T., Kurita, J., & Yuasa, K. (2014). Differences in the susceptibility of Japanese indigenous and domesticated Eurasian common carp (Cyprinus carpio), identified by mitochondrial DNA typing, to cyprinid herpesvirus 3 (CyHV-3). *Veterinary Microbiology, 171*(1), 31-40. doi: https://doi.org/10.1016/j.vetmic.2014.03.002

Ito, T., Sano, M., Kurita, J., Yuasa, K., & Iida, T. (2007). Carp Larvae Are Not Susceptible to Koi Herpesvirus. *魚病研究, 42*(2), 107-109. doi: 10.3147/jsfp.42.107

Jeffery, K. R., Bateman, K., Bayley, A., Feist, S. W., Hulland, J., Longshaw, C., . . . Way, K. (2007). Isolation of a cyprinid herpesvirus 2 from goldfish, Carassius auratus (L.), in the UK. *Journal of Fish Diseases, 30*(11), 649-656. doi: doi:10.1111/j.1365-2761.2007.00847.x

Jia, Z., Wang, S., Bai, S., Ge, Y., Li, C., Hu, X., . . . Shi, L. (2018). Survival Rate and Immunological Responses of Mirror Carp Selective Breeding Generations to CyHV-3. *Journal of the World Aquaculture Society*. doi: 10.1111/jwas.12511

Jung, S. J., & Miyazaki, T. (1995). Herpesviral haematopoietic necrosis of goldfish, Carassius auratus (L.). *Journal of Fish Diseases, 18*(3), 211-220. doi: doi:10.1111/j.1365-2761.1995.tb00296.x

Kamimura, S., Hagi, T., Kurata, S., Takatsu, K., Sogo, H., Hoshino, T., & Nakamura, K. (2007). Evaluation of quenching probe (QProbe)-PCR assay for quantification of the koi herpes virus (KHV). *Microbes and Environments, 22*(3), 223-231. doi: 10.1264/jsme2.22.223

Kancharla, S. R., & Hanson, L. A. (1996). Production and shedding of channel catfish virus (CCV) and thymidine kinase negative CCV in immersion exposed channel catfish fingerlings. *Diseases of aquatic organisms, 27*(1), 25-34.

Kane, D. A. (1998). Cell cycles and development in the embryonic zebrafish *Methods in cell biology* (Vol. 59, pp. 11-26): Elsevier.

Kempter, J., & Bergmann, S. M. (2007). Detection of koi herpesvirus (KHV) genome in wild and farmed fish from Northern Poland. *Aquaculture, 272*, S275. doi: https://doi.org/10.1016/j.aquaculture.2007.07.102

Kempter, J., Kielpinski, M., Panicz, R., Sadowski, J., Mysłowski, B., & Bergmann, S. (2012). Horizontal transmission of koi herpes virus (KHV) from potential vector species to common carp. *Bull Eur Assoc Fish Pathol, 32*, 212-219.

Kempter, J., Sadowski, J., Schutze, H., Fischer, U., Dauber, M., Fichtner, D., . . . Bergmann, S. M. (2009). KOI HERPES VIRUS: DO ACIPENSERID RESTITUTION PROGRAMS POSE A THREAT TO CARP FARMS IN THE DISEASE-FREE ZONES? *Acta Ichthyologica et Piscatoria, 39*(2), 119-126. doi: 10.3750/aip2009.39.2.06

Kempter, J., Sadowski, J., Schütze, H., Fischer, U., Dauber, M., Fichtner, D., . . . Bergmann, S. M. (2009). Koi herpes virus: do acipenserid restitution programs pose a threat to carp farms in the disease-free zones? *Acta Ichthyologica et Piscatoria, 39*, 119-126.

Kempter., J., Kielpinski, M., Panicz, R., & Sadowski, J. (2008). Determination of the carrier species and the infection susceptibility for Koi-Herpes-Virus infections among selected cyprinid fish

species and their hybrids acquired from open waters and culture sites of the Odra River drainage. *Wydawnictwo naukowe Akademii Rolniczej w Szczecinie (Publishers of Scientific Agricultural University in Szczecin), Szczecin.*

Kempterl, J., Hofsoel, P., Panicz, R., & Bergmann, S. (2014). First detection of anguillid herpesvirus 1 (AngI—IVl) in European eel (Anguilla an gnilla) and imported American eel. *Bull. Eur. Ass. Fish Pathol, 34*(3), 87.

Kielpinski, M., Kempter, J., Panicz, R., Sadowski, J., Schutze, H., Ohlemeyer, S., & Bergmann, S. M. (2010). Detection of KHV in Freshwater Mussels and Crustaceans from Ponds with KHV History in Common Carp (Cyprinus carpio). *Israeli Journal of Aquaculture-Bamidgeh, 62*(1), 28-37.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental dynamics, 203*(3), 253-310.

Kimura, T., Yoshimizu, M., & Tanaka, M. (1983). Susceptibility of different fry stages of representative salmonid species to Oncorhynchus masou virus (OMV). . *Fish Pathology, 17*(4), 251-258. doi: 10.3147/jsfp.17.251

Kurita, J., & Nakajima, K. (2012). Megalocytiviruses. *Viruses, 4*(4), 521-538. doi: 10.3390/v4040521

Kurobe, T., Marcquenski, S., & Hedrick, R. P. (2009). PCR assay for improved diagnostics of epitheliotropic disease virus (EEDV) in lake trout Salvelinus namaycush. *Diseases of aquatic organisms, 84*(1), 17-24.

Langdon, J. S., Humphrey, J. D., Williams, L. M., Hyatt, A. D., & Westbury, H. A. (1986). First virus isolation from Australian fish: an iridovirus-like pathogen from redfin perch, Perca fluviatilis L. *Journal of Fish Diseases, 9*(3), 263-268. doi: 10.1111/j.1365-2761.1986.tb01011.x

LaPatra, S., Misk, E., Al-Hussinee, L., & Lumsden, J. S. (2016). Chapter 18 - Rhabdoviruses of Fish. In F. S. B. Kibenge & M. G. Godoy (Eds.), *Aquaculture Virology* (pp. 267-297). San Diego: Academic Press.

Lein, I., & Holmefjord, I. (1992). Age at first feeding of Atlantic halibut larvae. *Aquaculture, 105*(2), 157-164. doi: https://doi.org/10.1016/0044-8486(92)90127-7

Levraud, J.-P., Boudinot, P., Colin, I., Benmansour, A., Peyrieras, N., Herbomel, P., & Lutfalla, G. (2007). Identification of the Zebrafish IFN Receptor: Implications for the Origin of the Vertebrate IFN System. *The Journal of Immunology, 178*(7), 4385-4394. doi: 10.4049/jimmunol.178.7.4385

Lin, L., Chen, S., Russell, D. S., Lohr, C. V., Milston-Clements, R., Song, T., . . . Jin, L. (2017). Analysis of stress factors associated with KHV reactivation and pathological effects from KHV reactivation. *Virus Research, 240*, 200-206. doi: 10.1016/j.virusres.2017.08.010

López-Muñoz, A., Roca, F. J., Sepulcre, M. P., Meseguer, J., & Mulero, V. (2010). Zebrafish larvae are unable to mount a protective antiviral response against waterborne infection by spring viremia of carp virus. *Developmental & Comparative Immunology, 34*(5), 546-552. doi: https://doi.org/10.1016/j.dci.2009.12.015

Masuda, R. (2009). *Behavioral ontogeny of marine pelagic fishes with the implications for the sustainable management of fisheries resources*: Terrapub.

Mateus, A. P., Power, D. M., & Canário, A. V. M. (2017). Chapter 8 - Stress and Disease in Fish A2 - Jeney, Galina *Fish Diseases* (pp. 187-220): Academic Press.

Matsui, K., Honjo, M., Kohmatsu, Y., Uchii, K., Yonekura, R., & Kawabata, Z. (2008). Detection and significance of koi herpesvirus (KHV) in freshwater environments. *Freshwater Biology, 53*(6), 1262-1272. doi: 10.1111/j.1365-2427.2007.01874.x

McCleary, S., Giltrap, M., Henshilwood, K., & Ruane, N. (2014). Detection of salmonid alphavirus RNA in Celtic and Irish Sea flatfish. *Diseases of aquatic organisms, 109*(1), 1-7.

McColl, K., Sunarto, A., Slater, J., Bell, K., Asmus, M., Fulton, W., . . . Hoad, J. (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*(9), 1141-1153.

McColl, K. A., Cooke, B. D., & Sunarto, A. (2014). Viral biocontrol of invasive vertebrates: Lessons from the past applied to cyprinid herpesvirus-3 and carp (Cyprinus carpio) control in Australia. *Biological Control, 72*(Supplement C), 109-117. doi: https://doi.org/10.1016/j.biocontrol.2014.02.014

McColl, K. A., Sunarto, A., & Holmes, E. C. (2016). Cyprinid herpesvirus 3 and its evolutionary future as a biological control agent for carp in Australia. *Virology Journal, 13*(1), 206. doi: 10.1186/s12985-016-0666-4

McColl, K. A., Sunarto, A., Slater, J., Bell, K., Asmus, M., Fulton, W., . . . Crane, M. S. J. (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*(9), 1141-1153. doi: 10.1111/jfd.12591

McLoughlin, M. F., & Graham, D. A. (2007). Alphavirus infections in salmonids – a review. *Journal of Fish Diseases, 30*(9), 511-531. doi: doi:10.1111/j.1365-2761.2007.00848.x

Meyer, K., Bergmann, S. M., van der Marel, M., & Steinhagen, D. (2012). Detection of Koi herpesvirus: impact of extraction method, primer set and DNA polymerase on the sensitivity of polymerase chain reaction examinations. *Aquaculture Research, 43*(6), 835-842. doi: 10.1111/j.1365-2109.2011.02897.xMiller, B., & Kendall, A. W. (2009). *Early life history of marine fishes*: University of California Press.

Minamoto, T., Honjo, M. N., & Kawabata, Z. (2009). Seasonal Distribution of Cyprinid Herpesvirus 3 in Lake Biwa, Japan. *Applied and Environmental Microbiology, 75*(21), 6900-6904. doi: 10.1128/aem.01411-09

Minamoto, T., Honjo, M.N.,Yamanaka, H., et al. (2011) Detection of cyprinid herpesvirus-3 DNA in lake plankton, RESEARCH IN VETERINARY SCIENCE, Volume 90 Issue 3: 530-532

Monaghan, S., Thompson, K., Adams, A., & Bergmann, S. M. (2015). Sensitivity of seven PCRs for early detection of koi herpesvirus in experimentally infected carp, Cyprinus carpio L., by lethal and non-lethal sampling methods. *Journal of Fish Diseases, 38*(3), 303-319.

Monaghan, S., Thompson, K., Adams, A., Kempter, J., & Bergmann, S. (2015). Examination of the early infection stages of koi herpesvirus (KHV) in experimentally infected carp, Cyprinus carpio L. using in situ hybridization. *Journal of Fish Diseases, 38*(5), 477-489.

Monaghan, S. J., Thompson, K. D., Adams, A., & Bergmann, S. M. (2015). Sensitivity of seven PCRs for early detection of koi herpesvirus in experimentally infected carp, Cyprinus carpio L., by lethal and non-lethal sampling methods. *Journal of Fish Diseases, 38*(3), 303-319. doi: 10.1111/jfd.12235

Munday, B. L., Kwang, J., & Moody, N. (2002). Betanodavirus infections of teleost fish: a review. *Journal of Fish Diseases, 25*(3), 127-142. doi: doi:10.1046/j.1365-2761.2002.00350.x

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research, 28*(12), e63-e63. doi: 10.1093/nar/28.12.e63

Novotny, L., Pokorova, D., Reschova, S., Vicenova, M., Axmann, R., Vesely, T., & Mikler, J. R. (2010). First clinically apparent koi herpesvirus infection in the Czech Republic. *Bulletin of the European Association of Fish Pathologists, 30*(3), 85-91.

Nusbaum, K., & Grizzle, J. (1987). Uptake of channel catfish virus from water by channel catfish and bluegills. *American Journal of Veterinary Research, 48*(3), 375-377.

Nylund, A., Krossøy, B., Devold, M., Aspehaug, V., Steine, N., & Hovland, T. (1999). Outbreak of ISA during first feeding of salmon fry (Salmo salar). *Bulletin of the European Association of Fish Pathologists (United Kingdom)*.

Oidtmann, B, Dixon, P., Way, K., et al. (2018) Risk of waterborne virus spread - review of survival of relevant fish and crustacean viruses in the aquatic environment and implications for control measures, REVIEWS IN AQUACULTURE, Volume: 10; Issue 3: 641-669

OIE. (2017a). Criteria for listing species as susceptible to infection with a specific pathogen *OIE - Aquatic Animal Health Code*: OIE.
OIE. (2017b). Koi Herpes Virus Disease *Manual of Diagnostic Tests for Aquatic Animals*: OIE.
OIE. (2018). *Manual of Diagnostic Tests for Aquatic Animals*

(2018). Paris, France: World Organization for Animal Health (OIE).
Ortuño, J., Esteban, M. A., & Meseguer, J. (2001). Effects of short-term crowding stress on the gilthead seabream (Sparus aurata L.) innate immune response. *Fish & Shellfish Immunology, 11*(2), 187-197. doi: https://doi.org/10.1006/fsim.2000.0304
Osse, J., & Van den Boogaart, J. (1995). *Fish larvae, development, allometric growth, and the aquatic environment.* Paper presented at the ICES Marine Science Symposia.
Colin R. Parrish, C.R, Holmes C.R., Morens D.M., Park E. et al (2008) Cross-Species Virus Transmission and the Emergence of New
Epidemic Diseases, MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, p. 457–470 Vol. 72, No. 3 1092-2172/08/$08.000 doi:10.1128/MMBR.00004-08
Perelberg, A., Smirnov, M., Hutoran, M., Diamant, A., Bejerano, Y., & Kotler, M. (2003). Epidemilogical Description Of A New Viral Disease Afflicting Cultured Cyprinus Carpio In Israel.
Perelberg, A., Smirnov, M., Hutoran, M., Diamant, A., Bejerano, Y., & Kotler, M. (2003). Epidemiological description of a new viral disease afflicting cultured Cyprinus carpio in Israel. *Israeli Journal of Aquaculture-Bamidgeh, 55*(1), 5-12.
Piackova, V., Flajshans, M., Pokorova, D., Reschova, S., Gela, D., Cizek, A., & Vesely, T. (2013). Sensitivity of common carp, Cyprinus carpio L., strains and crossbreeds reared in the Czech Republic to infection by cyprinid herpesvirus 3 (CyHV-3; KHV). *Journal of Fish Diseases, 36*(1), 75-80. doi: 10.1111/jfd.12007
Pickering, C., & Byrne, J. (2014). The benefits of publishing systematic quantitative literature reviews for PhD candidates and other early-career researchers. *Higher Education Research & Development, 33*(3), 534-548. doi: 10.1080/07294360.2013.841651
Piepenburg, O., Williams, C. H., Stemple, D. L., & Armes, N. A. (2006). DNA Detection Using Recombination Proteins. *PLoS Biology, 4*(7), e204-1121. doi: 10.1371/journal.pbio.0040204
Pikarsky, E., Ronen, A., Abramowitz, J., Levavi-Sivan, B., Hutoran, M., Shapira, Y., . . . Kotler, M. (2004). Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. *Journal of Virology, 78*(17), 9544-9551. doi: 10.1128/jvi.78.17.9544-9551.2004
Pikulkaew, S., Meeyam, T., & Banlunara, W. (2009). The Outbreak of Koi Herpesvirus (KHV) in Koi (Cyprinus carpio koi) from Chiang Mai Province, Thailand. *Thai Journal of Veterinary Medicine, 39*(1), 53-58.
Plumb, J. A., & Hanson, L. A. (2011). Other Viral Diseases of Fish. In J. A. Plumb & L. A. Hanson (Eds.), *Health Maintenance and Principal Microbial Diseases of Cultured Fishes*.
Pokorova, D., Reschova, S., Hulova, J., Vicenova, M., Vesely, T., & Piackova, V. (2010). Detection of Cyprinid Herpesvirus-3 in Field Samples of Common and Koi Carp by Various Single-Round and Nested PCR Methods. *Journal of the World Aquaculture Society, 41*(5), 773-779. doi: 10.1111/j.1749-7345.2010.00419.x
Pokorova, D., Reschova, S., Hulova, J., Vicenova, M., Vesely, T., & Piackova, V. (2010). Detection of Cyprinid Herpesvirus-3 in Field Samples of Common and Koi Carp by Various Single-Round and Nested PCR Methods. *Journal of the World Aquaculture Society, 41*(5), 773-779.
Pokorova, D., Vesely, T., Piackova, V., Reschova, S., & Hulova, J. (2005). Current knowledge on koi herpesvirus (KHV): A review. *Veterinarni Medicina, 50*(4), 139-147.
Portz, D. E., Woodley, C. M., & Cech, J. J. (2006). Stress-associated impacts of short-term holding on fishes. *Reviews in Fish Biology and Fisheries, 16*(2), 125-170. doi: 10.1007/s11160-006-9012-z

Pospichal, A., Piackova, V., Pokorova, D., & Vesely, T. (2016). Susceptibility of stone loach (Barbatula barbatula) and hybrids between sterlet (Acipenser ruthenus) and beluga (Huso huso) to cyprinid herpesvirus 3. *Veterinarni Medicina, 61*(5), 249-255. doi: 10.17221/8879-VETMED

Pospichal, A., Pokorova, D., Vesely, T., & Piackova, V. (2018). Susceptibility of the topmouth gudgeon (Pseudorasbora parva) to CyHV-3 under no-stress and stress conditions. *Veterinarni Medicina, 63*(5), 229-239. doi: 10.17221/88/2017-VETMED

Prescott, M. A., Reed, A. N., Jin, L., & Pastey, M. K. (2016). Rapid Detection of Cyprinid Herpesvirus 3 in Latently Infected Koi by Recombinase Polymerase Amplification. *Journal of Aquatic Animal Health, 28*(3), 173-180. doi: 10.1080/08997659.2016.1185048

Radosavljević, V., Jeremić, S., Ćirković, M., Lako, B., Milićević, V., Potkonjak, A., & Nikolin, V. (2012). Common fish species in polyculture with carp as cyprinid herpes virus 3 carriers. *Acta Veterinaria, 62*(5), 675-681. doi: 10.2298/AVB1206675R

Raj, V. S., Fournier, G., Rakus, K., Ronsmans, M., Ouyang, P., Michel, B., . . . Vanderplasschen, A. (2011a). Skin mucus of Cyprinus carpio inhibits cyprinid herpesvirus 3 binding to epidermal cells. *Veterinary Research, 42*(1). doi: 10.1186/1297-9716-42-92

Raj, V. S., Fournier, G., Rakus, K., Ronsmans, M., Ouyang, P., Michel, B., . . . Vanderplasschen, A. (2011b). Skin mucus of Cyprinus carpio inhibits cyprinid herpesvirus 3 binding to epidermal cells. *Veterinary Research, 42*. doi: 10.1186/1297-9716-42-92

Rakus, K. L., Wiegertjes, G. F., Adamek, M., Siwicki, A. K., Lepa, A., & Irnazarow, I. (2009). Resistance of common carp (Cyprinus carpio L.) to Cyprinid herpesvirus-3 is influenced by major histocompatibility (MH) class II B gene polymorphism. *Fish & Shellfish Immunology, 26*(5), 737-743. doi: 10.1016/j.fsi.2009.03.001

Rathore, G., Kumar, G., Swaminathan, T.R., Swain, P., Koi Herpes Virus: A Review and Risk Assessment of Indian Aquaculture, J. Virol. 23(2):124–133, DOI 10.1007/s13337-012-0101-4

Reed, A., Lin, L., Ostertag-Hill, C., Wang, Q., Wu, Z., Miller-Morgan, T., & Jin, L. (2017). Detection of ORF6 protein associated with latent KHV infection. *Virology, 500*, 82-90. doi: 10.1016/j.virol.2016.09.030

Reed, A. M., Lin, L., Ostertag-Hill, C., Wang, Q., Wu, Z. X., Miller-Morgan, T., & Jin, L. (2017). Detection of ORF6 protein associated with latent KHV infection. *Virology, 500*, 82-90. doi: 10.1016/j.virol.2016.09.030

Reed, A. N., Izume, S., Dolan, B. P., LaPatra, S., Kent, M., Dong, J., & Jin, L. (2014). Identification of B Cells as a Major Site for Cyprinid Herpesvirus 3 Latency. *Journal of Virology, 88*(16), 9297-9309. doi: 10.1128/jvi.00990-14

Reno, P.W. (1998) Factors involved in the dissemination of disease in fish populations, Conference: Symposium on Pathogens and Diseases of Fish in Aquatic Ecosystems - Implications in Fisheries Management Location, JOURNAL OF AQUATIC ANIMAL HEALTH; Volume: 10; Issue: 2; 160-171

Rimstad, E., Dale, , O. B., D., B. H., , & Falk, K. (2011). Infectious salmon anaemia. In P. T. K. Woo, Bruno, D. W. (Ed.), *Fish diseases and disorders. Volume 3: viral, bacterial and fungal infections.* . Oslo, Norway.: Norwegian School of Veterinary Science,.

Ronsmans, M., Boutier, M., Rakus, K., Farnir, F., Desmecht, D., Ectors, F., . . . Vanderplasschen, A. (2014). Sensitivity and permissivity of Cyprinus carpio to cyprinid herpesvirus 3 during the early stages of its development: importance of the epidermal mucus as an innate immune barrier. *Veterinary Research, 45*. doi: 10.1186/s13567-014-0100-0

Ronsmans, M., Boutier, M., Rakus, K., Farnir, F., Desmecht, D., Ectors, F., . . . Vanderplasschen, A. (2014). Sensitivity and permissivity of Cyprinus carpio to cyprinid herpesvirus 3 during the early stages of its development: importance of the epidermal mucus as an innate immune barrier. *Veterinary Research, 45*(1), 100. doi: 10.1186/s13567-014-0100-0

Sadler, J., Marecaux, E., & Goodwin, A. E. (2008). Detection of koi herpes virus (CyHV-3) in goldfish, Carassius auratus (L.), exposed to infected koi. *Journal of Fish Diseases, 31*(1), 71-72. doi: 10.1111/j.1365-2761.2007.00830.x

Sano, M., Fukuda, H., & Sano, T. (1990). ISOLATION AND CHARACTERIZATION OF A NEW HERPESVIRUS FROM EEL. In F. O. Perkins & T. C. Cheng (Eds.), *Pathology in Marine Science* (pp. 15-31). San Diego: Academic Press.

Sano, M., Ito, T., Kurita, J., Yanai, T., Watanabe, N., Miwa, S., & Iida, T. (2004). First detection of koi herpesvirus in cultured common carp Cyprinus carpio in Japan. *Fish Pathology, 39*(3), 165-167. doi: 10.3147/jsfp.39.165

Sano, M., Nakai, T., & Fijan, N. (2011). Viral diseases and agents of warmwater fish. In P. T. K. Woo & D. W. Bruno (Eds.), *Fish diseases and disorders. Volume 3: viral, bacterial and fungal infections.* (Vol. Volume 3): CABI.

Sano, T., Fukuda, H., & Furukawa, M. (1985). Herpesvirus cyprini : Biological and Oncogenic Properties. *Fish Pathology, 20*(2-3), 381-388. doi: 10.3147/jsfp.20.381

Sano, T., Morita, N., Shima, N., & Akimoto, M. (1991). Herpesvirus cyprini: lethality and oncogenicity. *Journal of Fish Diseases, 14*(5), 533-543. doi: 10.1111/j.1365-2761.1991.tb00608.x

Schilling, T. F. (2002). The morphology of larval and adult zebrafish. *Zebrafish, 10*, 59-94.

Shapira, Y., Magen, Y., Zak, T., Koder, M., Hulata, G., & Levavi-Sivan, B. (2005). Differential resistance to koi herpes virus (KHV)/carp interstitial nephritis and gill necrosis virus (CNGV) among common carp (Cyprinus carpio L.) strains and crossbreds. *Aquaculture, 245*(1-4), 1-11. doi: 10.1016/j.aquaculture.2004.11.038

Sharma, J. G., & Chakrabarti, R. (1999). Larval Rearing of Common Carp Cyprinus carpio: A Comparision Between Natural and Artificial Diets Under Three Stocking Densities. *Journal of the World Aquaculture Society, 30*(4), 490-495. doi: doi:10.1111/j.1749-7345.1999.tb00997.x

Shchelkunov, I. S., Shchelkunova, T. I., Shchelkunov, A. I., Kolbassova, Y. P., Didenko, L. V., & Bykovsky, A. P. (2009). First detection of a viral agent causing disease in farmed sturgeon in Russia. *Diseases of aquatic organisms, 86*(3), 193. doi: 10.3354/dao02124

Shimizu, T., Yoshida, N., Kasai, H., & Yoshimizu, M. (2006). Survival of koi herpesvirus (KHV) in environmental water. *Fish Pathology, 41*(4), 153-157. doi: 10.3147/jsfp.41.153

Siwicki, A., Malaczewska, J., Lepa, A., Kazun, B., Kazun, K., Glabski, E., & Wojcik, R. (2009). EFFECT OF CYPRINID HERPESVIRUS-3 (CYHV-3) ON INNATE IMMUNITY IN CARP, TENCH AND SHEATFISH. *Journal of Comparative Pathology, 141*(4), 309-309.

Skall, H. F., Olesen, N. J., & Mellergaard, S. (2005). Viral haemorrhagic septicaemia virus in marine fish and its implications for fish farming – a review. *Journal of Fish Diseases, 28*(9), 509-529. doi: doi:10.1111/j.1365-2761.2005.00654.x

Smail, D. A., & Snow, M. (2011). Viral haemorrhagic septicaemia. In P. T. K. Woo, Bruno, D. W. (Ed.), *Fish diseases and disorders. Volume 3: viral, bacterial and fungal infections.* : CABI

Snow, M., Black, J., Matejusova, I., McIntosh, R., Baretto, E., Wallace, I., & Bruno, D. (2010). Detection of salmonid alphavirus RNA in wild marine fish: implications for the origins of salmon pancreas disease in aquaculture. *Diseases of aquatic organisms, 91*(3), 177-188.

Soliman, H., & El-Matbouli, M. (2005). An inexpensive and rapid diagnostic method of Koi Herpesvirus (KHV) infection by loop-mediated isothermal amplification. *Virology Journal, 2*. doi: 10.1186/1743-422X-2-83

Soliman, H., & El-Matbouli, M. (2010). Loop mediated isothermal amplification combined with nucleic acid lateral flow strip for diagnosis of cyprinid herpes virus-3. *Molecular and Cellular Probes, 24*(1), 38-43. doi: 10.1016/j.mcp.2009.09.002

Soliman, H., & El-Matbouli, M. (2018). Rapid detection and differentiation of carp oedema virus and cyprinid herpes virus-3 in koi and common carp. *Journal of Fish Diseases*. doi: 10.1111/jfd.12774

St-Hilaire, S., Beevers, N., Way, K., Deuff, R., Martin, P., & Joiner, C. (2005). Reactivation of koi herpesvirus infections in common carp Cyprinus carpio. *Diseases of aquatic organisms, 67*(1), 15-23.

St-Hilaire, S., Beevers, N., Way, K., Le Deuff, R. M., Martin, P., & Joiner, C. (2005). Reactivation of koi herpesvirus infections in common carp Cyprinus carpio. *Diseases of aquatic organisms, 67*(1-2), 15.

Stephens, F., Raidal, Sr., & Jones, B. (2004). Haematopoietic necrosis in a goldfish (Carassius auratus) associated with an agent morphologically similar to herpesvirus. *Australian Veterinary Journal, 82*(3), 167-169. doi: 10.1111/j.1751-0813.2004.tb12650.x

Takahara, T., Honjo, M. N., Uchii, K., Minamoto, T., Doi, H., Ito, T., & Kawabata, Z. (2014). Effects of daily temperature fluctuation on the survival of carp infected with Cyprinid herpesvirus 3. *Aquaculture, 433*, 208-213. doi: 10.1016/j.aquaculture.2014.06.001

Thresher, R. E., Allman, J., & Stremick-Thompson, L. (2018). Impacts of an invasive virus (CyHV-3) on established invasive populations of common carp (Cyprinus carpio) in North America. *Biological Invasions*. doi: 10.1007/s10530-017-1655-2

Traxler, G., & Rankin, J. (1989). An infectious hematophoietic necrosis epizootic in sockeye salmon Oncorhynchus nerka in Weaver Creek spawning channel, Fraser River system, B.C., Canada. *Diseases of aquatic organisms, 6*, 221-226. doi: 10.3354/dao006221

Ueno, Y., Kitao, T., Chen, S.-N., Aoki, T., & Kou, G.-H. (1992). Characterization of A Herpes-like Virus Isolated from Cultured Japanese Eels in Taiwan. *Fish Pathology, 27*(1), 7-17. doi: 10.3147/jsfp.27.7

Vadstein, O., Bergh, Ø., Gatesoupe, F.-J., Galindo-Villegas, J., Mulero, V., Picchietti, S., . . . Bossier, P. (2013). Microbiology and immunology of fish larvae. *Reviews in Aquaculture, 5*(s1), S1-S25. doi: doi:10.1111/j.1753-5131.2012.01082.x

van Beurden, S. J., Engelsma, M. Y., Roozenburg, I., Voorbergen-Laarman, M. A., van Tulden, P. W., Kerkhoff, S., . . . Haenen, O. L. M. (2012). Viral diseases of wild and farmed European eel Anguilla anguilla with particular reference to the Netherlands. *Diseases of aquatic organisms, 101*(1), 69-86.

Varsamos, S., Flik, G., Pepin, J. F., Bonga, S. E. W., & Breuil, G. (2006). Husbandry stress during early life stages affects the stress response and health status of juvenile sea bass, Dicentrarchus labrax. *Fish & Shellfish Immunology, 20*(1), 83-96. doi: https://doi.org/10.1016/j.fsi.2005.04.005

Vazzana, M., Cammarata, M., Cooper, E. L., & Parrinello, N. (2002). Confinement stress in sea bass (Dicentrarchus labrax) depresses peritoneal leukocyte cytotoxicity. *Aquaculture, 210*(1), 231-243. doi: https://doi.org/10.1016/S0044-8486(01)00818-3

Waltzek, T. B., Kelley, G. O., Stone, D. M., Way, K., Hanson, L., Fukuda, H., . . . Hedrick, R. P. (2005). Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family Herpesviridae. *Journal of General Virology, 86*, 1659-1667. doi: 10.1099/vir.0.80982-0

Wang, C. S., Shih, H. H., Ku, C. C., & Chen, S. N. (2003). Studies on epizootic iridovirus infection among red sea bream, Pagrus major (Temminck & Schlegel), cultured in Taiwan. *Journal of Fish Diseases, 26*(3), 127-133. doi: doi:10.1046/j.1365-2761.2003.00441.x

Wang, Y., Zeng, W., Li, Y., Liang, H., Liu, C., Pan, H., . . . Wang, Q. (2015). Development and characterization of a cell line from the snout of koi (Cyprinus carpio L.) for detection of koi herpesvirus. *Aquaculture, 435*, 310-317. doi: 10.1016/j.aquaculture.2014.10.006

Wang, Y., Zeng, W., Wang, Q., Li, Y., Bergmann, S. M., Zheng, S., . . . Lee, P. (2018). Establishment and characterization of a new cell line from koi brain (Cyprinus carpio L.). *Journal of Fish Diseases, 41*(2), 357-364. doi: 10.1111/jfd.12738

Watson, L. R., Yun, S. C., Groff, J. M., & Hedrick, R. P. (1995). Characteristics and pathogenicity of a novel herpesvirus isolated from adult and subadult white sturgeon Acipensertransmontanus. *Diseases of aquatic organisms, 22*(3), 199-210.

Whittington, R. J., Becker, J. A., & Dennis, M. M. (2010). Iridovirus infections in finfish – critical review with emphasis on ranaviruses. *Journal of Fish Diseases, 33*(2), 95-122. doi: doi:10.1111/j.1365-2761.2009.01110.x

Whittington, R. J., Reddacliff, L. A., Marsh, I., Kearns, C., Zupanovic, Z., & Callinan, R. B. (1999). Further observations on the epidemiology and spread of epizootic haematopoietic necrosis virus (EHNV) in farmed rainbow trout Oncorhynchus mykiss in southeastern Australia and a recommended sampling strategy for surveillance. *Diseases of aquatic organisms, 35*(2), 125. doi: 10.3354/dao035125

Whitworth, K. L., Baldwin, D. S., & Kerr, J. L. (2012). Drought, floods and water quality: Drivers of a severe hypoxic blackwater event in a major river system (the southern Murray–Darling Basin, Australia). *Journal of Hydrology, 450-451*, 190-198. doi: https://doi.org/10.1016/j.jhydrol.2012.04.057

Wolf, K., Darlington, R. W., Taylor, W. G., Quimby, M. C., & Nagabayashi, T. (1978). Herpesvirus salmonis: Characterization of a New Pathogen of Rainbow Trout. *Journal of Virology, 27*(3), 659-666.

Wolf, K., & Smith, C. E. (1981). Herpesvirus salmonis: pathological changes in parenterally-infected rainbow trout, Salmo gairdneri Richardson, fry. *Journal of Fish Diseases, 4*(6), 445-457. doi: doi:10.1111/j.1365-2761.1981.tb01158.x

Yin, Z., Lam, T. J., & Sin, Y. M. (1995). The effects of crowding stress on the non-specific immuneresponse in fancy carp (Cyprinus carpio L.). *Fish & Shellfish Immunology, 5*(7), 519-529. doi: https://doi.org/10.1016/S1050-4648(95)80052-2

Yoshino, M., Watari, H., Kojima, T., Ikedo, M., & Kurita, J. (2009). Rapid, sensitive and simple detection method for koi herpesvirus using loop-mediated isothermal amplification. *Microbiology and Immunology, 53*(7), 375-383. doi: 10.1111/j.1348-0421.2009.00145.x

Yuasa, K., Ito, T., & Sano, M. (2008). Effect of Water Temperature on Mortality and Virus Shedding in Carp Experimentally Infected with Koi Herpesvirus. *Fish Pathology, 43*(2), 83-85. doi: 10.3147/jsfp.43.83

Yuasa, K., Kurita, J., Kawana, M., Kiryu, I., Ohseko, N., & Sano, M. (2012). Development of mRNA-specific RT-PCR for the detection of koi herpesvirus (KHV) replication stage. *Diseases of aquatic organisms, 100*(1), 11-18.

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. *Diseases of aquatic organisms, 100*(1), 11-18.

Yuasa, K., & Sano, M. (2009). Koi Herpesvirus: Status of Outbreaks, Diagnosis, Surveillance, and Research. *Israeli Journal of Aquaculture-Bamidgeh, 61*(3), 169-179.

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*(1), 37-39. doi: 10.3147/jsfp.40.37

Yuasa, K., Sano, M., & Oseko, N. (2013). Goldfish is Not a Susceptible Host of Koi Herpesvirus (KHV) Disease. *Fish Pathology, 48*(2), 52-55. doi: 10.3147/jsfp.48.52

Zapata, A., Diez, B., Cejalvo, T., Gutiérrez-de Frías, C., & Cortés, A. (2006). Ontogeny of the immune system of fish. *Fish & Shellfish Immunology, 20*(2), 126-136. doi: https://doi.org/10.1016/j.fsi.2004.09.005

Zhang, S., Wang, Z., & Wang, H. (2013). Maternal immunity in fish. *Developmental & Comparative Immunology, 39*(1), 72-78. doi: https://doi.org/10.1016/j.dci.2012.02.009

10. Appendices

Appendix 1. Database of articles that investigated the susceptibility/resistance of fish to CyHV-3.

Title	Authors	Year	Publication
Epidemilogical Description Of A New Viral Disease Afflicting Cultured Cyprinus Carpio In Israel	Perelberg, Ayana Smirnov, Margarita Hutoran, Marina Diamant, Ariel Bejerano, Yitzhak Kotler, Moshe	2003	Israeli Journal of Aquaculture - BAMIGDEH
Differential resistance to koi herpes virus (KHV)/carp interstitial nephritis and gill necrosis virus (CNGV) among common carp (Cyprinus carpio L.) strains and crossbreds	Shapira, Y. Magen, Y. Zak, T. Kotler, M. Hulata, G. Levavi-Sivan, B.	2005	Aquaculture
Reactivation of koi herpesvirus infections in common carp Cyprinus carpio	St-Hilaire, S. Beevers, N. Way, K. Le Deuff, R. M. Martin, P. Joiner, C.	2005	Diseases of Aquatic Organisms
Susceptibility of koi carp, common carp, goldfish, and goldfish x common carp hybrids to cyprinid herpesvirus-2 and herpesvirus-3	Hedrick, R. P. Waltzek, T. B. McDowell, T. S.	2006	Journal of Aquatic Animal Health
Similarities and heterogenicity of koi herpes virus (KHV) genome detected in ornamental fish without clinical signs	Bergmann, S. M. Stumpf, P. Schutze, H. Fichtner, D. Sadowski, J. Kempter, J.	2007	Aquaculture
Detection of cyprinid herpesvirus type 3 in goldfish cohabiting with CyHV-3-infected koi carp (Cyprinus carpio koi)	El-Matbouli, M. Saleh, M. Soliman, H.	2007	Veterinary Record
Detection of koi herpesvirus (KHV) genome in wild and farmed fish from Northern Poland	Kempter, J. Bergmann, S. M.	2007	Aquaculture
Difference in the susceptibility of strains and developmental stages of carp to koi herpesvirus	Ito, T. Kurita, J. Sano, M. Iida, T.	2007	Fish Pathology
Carp larvae are not susceptible to Koi herpesvirus	Ito, T. Sano, M. Kurita, J. Yuasa, K. Iida, T.	2007	Fish Pathology
Detection of koi herpes virus (CyHV-3) in goldfish, Carassius auratus (L.), exposed to infected koi	Sadler, J. Marecaux, E. Goodwin, A. E.	2008	Journal of Fish Diseases

Effect of Water Temperature on Mortality and Virus Shedding in Carp Experimentally Infected with Koi Herpesvirus	Yuasa, K. Ito, T. Sano, M.	2008	Fish Pathology
Detection of koi herpes virus (KHV) genome in apparently healthy fish	Bergmann, SM Schütze, H Fischer, U Fichtner, D Riechardt, M Meyer, K Schrudde, D Kempter, J	2009	Bulletin of the European Association of Fish Pathologists
Comparison of the resistance of selected families of common carp, Cyprinus carpio L., to koi herpesvirus: Preliminary study	Dixon, P. F. Joiner, C. L. Way, K. Reese, R. A. Jeney, G. Jeney, Z.	2009	Journal of Fish Diseases
Koi herpes virus: do acipenserid restitution programs pose a threat to carp farms in the disease-free zones?	Kempter, J. Sadowski, J. Schutze, H. Fischer, U. Dauber, M. Fichtner, D. Panicz, R. Bergmann, S. M.	2009	Acta Ichthyologica Et Piscatoria
Resistance of common carp (Cyprinus carpio L.) to Cyprinid herpesvirus-3 is influenced by major histocompatibility (MH) class II B gene polymorphism	Rakus, K. L. Wiegertjes, G. F. Adamek, M. Siwicki, A. K. Lepa, A. Irnazarow, I.	2009	Fish & Shellfish Immunology
EFFECT OF CYPRINID HERPESVIRUS-3 (CYHV-3) ON INNATE IMMUNITY IN CARP, TENCH AND SHEATFISH	Siwicki, A. Malaczewska, J. Lepa, A. Kazun, B. Kazun, K. Glabski, E. Wojcik, R.	2009	Journal of Comparative Pathology
Goldfish (Carassius auratus auratus) is a susceptible species for koi herpesvirus (KHV) but not for KHV disease (KHVD)	Bergmann, S. M. Lutze, P. Schutze, H. Fischer, U. Dauber, M. Fichtner, D. Kempter, J.	2010	Bulletin of the European Association of Fish Pathologists
Susceptibility of koi x crucian carp and koi x goldfish hybrids to koi herpesvirus (KHV) and the development of KHV disease (KHVD)	Bergmann, S. M. Sadowski, J. Kielpinski, M. Bartlomiejczyk, M. Fichtner, D.	2010	Journal of Fish Diseases

	Riebe, R. Lenk, M. Kempter, J.		
Detection of KHV in Freshwater Mussels and Crustaceans from Ponds with KHV History in Common Carp (Cyprinus carpio)	Kielpinski, M. Kempter, J. Panicz, R. Sadowski, J. Schutze, H. Ohlemeyer, S. Bergmann, S. M.	2010	The Israeli Journal of Aquaculture
Detection of koi herpesvirus (KHV) after re-activation in persistently infected common carp (Cyprinus carpio L.) using non-lethal sampling methods	Bergmann, S. M. Kempter, J.	2011	Bulletin of the European Association of Fish Pathologists
Investigation of Koi Herpesvirus Latency in Koi	Eide, K. E. Miller-Morgan, T. Heidel, J. R. Kent, M. L. Bildfell, R. J. LaPatra, S. Watson, G. Jin, L.	2011	Journal of Virology
Transmission of Cyprinid herpesvirus-3 (CyHV-3) from goldfish to naïve common carp by cohabitation	El-Matbouli, M. Soliman, H.	2011	Research in Veterinary Science
Feeding Cyprinus carpio with infectious materials mediates cyprinid herpesvirus 3 entry through infection of pharyngeal periodontal mucosa	Fournier, G. Boutier, M. Raj, V. S. Mast, J. Parmentier, E. Vanderwalle, P. Peeters, D. Lieffrig, F. Famir, F. Gillet, L. Vanderplasschen, A.	2012	Veterinary Research
Horizontal transmission of koi herpes virus (KHV) from potential vector species to common carp	Kempter, J. Kielpinski, M. Panicz, R. Sadowski, J. Myslowski, B. Bergmann, S. M.	2012	Bulletin of the European Association of Fish Pathologists
Common fish species in polyculture with carp as cyprinid herpes virus 3 carriers	Radosavljević, V. Jeremić, S. Ćirković, M. Lako, B. Milićević, V. Potkonjak, A. Nikolin, V.	2012	Acta Veterinaria

Development of mRNA-specific RT-PCR for the detection of koi herpesvirus (KHV) replication stage	Yuasa, K. Kurita, J. Kawana, M. Kiryu, I. Ohseko, N. Sano, M.	2012	Diseases of Aquatic Organisms
Do wild fish species contribute to the transmission of koi herpesvirus to carp in hatchery ponds?	Fabian, M. Baumer, A. Steinhagen, D.	2013	Journal of Fish Diseases
Sensitivity of common carp, Cyprinus carpio L., strains and crossbreeds reared in the Czech Republic to infection by cyprinid herpesvirus 3 (CyHV-3; KHV)	Piackova, V. Flajshans, M. Pokorova, D. Reschova, S. Gela, D. Cizek, A. Vesely, T.	2013	Journal of Fish Diseases
Goldfish is not a susceptible host of koi herpesvirus (KHV) disease	Yuasa, K. Sano, M. Oseko, N.	2013	Fish Pathology
Investigating the interactions of Cyprinid herpesvirus-3 with host proteins in goldfish Carassius auratus	Gotesman, M. Abd-Elfattah, A. Kattlun, J. Soliman, H. El-Matbouli, M.	2014	Journal of Fish Diseases
Differences in the susceptibility of Japanese indigenous and domesticated Eurasian common carp (Cyprinus carpio), identified by mitochondrial DNA typing, to cyprinid herpesvirus 3 (CyHV-3)	Ito, T. Kurita, J. Yuasa, K.	2014	Veterinary Microbiology
Sensitivity and permissivity of Cyprinus carpio to cyprinid herpesvirus 3 during the early stages of its development: importance of the epidermal mucus as an innate immune barrier	Ronsmans, M. Boutier, M. Rakus, K. Farnir, F. Desmecht, D. Ectors, F. Vandecan, M. Lieffrig, F. Melard, C. Vanderplasschen, A.	2014	Veterinary Research
Effects of daily temperature fluctuation on the survival of carp infected with Cyprinid herpesvirus 3	Takahara, T. Honjo, M. N. Uchii, K. Minamoto, T. Doi, H. Ito, T. Kawabata, Z.	2014	Aquaculture
Is There Any Species Specificity in Infections with Aquatic Animal herpesviruses?--The koi herpesvirus (KHV): An Alloherpesvirus model	Bergmann, Sven M. Cieslak, Michael Fichtner, Dieter Dabels, Juliane	2016	Fisheries and Aquaculture Journal

	Monaghan, Sean J. Wang, Qing Zeng, Weiwei Kempter, Jolanta		
Transmission of Cyprinid herpesvirus 3 by wild fish species - results from infection experiments	Fabian, M. Baumer, A. Adamek, M. Steinhagen, D.	2016	Journal of Fish Diseases
Susceptibility of stone loach (Barbatula barbatula) and hybrids between sterlet (Acipenser ruthenus) and beluga (Huso huso) to cyprinid herpesvirus 3	Pospichal, A. Piackova, V. Pokorova, D. Vesely, T.	2016	Veterinarni Medicina
Koi herpesvirus infection in experimentally infected common carp Cyprinus carpio (Linnaeus, 1758) and three potential carrier fish species Carassius carassius (Linnaeus, 1758); Rutilus rutilus (Linnaeus, 1758); and Tinca tinca (Linnaeus, 1758) by quantitative real-time PCR and in-situ hybridization	Gaede, L. Steinbruck, J. Bergmann, S. M. Jager, K. Grafe, H. Schoon, H. A. Speck, S. Truyen, U.	2017	Journal of Applied Ichthyology
Analysis of stress factors associated with KHV reactivation and pathological effects from KHV reactivation	Lin, L. Chen, S. Russell, D. S. Lohr, C. V. Milston-Clements, R. Song, T. Miller-Morgan, T. Jin, L.	2017	Virus Research
Cyprinid herpesvirus 3 as a potential biological control agent for carp (Cyprinus carpio) in Australia: susceptibility of non-target species	McColl, K. A. Sunarto, A. Slater, J. Bell, K. Asmus, M. Fulton, W. Hall, K. Brown, P. Gilligan, D. Hoad, J. Williams, L. M. Crane, M. S.	2017	Journal of Fish Diseases
Survival Rate and Immunological Responses of Mirror Carp Selective Breeding Generations to CyHV-3	Jia, Z. Wang, S. Bai, S. Ge, Y. Li, C. Hu, X. Shang, M. Zhang, J. Li, B. Shi, L.	2018	Journal of the World Aquaculture Society

Susceptibility of the topmouth gudgeon (*Pseudorasbora parva*) to CyHV-3 under no-stress and stress conditions	Pospichal, A. Pokorova, D. Vesely, T. Piackova, V.	2018	Veterinární Medicína

Appendix 2. Database of articles that have compared the performance of various tests used in the diagnosis of CyHV-3

				Diagnostic tools developed/appraised			
Title	**Authors**	**Year**	**Publication**	**Clinical techniques, isolation in cell culture, electron microscopy, virus detection assays, in situ DNA probes**	**PCR/Molecular methods**	**Serology**	**Other/comments**
Initial characteristics of koi herpesvirus and development of a polymerase chain reaction assay to detect the virus in koi, *Cyprinus carpio* koi	Gilad, O. Yun, S. Andree, K. B. Adkison, M. A. Zlotkin, A. Bercovier, H. Eldar, A. Hedrick, R. P.	2002	Diseases of Aquatic Organisms	Virus isolation in cell culture	c-PCR		c-PCR (100% positive samples) outperformed virus isolation (82% positive samples)
Development and comparison of techniques for the diagnosis of koi herpesvirus (KHV)	Le Deuff, RM Way, K Ecclestone, L Dixon, PF Betts, AM Stone, DM Gilad, O Hedrick, RP	2002	Abstract of 10th International Conference of the European Association of Fish Pathologists **(POSTER PRESENTATION)**	Cell culture isolation. Light microscopy, Electron Microscopy	c-PCR, n-PCR,	ISH	c-PCR (50% positive sites) outperformed virus isolation (40% positive sites)
The emergence of koi herpesvirus and its significance to European aquaculture	Haenen, OLM Way, K Bergmann, SM Ariel, Ellen	2004	Bulletin of the European Association of Fish Pathologists **(REVIEW ARTICLE)**	In-situ hybridisation (ISH), Immunofluorescent assay (IFAT), Immunohistochemical assays, clinical pathology, histopathology, virus isolation	c-PCR	Enzyme Linked-Immunosorbent Assays (ELISA) - antibodies	•Clinical pathology and histopathology considered useful for presumptive diagnosis. PCR considered most sensitive test for confirmative diagnosis. •Recommends using at least 2 different diagnostic tests. •Microscopy not considered specific. •Electron microscopy only useful in fish with high virus load.

							•Isolation in cell culture less sensitive than PCR. •c-PCR considered effective but not validated at time of review. •q-PCR not validated at the time of review. •ELISA still under development at time of review. •ISH and IFAT mentioned but not validated at time of review.
Cloning of the koi herpesvirus (KHV) gene encoding thymidine kinase and its use for a highly sensitive PCR based diagnosis	Bercovier, H. Fishman, Y. Nahary, R. Sinai, S. Zlotkin, A. Eyngor, M. Gilad, O. Eldar, A. Hedrick, R. P.	2005	BMC Microbiology	Virus isolation in cell culture	Bercovier c-PCR, Gilad c-PCR, Gray c-PCR		Bercovier c-PCR was as sensitive as virus isolation in cell culture and more sensitive than Gilad c-PCR or Gray c-PCR. Bercovier c-PCR and isolation in cell culture identified CyHV-3 in sick fish and in fish cohabitating with sick fish but only in 9 out of 13 fish that had survived CyHV-3 exposure. Bercovier c-PCR had greater analytical responsiveness than that of Gilad c-PCR or Gray c-PCR
Detection of carp interstitial nephritis and gill necrosis virus in fish droppings	Dishon, A. Perelberg, A. Bishara-Shieban, J. Ilouze, M. Davidovich, M. Werker, S. Kotler, M.	2005	Applied and Environmental Microbiology	Virus isolation in cell culture	c-PCR		•ELISA targeting viral antigen also assessed. •CyHV-3 DNA was observed by c-PCR and CyHV-3 antigens were observed by ELISA, in fish droppings. •Fish droppings contained active virus that could be isolated from cell culture.
Current knowledge on koi herpesvirus (KHV): A review	Pokorova, D. Vesely, T. Piackova, V. Reschova, S. Hulova, J.	2005	Veterinarni Medicina **(REVIEW ARTICLE)**	Clinical signs, histology, virus isolation in cell culture,	c-PCR		c-PCR considered to be more sensitive than virus isolation, especially in frozen samples and gills samples that are prone to bacterial contamination.

							Gilad q-PCR mentioned as promising new technique. Gunimaladevi LAMP assay mentioned as potential diagnostic technique. Antigen based ELISA also mentioned. Antibody based ELISA mentioned as useful to show previous exposure to CyHV-3 but not current infection status.
Diagnosis of koi herpesvirus (KHV) disease in Japan	Sano, Motohiko Ito, Takafumi Kurita, Jun Miwa, Satoshi Iida, Takaji	2005	Bull. Fish. Res. Agen. Supplement No **(REVIEW ARTICLE)**	Epidemiology, clinical observations, gross observations, virus isolation in cell culture	Gray c-PCR, Gilad c-PCR		Diagnostic protocol for CyHV-3 was outlined with the following steps: 1.Epidemiology, clinical observations, gross observations 2.Gray c-PCR 3.Gray c-PCR + Gilad c-PCR 4.Virus isolation (attempted but not relied upon for diagnosis)
Improvement of a PCR method with the Sph 1-5 primer set for the detection of koi herpesvirus (KHV)	Yuasa, K. Sano, M. Kurita, J. Ito, T. Iida, T.	2005	Fish Pathology		Gray c-PCR, Gilad c-PCR, (New) Yuasa c-PCR		Yuasa c-PCR was developed from Gray c-PCR. The new Yuasa c-PCR outperformed Gray c-PCR and very was similar to Gilad c-PCR, both detected 19 positive samples out of 21 assessed.
Detection of Cyprinid herpesvirus-3 (CyHV-3) DNA in infected fish tissues by nested polymerase chain reaction	El-Matbouli, M. Rucker, U. Soliman, H.	2007	Diseases of Aquatic Organisms		Gilad c-PCR, Gray c-PCR, (New) El-Matbouli n-PCR		New n-PCR described using primers from protein coding regions. The new n-PCR was found to be 10 times more responsive that Gilad c-PCR. It is also claimed to have identified CyHV-3 in samples that were previously negative by Gilad c-PCR (results not shown).
Evaluation of quenching probe (QProbe)-PCR assay for quantification of the koi herpes virus (KHV)	Kamimura, S. Hagi, T. Kurata, S. Takatsu, K. Sogo, H.	2007	Microbes and Environments		Quenching probe PCR (qp-PCR)		A new qp-PCR technique was developed. This method had similar performance to Gilad q-PCR. However, the authors claim that the qp-PCR eliminates false positives.

	Hoshino, T. Nakamura, K.						
Tests for the presence of koi herpesvirus (KHV) in common carp (Cyprinus carpio carpio) and koi carp (Cyprinus carpio koi) in the Czech Republic	Pokorova, D. Piackova, V. Cizek, A. Reschova, S. Hulova, J. Vicenova, M. Vesely, T.	2007	Veterinarni Medicina	Virus culture	Gilad c-PCR, Bercovier c-PCR		Gilad c-PCR did not detect CyHV-3 DNA in any samples of pooled organs, while Bercovier c-PCR detected CyHV-3 in 5/15 samples. Isolation in cell culture did not detect any CPE in cell culture.
Detection of koi herpes virus (KHV) genome in apparently healthy fish	Bergmann, SM Schütze, H Fischer, U Fichtner, D Riechardt, M Meyer, K Schrudde, D Kempter, J	2009	Bulletin of the European Association of Fish Pathologists	Isolation in cell culture	Gilad c-PCR, Gray c-PCR, Bercovier c-PCR, Bergmann n-PCR	ISH	Gilad c-PCR, Gray c-PCR and Bercvier c-PCR failed to detect CyHV-3 DNA, while Bergmann n-PCR detected CyHV-3 in a number of samples from species other than common carp. Isolation in cell culture was always negative. ISH detected CyHV-3 in two non-common carp species.
Koi herpes virus: do acipenserid restitution programs pose a threat to carp farms in the disease-free zones?	Kempter, J Sadowski, J. Schütze, H. Fischer, U Dauber, M Fichtner, D Panicz, R. Bergmann, S. M.	2009	Acta Ichthyologica et Piscatoria				
Koi herpesvirus: Status of outbreaks, diagnosis, surveillance, and research	Yuasa, K. Sano, M.	2009	Israeli Journal of Aquaculture – Bamidgeh **(REVIEW ARTICLE)**	Virus isolation in cell culture,	Gray c-PCR, Gilad c-PCR, Yuasa c-PCR , Bercovier c-PCR, Kanchanakhan n-PCR, Gilad q-PCR, Yoshino	ELISA	Virus isolation in cell culture considered difficult due to low susceptibility of available cell lines. All PCRs except Gray c-PCR considered to have similar performance. Kanchanakhan n-PCR and Gilad q-PCR considered more sensitive than c-PCRs.

					LAMP, Yuasa rt-PCR		Kanchanakhan n-PCR had higher risk of contamination. Yoshino LAMP considered more sensitive than Yuasa c-PCR and Bercovier c-PCR. ELISA considered useful to detect previous CyHV-3 exposure but technique requires refinement. Diagnostic protocol for CyHV-3 was outlined with the following steps. 1.Epidemiology, clinical observations, gross observations 2.Yuasu c-PCR OR Yoshino LAMP 3.Virus isolation (attempted but not relied upon for diagnosis) 4. Yuasa c-PCR + Gilad c-PCR
Goldfish (Carassius auratus auratus) is a susceptible species for koi herpesvirus (KHV) but not for KHV disease (KHVD)	Bergmann, SM Lutze, P Schütze, H Fischer, U Dauber, M Fichtner, D Kempter, J	2010	Bulletin of the European Association of Fish Pathologists	Clinical signs	Gilad c-PCR, Gray c-PCR, Bergmann n-PCR, ISH		IFAT of viral proteins also used. Common carp showed typical clinical signs. Goldfish displayed weak clinical signs – swollen abdomen, pronounced lateral line. Virus isolation in cell culture failed. Gilad c-PCR and Gray c-PCR positive for pooled tissue samples but negative from gill samples from survivors. Bergmann n-PCR detected CyHV-3 in gill samples from survivors. CyHV-3 detected in goldfish organs by Bergmann n-PCR and Gilad c-PCR. Bergmann n-PCR and Gilad c-PCR gave similar results for koi. After 21 days post infection, healthy goldfish and koi always gave negative results by Gilad c-PCR and Gray c-PCR but Bergmann n-PCR was able to detect CyHV-3 in these samples. ISH and IFAT

							also confirmed the presence of CyHV-3 in koi and goldfish. CyHV-3 was identified inside koi and goldfish cell by ISH. By IFAT, more CyHV-3 was identified in koi leucocytes after immersion than IP injection of CyHV-3, goldfish had equal amounts of CyHV-3 by either rout of infection.
Investigation on the diagnostic sensitivity of molecular tools used for detection of koi herpesvirus	Bergmann, S. M. Riechardt, M. Fichtner, D. Lee, P. Kempter, J.	2010	Journal of Virological Methods		12 Different PCR assays		Reviewed in detail in text
Detection of Cyprinid Herpesvirus-3 in Field Samples of Common and Koi Carp by Various Single-Round and Nested PCR Methods	Pokorova, D Reschova, S Hulova, J Vicenova, M Vesely, T Piackova, V	2010	Journal of the World Aquaculture Society		Various c-PCR and n-PCRs.		Reviewed in detail in text.
Loop mediated isothermal amplification combined with nucleic acid lateral flow strip for diagnosis of cyprinid herpes virus-3	Soliman, H. El-Matbouli, M.	2010	Molecular and Cellular Probes		LAMP, Bercovier c-PCR		LAMP had similar analytical responsiveness to Bercovier c-PCR. LAMP reported to have comparable diagnostic sensitivity to Bercovier c-PCR
CYHV-3 Infection dynamics in common carp (Cyprinus carpio) - evaluation	Matras, M. Antychowicz, J. Castric, J. Bergmann, S. M.	2012	Bulletin of the Veterinary Institute in Pulawy	Clinical signs, Virus isolation in cell culture	Gilad c-PCR	Serum neutralisation test (SNT)	Gilad c-PCR was effective in detecting CyHV-3 DNA during early infection and active infection but was less effective in recovered fish. Skin was the most effective site for Gilad c-PCR during

of diagnostic methods							early infection, all tissues were equally effective during active infection. Virus isolation in cell culture was somewhat effective during active infection. SNT was effective during later infection and in recovered fish.
Detection of Koi herpesvirus: Impact of extraction method, primer set and DNA polymerase on the sensitivity of polymerase chain reaction examinations	Meyer, K. Bergmann, S. M. van der Marel, M. Steinhagen, D.	2012	Aquaculture Research		c-PCR, nested PCR, q-PCR		Reviewed in detail in text
Koi Herpes Virus: A Review and Risk Assessment of Indian Aquaculture	Rathore, G. Kumar, G. Swaminathan, T. R. Swain, P.	2012	Indian Journal of Virology **(REVIEW ARTICLE)**	Clinical signs, histopathology, virus isolation in cell culture	Bercovier c-PCR, Bergmann n-PCR, Gilad q-PCR, LAMP, Bergmann sn-PCR	ELISA	Virus isolation in cell culture not considered to be as sensitive as PCR and therefore not considered to be reliable. Gilad q-PCR considered gold standard for virus quantification. Bercovier c-PCR and Bergmann n-PCR reported to have the same sensitivity as Gilad q-PCR. Bergmann sn-PCR also considered to be highly sensitive. Soliman and El-Matbouli (2010) LAMP assay also considered to be highly sensitive. Commercial Loopamp DNA amplification kit mentioned that claims to be as sensitive as Bercovier c-PCR. Antigen ELISA considered to be useful method to detect CyHV-3.

							Antibody ELISA useful to detect previous exposure to CyHV-3 but not to diagnose current infection status.
Development of mRNA-specific RT-PCR for the detection of koi herpesvirus (KHV) replication stage	Yuasa, K. Kurita, J. Kawana, M. Kiryu, I. Oseko, N. Sano, M.	2012	Diseases of Aquatic Organisms		Bercovier c-PCR, Yuasa c-PCR, Gilad q-PCR, (New) Yuasa rt-PCR		rt-PCR developed to detect mRNA from replicating CyHV-3 and not genomic CyHV-3 DNA. c-PCR but not Yuasa rt-PCR detected CyHV-3 at 0 hours post exposure. But rt-PCR detected CyHV-3 mRNA at 24 hours post exposure, presumably as the virus had commenced replication.
Laboratory validation of a lateral flow device for the detection of CyHV-3 antigens in gill swabs	Vrancken, R. Boutier, M. Ronsmans, M. Reschner, A. Leclipteux, T. Lieffrig, F. Collard, A. Mélard, C. Wera, S. Neyts, J. Goris, N. Vanderplasschen, A.	2013	Journal of Virological Methods	Mortality	Bercovier c-PCR		Lateral flow device (LFD) was developed for detection of viral glycoprotein ORF 65. The LFD had good analytical responsiveness. Diagnostic sensitivity was 52.65 and 72.25 compared to Bercovier c-PCR, depending on viral isolate. When mortality was used as reference, sensitivity was 67% and 93.3%. LFD detected CyHV-3 in all freshly dead fish.
Cyprinid Herpesvirus 3: An Archetype of Fish Alloherpesviruses	Boutier, M. Ronsmans, M. Rakus, K. Jazowiecka-Rakus, J. Vancsok, C. Morvan, L. Penranda, M. M. D. Stone, D. M. Way, K.	2015	Advances in Virus Research, Vol 93 **(REVIEW ARTICLE)**	Clinical signs, Histopathology, isolation in cell culture, Lateral flow device,	Gilad c-PCR, Gray c-PCR, Yuas c-PCR, Hutoron c-PCR, Ishioka c-PCR, Gilad qPCR, Bergmann n-PCR, LAMP, Yuasa rt-PCR	Fluorescent antibody test (FAT), ELISA, Immuno Fluorescence (IF), In situ hybridisation (ISH)	16 cell lines are listed that have previously been found to be susceptible to CPE from CYHV-3 and an additional 2 that have been considered susceptible but without CPE. Bercovier c-PCR considered more responsive than other c-PCRs. Gilad q-PCR widely acknowledged to be the most responsive PCR method. Bergmann n-PCR considered to have similar responsiveness to Gilad q-PCR.

	van Beurden, S. J. Davison, A. J. Vanderplasschen, A.						LAMP considered good for pond-side diagnosis. CCB and KF-1 cell lines recommended for virus isolation in cell culture but this technique is less sensitive than PCR. FAT, immunoperoxidase staining and ELISA available but not widely favoured by diagnostic laboratories. IF and ISH also briefly mentioned.
Sensitivity of seven PCRs for early detection of koi herpesvirus in experimentally infected carp, Cyprinus carpio L., by lethal and non-lethal sampling methods	Monaghan, SJ Thompson, KD Adams, Alexandra Bergmann, Sven M	2015	Journal of fish diseases		Seven different PCR assays*		Reviewed in detail in text
Validation of a KHV antibody enzyme-linked immunosorbent assay (ELISA)	Bergmann, S. M. Wang, Q. Zeng, W. Li, Y. Wang, Y. Matras, M. Reichert, M. Fichtner, D. Lenk, M. Morin, T. Olesen, N. J. Skall, H. F. Lee, P. Y. Zheng, S. Monaghan, S. Reiche, S.	2017	Journal of Fish Diseases			SNT, ELISA*, IFAT	ELISA reported to have good diagnostic and analytical sensitivity and specificity for fish with or without clinical clinical signs.

	Fuchs, W. Kotler, M. Way, K. Bräuer, G. Böttcher, K. Kappe, A. Kielpinska, J.						
Diagnostic validation of three test methods for detection of cyprinid herpesvirus 3 (CyHV-3)	Clouthier, S. C. McClure, C. Schroeder, T. Desai, M. Hawley, L. Khatkar, S. Lindsay, M. Lowe, G. Richard, J. Anderson, E. D.	2017	Diseases of Aquatic Organisms	Isolation in cell culture	q-PCR, c-PCR		Reviewed in detail in text.
Koi Herpes Virus Disease	OIE	2017	Manual of Diagnostic Tests for Aquatic Animals **(REVIEW and STANDARDS PAPER)**	Clinical signs, histology, electron microscopy, virus isolation in cell culture	Bercovier c-PCR, Yuasa c-PCR, Gilad q-PCR,	ELISA	Clinical signs and histology not considered pathognomonic. Electron microscopy not considered reliable. Virus isolation in cell culture not considered a reliable diagnostic technique. Immunoperoxidase staining mentioned but positive results may occur due to cross reactions with related viruses or proteins. Immunofluorescence (IF), IFAT and ELISA also mentioned but not considered to have been thoroughly compared with other methods. Bercovier c-PCR and Yuasa c-PCR are considered to be most sensitive methods for fresh tissue samples from

							clinically diseased carp and may allow detection of subclinical levels of CyHV-3. Gilad q-PCR considered capable of detecting very low copy numbers of target NA sequences and minimises contamination risk. Considerable methodological recommendations presented to prevent false positives and false negatives from PCR procedures. ELISA tests reliably detect anti-CyHV-3 antibodies at high serum dilutions but antibodies may cross-react with CyHV-1. Detection of antibodies considered useful to establish previous exposure to CyHV-3 but may not accurately reflect the viral status of fish.

1 ## Appendix 3 References citing undiagnosed mortalities and/or false positive test results during susceptibility testing of fish to CyHV-3.

Title	Authors	Year	Publication	Reporting of false positives and the occurrence of positive diagnostic results in known negatives	Occurrence of undiagnosed mortalities and mortalities in negative control fish and non-common carp species
Epidemilogical Description Of A New Viral Disease Afflicting Cultured Cyprinus Carpio In Israel	Perelberg, Ayana Smirnov, Margarita Hutoran, Marina Diamant, Ariel Bejerano, Yitzhak Kotler, Moshe	2003	Israeli Journal of Aquaculture - BAMIGDEH	None reported	Mortalities were only observed in fish exposed to the virus and not in negative controls (injection and cohabitation). Mortalities were not observed in species other common carp.
Differential resistance to koi herpes virus (KHV)/carp interstitial nephritis and gill necrosis virus (CNGV) among common carp (Cyprinus carpio L.) strains and crossbreds	Shapira, Y. Magen, Y. Zak, T. Kotler, M. Hulata, G. Levavi-Sivan, B.	2005	Aquaculture	None reported	No mortality was observed in naïve fish (not exposed to the virus) in controlled exposure trials.
Reactivation of koi herpesvirus infections in common carp Cyprinus carpio	St-Hilaire, S. Beevers, N. Way, K. Le Deuff, R. M. Martin, P. Joiner, C.	2005	Diseases of Aquatic Organisms	None reported for PCR, **1 reported for ELISA**	**Small numbers of mortalities occurred in negative control tanks and were exceeded in number in exposure tanks. In trial "*KHV Exposure 1*" mortalities occurred in negative control fish.** **Mortalities and moribund fish were subjected to post-mortem examination for bacterial infection, and ectoparasites and assessed by PCR for the presence of CyHV-3 DNA.**

					No bacterial pathogens were observed. The ectoparasite *Ichthyophthirius multifilliis* was observed on wet mounts of skin and gills. Fish were PCR negative for CyHV-3. **In trial "*KHV Exposure 2*", mortalities also occurred in negative control fish. These were examined for clinical signs, external parasites, and by PCR. Skin ulcerations were observed on dead fish but no external parasites were observed and PCR did not detect CyHV-3 DNA. No further diagnostic evidence is provided and these mortalities remain undiagnosed.**
Susceptibility of koi carp, common carp, goldfish, and goldfish x common carp hybrids to cyprinid herpesvirus-2 and herpesvirus-3	Hedrick, R. P. Waltzek, T. B. McDowell, T. S.	2006	Journal of Aquatic Animal Health	None reported	No mortalities were observed in negative controls or in non-carp species (goldfish). All mortalities in fish exposed to CyHV-3 were assessed for evidence of ectoparasites by microscopy of skin and gill scrapings, of bacterial infection by streaking from the head kidney onto blood agar plates and by histology. A single mortality that was exposed to CyHV-3 but negative by Gilad c-PCR and CPE was found to have *Aeromonas* infection
Similarities and heterogenicity of koi	Bergmann, S. M. Stumpf, P.	2007	Aquaculture	Insufficient detail presented	Insufficient detail presented

herpes virus (KHV) genome detected in ornamental fish without clinical signs	Schutze, H. Fichtner, D. Sadowski, J. Kempter, J.				
Detection of cyprinid herpesvirus type 3 in goldfish cohabiting with CyHV-3-infected koi carp (Cyprinus carpio koi)	El-Matbouli, M. Saleh, M. Soliman, H.	2007	Veterinary Record	Insufficient detail presented	No mortalities were observed in non-carp species (goldfish). Negative controls were not used.
Detection of koi herpesvirus (KHV) genome in wild and farmed fish from Northern Poland	Kempter, J. Bergmann, S. M.	2007	Aquaculture	Insufficient detail presented	No mortalities observed
Carp larvae are not susceptible to Koi herpesvirus	Ito, T. Sano, M. Kurita, J. Yuasa, K. Iida, T.	2007	Fish Pathology	None reported	**Trial 1: In negative control larvae, 2/20 larvae died compared to 3/40 in larvae exposed to the virus. In negative control juveniles, 1/10 fish died, compared to 20/29 in juveniles exposed to the virus. PCR was the only diagnostic tool used to determine the presence/absence of the virus.**
Detection of koi herpes virus (CyHV-3) in goldfish, Carassius auratus (L.), exposed to infected koi	Sadler, J. Marecaux, E. Goodwin, A. E.	2008	Journal of Fish Diseases	None reported	No mortalities reported in non-carp species (goldfish)
Effect of Water Temperature on Mortality and Virus Shedding in Carp	Yuasa, K. Ito, T. Sano, M.	2008	Fish Pathology	None reported - PCR only performed on dead fish	Negative controls were not used.

Experimentally Infected with Koi Herpesvirus					
Detection of koi herpes virus (KHV) genome in apparently healthy fish	Bergmann, SM Schütze, H Fischer, U Fichtner, D Riechardt, M Meyer, K Schrudde, D Kempter, J	2009	Bulletin of the European Association of Fish Pathologists	No False positives were reported from negative controls - negative controls were always negative.	No mortalities occurred in non-common carp species
Comparison of the resistance of selected families of common carp, Cyprinus carpio L., to koi herpesvirus: Preliminary study	Dixon, P. F. Joiner, C. L. Way, K. Reese, R. A. Jeney, G. Jeney, Z.	2009	Journal of Fish Diseases	None reported. No negative controls reported	Negative controls were not used/reported
Koi herpes virus: do acipenserid restitution programs pose a threat to carp farms in the disease-free zones?	Kempter, J. Sadowski, J. Schutze, H. Fischer, U. Dauber, M. Fichtner, D. Panicz, R. Bergmann, S. M.	2009	Acta Ichthyologica Et Piscatoria	None reported	**This was not an exposure trial and negative controls were not used. Mortalities were observed in sturgeon collected from polyculture farms. Clinical signs were reported. PCR tests revealed the presence of CyHV-3 DNA. CyHV-3 also identified by iIFAT and ISH.**
Resistance of common carp (Cyprinus carpio L.) to Cyprinid herpesvirus-3 is influenced by major histocompatibility (MH) class II B gene polymorphism	Rakus, K. L. Wiegertjes, G. F. Adamek, M. Siwicki, A. K. Lepa, A. Irnazarow, I.	2009	Fish & Shellfish Immunology	Diagnostic PCR data is not reported for negative controls	Mortality data is not reported for negative controls.

Goldfish (Carassius auratus auratus) is a susceptible species for koi herpesvirus (KHV) but not for KHV disease (KHVD)	Bergmann, S. M. Lutze, P. Schutze, H. Fischer, U. Dauber, M. Fichtner, D. Kempter, J.	2010	Bulletin of the European Association of Fish Pathologists	No false positives from negative controls for PCR, ISH or IFAT	No mortality occurred in non-common carp species (gold fish)
Susceptibility of koi x crucian carp and koi x goldfish hybrids to koi herpesvirus (KHV) and the development of KHV disease (KHVD)	Bergmann, S. M. Sadowski, J. Kielpinski, M. Bartlomiejczyk, M. Fichtner, D. Riebe, R. Lenk, M. Kempter, J.	2010	Journal of Fish Diseases	No false positive PCRs occurred in negative controls	Negative controls were used but mortality data is not presented.
Detection of KHV in Freshwater Mussels and Crustaceans from Ponds with KHV History in Common Carp (Cyprinus carpio)	Kielpinski, M. Kempter, J. Panicz, R. Sadowski, J. Schutze, H. Ohlemeyer, S. Bergmann, S. M.	2010	The Israeli Journal of Aquaculture	Negative controls were used and were always negative. i.e. No false positives.	Not appropriate: This was not an exposure trial and only live mussels and filter feeding crustaceans were collected.
Detection of koi herpesvirus (KHV) after re-activation in persistently infected common carp (Cyprinus carpio L.) using non-lethal sampling methods	Bergmann, S. M. Kempter, J.	2011	Bulletin of the European Association of Fish Pathologists	Negative controls were used and were always negative. i.e. No false positives.	No mortalities occurred in negative controls
Investigation of Koi Herpesvirus Latency in Koi	Eide, K. E. Miller-Morgan, T. Heidel, J. R.	2011	Journal of Virology	Negative controls were not used	Negative controls were not used

	Kent, M. L. Bildfell, R. J. LaPatra, S. Watson, G. Jin, L.				
Transmission of Cyprinid herpesvirus-3 (CyHV-3) from goldfish to naïve common carp by cohabitation	El-Matbouli, M. Soliman, H.	2011	Research in Veterinary Science	No false positives occurred in negative control gold fish	No mortalities in non-common carp species (gold fish) or negative controls
Feeding Cyprinus carpio with infectious materials mediates cyprinid herpesvirus 3 entry through infection of pharyngeal periodontal mucosa	Fournier, G. Boutier, M. Raj, V. S. Mast, J. Parmentier, E. Vanderwalle, P. Peeters, D. Lieffrig, F. Famir, F. Gillet, L. Vanderplasschen, A.	2012	Veterinary Research	Negative controls were not used	Negative controls were not used
Horizontal transmission of koi herpes virus (KHV) from potential vector species to common carp	Kempter, J. Kielpinski, M. Panicz, R. Sadowski, J. Myslowski, B. Bergmann, S. M.	2012	Bulletin of the European Association of Fish Pathologists	No False positives were reported from negative controls - negative controls were always negative.	No mortalities in non-carp species or negative controls
Common fish species in polyculture with carp as cyprinid herpes virus 3 carriers	Radosavljević, V. Jeremić, S. Ćirković, M. Lako, B.	2012	Acta Veterinaria	No specific mention of negative controls	No mortalities in non-carp species or negative controls

	Milićević, V. Potkonjak, A. Nikolin, V.				
Development of mRNA-specific RT-PCR for the detection of koi herpesvirus (KHV) replication stage	Yuasa, K. Kurita, J. Kawana, M. Kiryu, I. Ohseko, N. Sano, M.	2012	Diseases of Aquatic Organisms	Negative controls are not mentioned. The majority of PCR testing was performed on cultivated virus	Negative controls are not mentioned. The majority of PCR testing was performed on cultured virus
Do wild fish species contribute to the transmission of koi herpesvirus to carp in hatchery ponds?	Fabian, M. Baumer, A. Steinhagen, D.	2013	Journal of Fish Diseases	Naïve common carp tested negative to CyHV-3 by PCR.	Mortalities are not reported in common carp prior to virus exposure or in non-carp species.
Sensitivity of common carp, Cyprinus carpio L., strains and crossbreeds reared in the Czech Republic to infection by cyprinid herpesvirus 3 (CyHV-3; KHV)	Piackova, V. Flajshans, M. Pokorova, D. Reschova, S. Gela, D. Cizek, A. Vesely, T.	2013	Journal of Fish Diseases	Negative controls are not reported	Negative controls are not reported
Goldfish is not a susceptible host of koi herpesvirus (KHV) disease	Yuasa, K. Sano, M. Oseko, N.	2013	Fish Pathology	When goldfish were assessed by PCR prior to exposure, no false positives were observed.	**Mortality occurred in Ryukin goldfish exposed to CyHV-3, with affected fish exhibiting upset swimming behaviour near the water surface. The authors not that this variety of goldfish is known to be sensitive to temperature fluctuations, similar to those applied in the study, with affected fish showing upset swimming behaviour.**

Differences in the susceptibility of Japanese indigenous and domesticated Eurasian common carp (Cyprinus carpio), identified by mitochondrial DNA typing, to cyprinid herpesvirus 3 (CyHV-3)	Ito, T. Kurita, J. Yuasa, K.	2014	Veterinary Microbiology	False positives were not observed in any negative control fish by Gilad c-PCR.	No mortalities were observed in negative control fish.
Sensitivity and permissivity of Cyprinus carpio to cyprinid herpesvirus 3 during the early stages of its development: importance of the epidermal mucus as an innate immune barrier	Ronsmans, M. Boutier, M. Rakus, K. Farnir, F. Desmecht, D. Ectors, F. Vandecan, M. Lieffrig, F. Melard, C. Vanderplasschen, A.	2014	Veterinary Research	False positives for IVIS are difficult to determine as a positive result is based on samples returning a radiance value greater than the mean of mock inoculated fish plus three standard deviations. It is not specified how many mock inoculated fish fell outside of this range.	**Some mortality was observed in mock infected fish <~10% compared with infected fish ~50-100%**
Effects of daily temperature fluctuation on the survival of carp infected with Cyprinid herpesvirus 3	Takahara, T. Honjo, M. N. Uchii, K. Minamoto, T. Doi, H. Ito, T. Kawabata, Z.	2014	Aquaculture	Negative controls don't appear to have been used.	Negative controls don't appear to have been used.
Is There Any Species Specificity in Infections with Aquatic Animal herpesviruses?--The koi	Bergmann, Sven M. Cieslak, Michael Fichtner, Dieter Dabels, Juliane Monaghan, Sean J.	2016	Fisheries and Aquaculture Journal	No false positive (Gilad q-PCR, Bergmann sn-PCR, SNT, ELISA) were observed in common carp or rainbow trout on the first day post exposure.	No mortality in non-carp species (rainbow trout)

herpesvirus (KHV): An Alloherpesvirus model	Wang, Qing Zeng, Weiwei Kempter, Jolanta				
Transmission of Cyprinid herpesvirus 3 by wild fish species - results from infection experiments	Fabian, M. Baumer, A. Adamek, M. Steinhagen, D.	2016	Journal of Fish Diseases	No false positives were observed in carp or non-carp species prior to exposure to CyHV-3.	No mortality was observed in non-carp species.
Susceptibility of stone loach (Barbatula barbatula) and hybrids between sterlet (Acipenser ruthenus) and beluga (Huso huso) to cyprinid herpesvirus 3	Pospichal, A. Piackova, V. Pokorova, D. Vesely, T.	2016	Veterinarni Medicina	No False positives were observed in carp or non-carp species in negative control groups.	**Mortality in non-carp species during the first round of experimental testing was: Stone loach 0/10 and 1/10; Sterbel, 1/10 and 0/10; and in the secondary transmission trial was: Stone loach 10/10; Sterbel 0/10. PCR testing of dead fish did not reveal the presence of CyHV-3 DNA. The high rate of mortality observed in stone loach in the secondary transmission trial was deemed to be due to compromised function of the biological filters that led to a higher concentration of nitrites (~1.73 mg L^{-1})**
Koi herpesvirus infection in experimentally infected common carp Cyprinus carpio (Linnaeus, 1758) and three potential carrier fish species Carassius carassius (Linnaeus, 1758); Rutilus rutilus	Gaede, L. Steinbruck, J. Bergmann, S. M. Jager, K. Grafe, H. Schoon, H. A. Speck, S. Truyen, U.	2017	Journal of Applied Ichthyology	CyHV-3 DNA was not detected in any negative control fish. CyHV-3 was not detected by ISH in any negative controls.	Mortality was not reported in any negative controls or non-carp species.

(Linnaeus, 1758); and Tinca tinca (Linnaeus, 1758) by quantitative real-time PCR and in-situ hybridization					
Analysis of stress factors associated with KHV reactivation and pathological effects from KHV reactivation	Lin, L. Chen, S. Russell, D. S. Lohr, C. V. Milston-Clements, R. Song, T. Miller-Morgan, T. Jin, L.	2017	Virus Research	Negative controls were not used	Negative controls/non-carp species were not used. No mortalities were observed.
Cyprinid herpesvirus 3 as a potential biological control agent for carp (Cyprinus carpio) in Australia: susceptibility of non-target species	McColl, K. A. Sunarto, A. Slater, J. Bell, K. Asmus, M. Fulton, W. Hall, K. Brown, P. Gilligan, D. Hoad, J. Williams, L. M. Crane, M. S.	2017	Journal of Fish Diseases	**False positive q-PCR were observed in a small number of samples from NTS in negative controls (5/353). False positive q-PCRs were observed in a large number of negative control carp in trial 1 (7/8) but less frequently in the remaining 8 trials (1/19). The were no positive rt-PCR results from negative control fish. Positive q-PCR results were obtained for a large number of NTS (104/921) the authors seem to imply that these are false positives.**	**Undiagnosed mortalities were observed in a number of NTS exposed to CyHV-3. These were disregarded in most species as the mortalities were matched in the negative controls. However, mortalities were observed in rainbow trout (O. Mykiss), sea mullet (M. cephalus), silver perch (B. bidyanus), Peron's tree frog (L. peronii). These mortalities were noted to occur ealier or much later than in carp. They were all tested by PCR, some were weakly positive but negative by RT-PCR. Clinical signs and histology revealed no evidence of viral infection although no further detail was provided.**

Survival Rate and Immunological Responses of Mirror Carp Selective Breeding Generations to CyHV-3	Jia, Z. Wang, S. Bai, S. Ge, Y. Li, C. Hu, X. Shang, M. Zhang, J. Li, B. Shi, L.	2018	Journal of the World Aquaculture Society	PCR data not directly reported in results	Negative control groups were not used. All groups of fish were exposed to the virus and all groups experienced mortality at differing levels.
Susceptibility of the topmouth gudgeon (*Pseudorasbora parva*) to CyHV-3 under no-stress and stress conditions	Pospichal, A. Pokorova, D. Vesely, T. Piackova, V.	2018	Veterinární Medicína	Gilad q-PCR did not identify CyHV-3 DNA in any negative controls. i.e. no false positives.	No mortality was observed in negative controls or non-carp species.

1

2

Appendix 4. Unexplained mortalities and false positives in previous CyHV-3 infection susceptibility/resistance testing in the NCCP.

When mortalities are observed in non-common carp species or negative control groups, during laboratory NTS CyHV-3 exposure testing, it appears to be normal that additional investigative diagnostic procedures are performed in order to attempt to identify causal or associated factors. This may provide an alternate explanation for the occurrence of mortalities (i.e. other than nviral exposure) and may help to provide evidence that the deaths were caused by something other than CyHV-3.

False positive results from direct detection or diagnostic procedures are rarely reported in studies that have assessed susceptibility/resistance to CyHV-3. It is not clear why this is the case but it is clear that every effort must be made to prevent the occurrence of false positives, even if this means repeating exposure trials or diagnostic procedures.

Of those studies identified by our systematic review of the literature, the work of McColl et al. (2017) reported the most frequent occurrence of undiagnosed mortalities and false positives. As this study was also performed as a foundational piece of work for the NCCP, we feel that it warrants a special section in this review. The occurrence of undiagnosed mortalities and false positives in exposure trials performed by McColl et al. (2017) will be discussed in *Chapter 4.4. Undiagnosed mortalities & false positives in previous NTS testing in the NCCP.* Based on the information presented, potential strategies to overcome these problems in any future resistance testing will be presented. This includes the development of a matrix that considers all diagnostic evidence available to aid in determining of the resistance status of the host.

Occurrence of unexplained mortalities and false positives in CyHV-3 resistance testing.

Unexplained mortalities and false positives have been observed in studies that have assessed the susceptibility of fish to CyHV-3. McColl et al. (2017) reported a greater number and so discussion of the work will guide the development of protocols designed to overcome the occurrence and implications of undiagnosed mortalities and false positives for future studies.

Unexplained mortalities

McColl et al. (2017) observed low to high levels of mortality in 12 NTS and in common carp exposed to CyHV-3 as well as in negative control groups (i.e. not exposed to the virus). In most NTS the number of mortalities observed in CyHV-3-exposed fish was matched or exceeded by the magnitude of mortalities occurring in the negative control groups, suggesting that deaths may have been due to factors un-related to CyHV-3 exposure.

To determine if mortalities were occurring due to CyHV-3 infection, dead animals were examined for gross pathology that could be specifically attributed to CyHV-3 infection in common carp and tissues assessed by Gilad q-PCR. Tissues assessed positive for CyHV-3 by Gilad q-PCR, were also assessed by Yuasa rt-PCR. Tissues from moribund and some healthy animals were also assessed to identify the presence of any histopathology consistent with CyHV-3 infection in common carp.

Additional investigative diagnostic procedures designed to identify an alternative cause of death other than CyHV-3 do not appear to have been performed on mortalities and no alternative cause of death is reported for any mortalities that occurred in the study. As such, all mortalities reported in this study, other than those reported to be due to CyHV-3 infection, can be considered to be undiagnosed mortalities. And while some evidence is provided to support the assertion that CyHV-3

was not involved in these mortalities, this notion is considerably weakened by the inability to provide a clear diagnosis for an alternative cause of death.

This is most concerning for NTS cohorts in the work, in which the number of mortalities in CyHV-3-exposed fish exceeded that in negative control groups. This was the case for rainbow trout (*O. Mykiss*), sea mullet (*M. cephalus*), silver perch (*B. bidyanus*) and Peron's tree frog tadpoles (*L. peronii*). The authors state that mortalities in these groups did not present as being typical of CyHV-3 infection in carp: the mortalities generally (although not always) occurred earlier or much later than in CyHV-3-affected carp[1]; they were generally (although not always) negative when tested by Gilad q-PCR; those that were positive by Gilad q-PCR were negative when assessed by Yuasa rt-PCR; and it was reported that no significant lesions were found to account for mortalities in viral challenged NTS.

However, no explanation is offered as to why significant levels of mortality occurred in some species when exposed to CyHV-3 while very low or no mortalities were observed in animals not exposed to the virus. McColl et al. (2017) provide no explanation for these fish deaths and they remain undiagnosed mortalities.

While it is unclear what, if any, efforts were made to diagnose an alternative cause of death in NTS exposed to CyHV-3, clear efforts were made to rule out CyHV-3 infection as a cause of death. Pathological examinations (including histology), Gilad q-PCR and Yuasa rt-PCR were undertaken to determine if CyHV-3 was present and causing clinical signs.

Pathological examinations, including histopathology, were performed on all moribund animals and on some healthy fish. Fish that died were not subjected to histolopathological examination. Diagnostic investigation revealed no gross or histological lesions consistent with a viral infection in any NTS. This provides some evidence that CyHV-3 was not causing clinical signs in moribund and healthy NTS and based on this diagnostic information it was assummed that CyHV-3 was not responsible for the mortalities observed. However, as dead animals do not appear to have been subjected to histopathology, it is difficult to confirm, that CyHV-3 was not responsible for mortalities.

The Gilad q-PCR was also performed to rule out CyHV-3 as a cause of death in NTS. Samples tested were as follows: NTS on day 0 post challenge ; all animals surviving at the end of each trial; some healthy NTS collected at various time points in the post challenge period; some moribund animals; and all mortalities. CyHV-3 DNA was detected in 104 of 921 samples collected from viral challenged NTS. The results for the Gilad q-PCR testing was not published for individual species, and it was not specified if positive tests originated from dead, moribund or healthy animals. Rather, Gilad q-PCR data is only shown as the total number of positive samples identified in each trial, with multiple NTS assessed in each trial.

In those trials in which the mortality rate of NTS exposed to CyHV-3 exceeded that in negative control groups, variable Gilad q-PCR data was reported.

In trials assessing rainbow trout and sea mullet, only a few samples tested positive for the presence of CyHV-3 by Gilad q-PCR. The positive prevelance (by Gilad q-PCR) for these species was 3.2% and

[1] Divergent mortality patterns have also been observed in different strains and hybrids of common carp that are susceptible to CyHV-3 (S. M. Bergmann, J. Sadowski, M. Kielpinski, et al., 2010; Takafumi Ito, Kurita, & Yuasa, 2014; Piackova et al., 2013).

2.2% respectively. This is less than that observed in negative control common carp that returned 5.3% positive results. Interpretation of these results is complicated by the fact that negative control (experimentally un-exposed) fish tested positive to CyHV-3 but may indicate that the PCR positive NTS were not killed by the presence of CyHV-3 virus. Contamination of CyHV-3 genomic DNA may be one alternative explaianation for the positive PCR results.

Further complicating the trial results were the positive Gilad q-PCR results in trials involving silver perch and Peron's tree frog tadpoles. Results indicating prevelance of infection of 41.9% and 15.1% respectively, exceeded the rate of positive Gilad q-PCR (plausibly false positive) results observed in negative control common carp (5.3%).

Yuasa rt-PCR was also undertaken to rule out CyHV-3 as a causative factor in mortalities. Yuasa rt-PCR was used as it specifically detects mRNA associated with viral replication and so can provide evidence of active infection, rather than the mere presence of viral DNA. When samples from NTS that had previously tested positive by Gilad q-PCR were subsequently assessed by Yuasa rt-PCR, no positive results were observed. This would suggest that an active, replicating CyHV-3 infection was not present and causing mortality. However, results from rt-PCR should be assessed with caution as the diagnostic sensitivity and specificity and analytical responsiveness of this test has not been appropriately validated for this purpose.

Some standard diagnostic techniques (histopathology and PCR) were applied by McColl et al. (2017) in an attempt to explain morbidity and mortalities with in CyHV-3 exposure trials and the interpretation of results were used to argue CyHV-3 was not present and not causing mortalities in NTS and negative control carp. However, their approach left considerable doubt as to the true cause of death in these animals. As a consequence the mortalities remained undiagnosed, making it considerably more difficult to demonstrate resistance of the NTS to CyHV-3 within these trials.

To overcome the problems associated with undiagnosed mortalities in trials attempting to demonstrate resistence to CyHV-3 infection after exposure in NTS, it is critical that all mortalities be subjected to a matrix of investigative diagnostic procedures to determine the true cause of death. This will reduce the occurrence of undiagnosed mortalities and if a cause of death other than CyHV-3 can be confirmed, this would greatly strengthen the assertion that CyHV-3 is not present and the cause of any mortalities.

<u>False positives</u>

McColl et al. (2017) refer directly to the occurrence of false positives during their investigation into the susceptibility of NTS to CyHV-3.. The authors observed a large number of positive Gilad q-PCR results in NTS exposed to CyHV-3 as well as in negative control groups of NTS and common carp. The authors discus a range of alternate explanations for the high number of positive results, before concluding that the Gilad q-PCR method that they used repeatedly yielded false positive results. This is a critical concern and casts considerable doubt over their application of the Gilad q-PCR assay.

As discussed, the reporting of false positives in studies assessing the susceptibility of fish to CyHV-3 is rare. Of the 39 studies assessed in this review, only McColl et al. (2017) report the frequent occurrence of false positives. And while false positives are generally acknowledged to be a potential problem with PCR methods (Clouthier et al., 2017), it is unusual for repeated false positives to be reported in published studies. This may indicate a systemic problem in the application of the Gilad q-

PCR when undertaken by McColl et al. (2017). Clearly, this should be addressed before any further NTS resistance testing is undertaken.

The frequent occurrence of positive Gilad q-PCR results in NTS exposed to CyHV-3 was discussed by McColl et al. (2017) in some detail. The authors initially offer two potential views to explain the positive Gilad q-PCR results from NTS exposed to CyHV-3. They state that it could possibly be explained either by low levels of replicating CyHV-3 in tissues from viral challenged animals, or by inadvertent contamination of samples with viral DNA during processing.

To counter the argument that viable CyHV-3 was present in NTS in which Gilad q-PCR data suggested the presence of the virus, McColl et al. (2017) assessed tissue samples using the Yuasa rt-PCR protocol. When Yuasa rt-PC was applied, all samples from NTS returned a negative result. As discussed in Chapter 3, Yuasa rt-PCR is used to determine the presence of viral mRNA associated with viral replication. As such, it can be used to distinguish between the presence of non-viable viral DNA (or latent infection) and active, replicating virus.

McColl et al. (2017) interpreted negative Yuasa rt-PCR results as evidence that viral replication was not occurring in any of the NTS assessed. They also assert that positive results attained by Gilad q-PCR were due to sample contamination and not the true presence of CyHV-3 DNA in tissues. This is a reasonable conclusion. However, the use of Yuasa rt-PCR to over-ride Gilad q-PCR data can only be considered valid if the Yuasa rt-PCR is known to have sufficient diagnostic sensitivity and analytical responsiveness to detect CyHV-3 mRNA in all samples that truly contain replicating virus.

As stated previously the diagnostic responsiveness of the Yuasa rt-PCR test is currently unknown and has not been validated in the published scientific literature. It is also worth noting that when using other PCR tests known to have high analytical responsiveness (e.g. Gilad q-PCR), CyHV-3 DNA can remain undetected when present at low concentration, even during active infection that is causing mortality (Monaghan, et al.,2015).

From this, the authors determine that the high rate of positive Gilad q-PCR results observed in NTS are most likely the result of procedural errors during sample processing. However, they acknowledge the unknown sensitivity of the Yuasa rt-PCR and offer a third, alternative view to explain the frequent occurrence of positive Gilad q-PCR results in NTS exposed to CyHV-3: That positive Gilad q-PCR results represent low-level persistent infections below the level of detection by Yuasa rt-PCR. The authors refute this explanation on the grounds that such infections would require an acute viral infection in the early stages following challenge. They assert that this would provide abundant viral mRNA for detection by Yuasa rt-PCR. Again, this is a reasonable interpretation. However, it is not conclusive and there are at least two alternative, possible explanations for the results that they observed.

Firstly, positive Gilad q-PCR results could also have been due to the presence of a latent CyHV-3 infection in which viral replication was not proceeding. Although the ability of CyHV-3 to undergo true latency is not well understood, there is substantive, indicative evidence that the virus can establish latent infection (Prescott et al., 2016; Reed et al., 2017; Reed et al., 2014), a characteristic of other closely related herpes viruses. If latency is a characteristic of CyHV-3, it would not be identified by Yuasa rt-PCR. It should be noted as well that during the initial phases to latency/subclinical CyHV-3 infection in carp, there is a burst of lytic infection and viral replication which may be detected if frequent tissue analysis is undertaken (Michel et al, 2010). Whether similar

biology of infection occurs in NTS is yet to be documented but carp infections may inform our assumptions in other species.

Secondly, the diagnostic sensitivity and analytical responsiveness of the Yuasa rt-PCR are not well understood. It is possible that the Yuasa rt-PCR is unable to detect replicating virus during low-level persistent infections. Based on the evidence provided by McColl et al. (2017), the Yuasa rt-PCR does appear to be either less diagnostically sensitive than the Gilad q-PCR. In samples from common carp that had been exposed to CyHV-3 and confirmed positive by Gilad q-PCR, only ~80% of samples were positive by Yuasa rt-PCR. This indicates a ~20% rate of false negative in Yuasa rt-PCR tests which may have been due to a lack of diagnostic sensitivity. or simply a poor choice of definitive diagnostic test. One test indicating presence of genomic DNA and the other detecting mRNA. If mRNA is present at below detectable levels (viral replication low or absent) then the test will be more often negative leading to a discrepancy in diagnostic result. Choosing 'Fit for Purpose' tests is essential.

There is no concrete evidence for either of these explanations and the true source of positive Gilad q-PCR results in non-common carp species exposed to CyHV-3 remains unknown. What is clear is that CyHV-3 DNA was detected in 11.3% of samples collected from non-common carp species exposed to the virus while in non-common carp species not exposed to the virus only 1.42% of samples returned positive results. And in individual trials, positive Gilad q-PCR results were observed in up to ~42% of samples collected from NTS exposed to CyHV-3.

To further support the argument that positive Gilad q-PCR results were the result of contamination during sample processing, McColl et al. (2017) state that seven of eight positive results occurred during Trial 1. And that sampling protocols were improved considerably in subsequent trials. However, this does not explain why ~33% of samples from a NTS (bony bream) returned positive q-PCR results in Trial 9, during which negative control fish did not return any positive results by Gilad q-PCR.

McColl et al. (2017) attributed the large number of positive Gilad q-PCR test results to sample contamination, leading to the occurrence of false positives. The occurrence of false positives in PCR testing is well known (Borst, et al., 2004). However, if a procedural error was to blame for positive Gilad q-PCR results, it is unclear why this problem was not rectified during the course of the study. The authors state that sampling protocols were improved considerably in later trials, and yet positive Gilad q-PCR results continued to be observed throughout the course of the study. This makes it difficult to accept that many positive Gilad q-PCR results observed in NTS were in fact false positives.

Based on the available evidence presented from the study, only a relatively small number of positive Gilad q-PCR results can be comprehensively shown to be false positives. Only those results obtained from negative control groups can confidently be considered to be false positives as the likelihood of such NTS having CyHV-3 DNA in their tissues from any source is extremely low. For NTS exposed to CyHV-3, it is impossible to confidently assert that positive Gilad q-PCR results were actually false positives.

The implication of this is that CyHV-3 DNA may have been present in NTS in all trials performed, and may have been present in a high proportion of samples for certain species. While this may be seen as evidence that some NTS can be infected by CyHV-3, the presence of viral DNA in tissues does not confirm infection or affect on NTS.

The presence of viral DNA in tissue samples from fish exposed experimentally to CyHV-3 implies only presence not viral infection. This may be especially true for gill samples collected from fish exposed to CyHV-3 by immersion as there is an increased risk that water-borne virions, or DNA fragments, may have attached to the external surface of gill tissue and been subsequently detected by the Gilad q-PCR assay. Furthermore, it is plausible that viral DNA detected in kidney and spleen tissue is due to physical introduction of CyHV-3 by inadvertent injection into these tissues via IP injection. However, based on Gilad q-PCR data alone, it is impossible to rule out that CyHV-3 infected some of the NTS assessed by McColl et al. (2017).

The possible ability of CyHV-3 to infect NTS in the NCCP, even in the absence of clinical signs, should not be taken lightly. While infection does not imply the ability for CyHV-3 to cause mortality or acute clinical signs in NTS, then by definition a spillover infection/species jump has occurred. Whether there is no onward transmission in the new host (dead-ends), or only short chains of onward transmission in the new host (Parrish et al. 2008) then CyHV-3 has overcome the first barrier to sustained transmission in the new host, and there is an elevated probability that it could eventually acquire the adaptations necessary for onward transmission in the new host. As evidence of transpecies viral adaptions emerge (Lee et al 2017; Daniels et al 2007) . this is a critical consideration for the NCCP.

Appendix 5. Database of articles that applied, assessed or discussed external stressors in susceptibility/resistance testing for CyHV-3.

Title	Authors	Year	Publication	Stressor Applied
Goldfish (Carassius auratus auratus) is a susceptible species for koi herpesvirus (KHV) but not for KHV disease (KHVD)	Bergmann, S. M. Lutze, P. Schutze, H. Fischer, U. Dauber, M. Fichtner, D. Kempter, J.	2010	Bulletin of the European Association of Fish Pathologists	Stress discussed
Susceptibility of koi x crucian carp and koi x goldfish hybrids to koi herpesvirus (KHV) and the development of KHV disease (KHVD)	Bergmann, S. M. Sadowski, J. Kielpinski, M. Bartlomiejczyk, M. Fichtner, D. Riebe, R. Lenk, M. Kempter, J.	2010	Journal of Fish Diseases	Stress discussed
Detection of koi herpesvirus (KHV) after re-activation in persistently infected common carp (Cyprinus carpio L.) using non-lethal sampling methods	Bergmann, S. M. Kempter, J.	2011	Bulletin of the European Association of Fish Pathologists	Netting
Investigation of Koi Herpesvirus Latency in Koi	Eide, K. E. Miller-Morgan, T. Heidel, J. R. Kent, M. L. Bildfell, R. J. LaPatra, S. Watson, G. Jin, L.	2011	Journal of virology	Temperature
Transmission of Cyprinid herpesvirus-3 (CyHV-3) from goldfish to naïve common carp by cohabitation	El-Matbouli, M. Soliman, H.	2011	Research in Veterinary Science	Temperature
Horizontal transmission of koi herpes virus (KHV) from potential vector species to common carp	Kempter, J. Kielpinski, M. Panicz, R. Sadowski, J. Myslowski, B. Bergmann, S. M.	2012	Bulletin of the European association of fish pathologists	Stress discussed
Do wild fish species contribute to the transmission of koi herpesvirus to carp in hatchery ponds?	Fabian, M. Baumer, A. Steinhagen, D.	2013	Journal of Fish Diseases	Netting

Goldfish is not a susceptible host of koi herpesvirus (KHV) disease	Yuasa, K. Sano, M. Oseko, N.	2013	Fish Pathology	Temperature
Effects of daily temperature fluctuation on the survival of carp infected with Cyprinid herpesvirus 3	Takahara, T. Honjo, M. N. Uchii, K. Minamoto, T. Doi, H. Ito, T. Kawabata, Z.	2014	Aquaculture	Temperature
Is There Any Species Specificity in Infections with Aquatic Animal herpesviruses?--The koi herpesvirus (KHV): An Alloherpesvirus model	Bergmann, Sven M. Cieslak, Michael Fichtner, Dieter Dabels, Juliane Monaghan, Sean J. Wang, Qing Zeng, Weiwei Kempter, Jolanta	2016	Fisheries and Aquaculture journal	Netting
Transmission of Cyprinid herpesvirus 3 by wild fish species - results from infection experiments	Fabian, M. Baumer, A. Adamek, M. Steinhagen, D.	2016	Journal of Fish Diseases	Netting
Koi herpesvirus infection in experimentally infected common carp Cyprinus carpio (Linnaeus, 1758) and three potential carrier fish species Carassius carassius (Linnaeus, 1758); Rutilus rutilus (Linnaeus, 1758); and Tinca tinca (Linnaeus, 1758) by quantitative real-time PCR and in-situ hybridization	Gaede, L. Steinbruck, J. Bergmann, S. M. Jager, K. Grafe, H. Schoon, H. A. Speck, S. Truyen, U.	2017	Journal of Applied Ichthyology	Netting
Analysis of stress factors associated with KHV reactivation and pathological effects from KHV reactivation	Lin, L. Chen, S. Russell, D. S. Lohr, C. V. Milston-Clements, R. Song, T. Miller-Morgan, T. Jin, L.	2017	Virus Research	Temperature

1

2

Appendix 6. Evidence of resistance status of non-common carp species to CyHV-3 reported in the published literature.

Host species are considered resistant where there is no evidence presented to suggest a host species can be infected by CyHV-3. Host species are considered to be not-resistant where any evidence is presented that suggests that CyHV-3 can infect the host. This may be the occurrence of clinical signs or evidence of the presence of CyHV-3 in host tissues.

Order	Common name	Family	*Genus species*	Resistant or Not-resistant	Clinical signs	Weight of evidence	Reference
Cypriniformes	Stone loach	Nemacheilidae	*Barbatula barbatula*	**Detected/ Uncertain**	None	Fish were exposed to CyHV-3-infected common carp and subsequently tested positive for the virus by CEFAS n-PCR. When naïve common carp were cohabitated with these fish, CEFAS n-PCR could not detect the virus in these common carp.	(Pospichal et al., 2016)
Cypriniformes	Goldfish	Cyprinidae	*Carassius auratus*	**Not-resistant**	None	CyHV-3 DNA, mRNA and specific proteins were found 7, 14, 45, 60 days and one year after injection with CyHV-3. And the virus was isolated after cohabitation. Specific methodology and comprehensive results are not presented.	(O. Haenen & Hedrick, 2006)
Cypriniformes	Goldfish	Cyprinidae	*Carassius auratus*	**Resistant**	None	No mortality after exposure to infected common carp. Common carp not infected after cohabitation with exposed goldfish.	(Ayana Perelberg et al., 2003)
Cypriniformes	Goldfish	Cyprinidae	*Carassius auratus*	**Resistant**	None	No disease or mortality was observed in goldfish exposed to CyHV-3 by IP injection. Gilad c-PCR was always negative for goldfish exposed to the virus.	(R. P. Hedrick et al., 2006)
Cypriniformes	Goldfish	Cyprinidae	*Carassius auratus*	**Not-resistant**	Leucopenia	CyHV-3 was detected by Gilad c-PCR, Hutoran c-PCR, Bergmann n-PCR, IFAT, ISH and clinical signs (including leucopenia) in goldfish exposed to the virus by immersion and IP injection. Goldfish could transmit CyHV-3 to common carp by cohabitation.	(S. Bergmann et al., 2010)
Cypriniformes	Goldfish	Cyprinidae	*Carassius auratus*	**Not-resistant**	None	CyHV-3 was detected by a novel RT-PCR and modified Bercovier c-PCR in goldfish that had cohabitated with CyHV-3 infected common carp. Goldfish could transmit CyHV-3 to common carp by cohabitation.	(Mansour El-Matbouli & Soliman, 2011)
Cypriniformes	Goldfish	Cyprinidae	*Carassius auratus*	**Not-resistant**	None	Goldfish were cohabitated with asymptomatic common carp that had been exposed to CyHV-3. CyHV-3 was later detected in these goldfish by Bercovier c-PCR. These goldfish were then cohabitated with naïve common carp and the virus was later detected in these common carp by Bervcovier c-PCR.	(Radosavljević et al., 2012)
Cypriniformes	Goldfish	Cyprinidae	*Carassius auratus*	**Resistant**	None	Goldfish were exposed to CyHV-3 by immersion and later cohabitated with naïve common carp. None of these common carp died following cohabitation. Furthermore, CyHV-3 was not detected in any of these goldfish or common carp when assessed by Bercovier c-PCR.	(Kei Yuasa et al., 2013)
Cypriniformes	Goldfish	Cyprinidae	*Carassius auratus*	**Not-resistant**	None	When goldfish were cohabitated with CyHV-3-infected common carp, the virus was subsequently detected in goldfish by Gilad q-PCR and also in carp that were cohabitated with these goldfish.	(Fabian et al., 2016)
Cypriniformes	Crucian carp	Cyprinidae	*Carassius carassius*	**Not-resistant**	None	CyHV-3 DNA, mRNA and specific proteins were found 7, 14, 45, 60 days and one year after injection with CyHV-3. And the virus was isolated after cohabitation. Specific methodology and comprehensive results are not presented.	(O. Haenen & Hedrick, 2006)
Cypriniformes	Crucian carp	Cyprinidae	*Carassius carassius*	**Not-resistant**	Mild mucoid plaques	After exposure by immersion, CyHV-3 was detected in fish by Gilad q-PCR. Mild mucoid plaques on the head and fins were observed by the end of the first week	(Gaede, Steinbrück, et al., 2017)

						post exposure to CyHV-3. CyHV-3 DNA was also detected by ISH in a range of tissues.	
Cypriniformes	Prussian carp	Cyprinidae	*Carassius auratus gibelio*	**Not-resistant**	None	Prussian carp were cohabitated with asymptomatic common carp that had been exposed to CyHV-3. CyHV-3 was later detected in grass these carp by Bercovier c-PCR. These grass carp were then cohabitated with naïve common carp and the virus was later detected in these common carp by Bervcovier c-PCR.	(Radosavljević et al., 2012)
Cypriniformes	Prussian carp	Cyprinidae	*Carassius carassius gibelio*	**Uncertain**	None	Wild fish were collected from carp farms in Saxony, Germany and CyHV-3 was not detected in their tissues by PCR (Adamek et al., 2012; Oren Gilad et al., 2004). When these fish were cohabitated with naïve common carp, CyHV-3 was not detected in these carp by PCR (Adamek et al., 2012; Oren Gilad et al., 2004).	(Fabian et al., 2013)
Cypriniformes	Grass carp	Cyprinidae	*Ctenopharyngodon idella*	**Resistant**	None	No mortality after exposure to infected common carp. Common carp not infected after cohabitation with exposed grass carp.	(Ayana Perelberg et al., 2003)
Cypriniformes	Grass carp	Cyprinidae	*Ctenopharyngodon idella*	**Not-resistant**	None	CyHV-3 DNA was detected by Bercovier c-PCR, in fish collected from farms in the Oder River catchment, Poland. CyHV-3 could be transmitted to common carp by cohabitation, which was subsequently detected by Bergmann n-PCR and CEFAS n-PCR.	(J Kempter et al., 2012)
Cypriniformes	Grass carp	Cyprinidae	*Ctenopharyngodon idella*	**Not-resistant**	None	Grass carp were cohabitated with asymptomatic common carp that had been exposed to CyHV-3. CyHV-3 was later detected in grass carp by Bercovier c-PCR. These grass carp were then cohabitated with naïve common carp and the virus was later detected in these common carp by Bervcovier c-PCR.	(Radosavljević et al., 2012)
Cypriniformes	Silver carp	Cyprinidae	*Hypophthalmichthys molitrix*	**Resistant**	None	No mortality after exposure to infected common carp. Common carp not infected after cohabitation with exposed silver carp.	(Ayana Perelberg et al., 2003)
Cypriniformes	Silver carp	Cyprinidae	*Hypophthalmichthys molitrix*	**Not-resistant**	None	CyHV-3 DNA was detected by Bercovier c-PCR, in fish collected from farms in the Oder River catchment, Poland. CyHV-3 could be transmitted to common carp by cohabitation, which was subsequently detected by Bergmann n-PCR and CEFAS n-PCR.	(J Kempter et al., 2012)
Cypriniformes	Silver carp	Cyprinidae	*Hypophthalmichthys molitrix*	**Not-resistant**	None	Silver carp were cohabitated with asymptomatic common carp that had been exposed to CyHV-3. CyHV-3 was later detected in these silver carp by Bercovier c-PCR. These silver carp were then cohabitated with naïve common carp and the virus was later detected in these common carp by Bervcovier c-PCR.	(Radosavljević et al., 2012)
Cypriniformes	Gudgeon	Cyprinidae	*Gobio gobio*	**Not-resistant**	None	Wild fish were collected from carp farms in Saxony, Germany and CyHV-3 was detected in their tissues by PCR (Adamek et al., 2012; Oren Gilad et al., 2004). When these fish were cohabitated with naïve common carp, CyHV-3 was detected in these carp by PCR (Adamek et al., 2012; Oren Gilad et al., 2004).	(Fabian et al., 2013)
Cypriniformes	Gudgeon	Cyprinidae	*Gobio gobio*	**Detected/ Uncertain**	None	Gudgeon were exposed to CyHV-3 by immersion. These fish were then cohabitated with naïve common carp. Following this, the virus could not be detected in gudgeon or common carp by Gilad q-PCR. When gudgeon were cohabitated with CyHV-3-infected common carp, the virus was subsequently detected in gudgeon by Gilad q-PCR but not in carp that were subsequently cohabitated with these gudgeon.	(Fabian et al., 2016)

Cypriniformes	Ide	Cyprinidae	*Leuciscus idus*	**Not-resistant**	None	Ide were exposed to CyHV-3 by immersion. These fish were then cohabitated with naïve common carp. Following this, the virus was detected in a single ide and a single common carp by Gilad q-PCR. When ide were cohabitated with CyHV-3-infected common carp, the virus was subsequently detected in ide by Gilad q-PCR but not in carp cohabitated with these ide.	(Fabian et al., 2016)
Cypriniformes	Common dace	Cyprinidae	*Leuciscus leuciscus*	**Detected/ Uncertain**	None	Wild fish were collected from carp farms in Saxony, Germany and CyHV-3 was detected in their tissues by PCR (Adamek et al., 2012; Oren Gilad et al., 2004). When these fish were cohabitated with naïve common carp, CyHV-3 was not detected in these carp by PCR (Adamek et al., 2012; Oren Gilad et al., 2004).	(Fabian et al., 2013)
Cypriniformes	Top mouth gudgeon	Cyprinidae	*Pseudorasbora parva*	**Not-resistant**	None	After cohabitation with CyHV-3-infected koi, fish tested positive for CyHV-3 by Gilad q-PCR. When these fish were cohabitated with common carp, a single common carp tested positive by Gilad q-PCR.	(Pospichal et al., 2018)
Cypriniformes	Common roach	Cyprinidae	*Rutilus rutilus*	**Not-resistant**	None	CyHV-3 DNA was detected by Bercovier c-PCR, in fish collected from farms in the Oder River catchment, Poland. CyHV-3 could be transmitted to common carp by cohabitation, which was subsequently detected by Bergmann n-PCR and CEFAS n-PCR.	(J Kempter et al., 2012)
Cypriniformes	Roach	Cyprinidae	*Rutilus rutilus*	**Detected/ Uncertain**	None	Wild fish were collected from carp farms in Saxony, Germany and CyHV-3 was detected in their tissues by PCR (Adamek et al., 2012; Oren Gilad et al., 2004). When these fish were cohabitated with naïve common carp, CyHV-3 was not detected in these carp by PCR (Adamek et al., 2012; Oren Gilad et al., 2004).	(Fabian et al., 2013)
Cypriniformes	Common roach	Cyprinidae	*Rutilus rutilus*	**Not-resistant**	Slight abrasions on fins	After exposure by immersion, CyHV-3 was detected in fish by Gilad q-PCR. Slight abrasions were observed on the fins during the first days post- infection.	(Gaede, Steinbrück, et al., 2017)
Cypriniformes	Rudd	Cyprinidae	*Scardinius erythrophthalmus*	**Not-resistant**	None	Wild fish were collected from carp farms in Saxony, Germany and CyHV-3 was detected in their tissues by PCR (Adamek et al., 2012; Oren Gilad et al., 2004). When these fish were cohabitated with naïve common carp, CyHV-3 was detected in these carp by PCR (Adamek et al., 2012; Oren Gilad et al., 2004).	(Fabian et al., 2013)
Cypriniformes	Rudd	Cyprinidae	*Scardinius erythrophthalmus*	**Resistant**	None	Rudd were exposed to CyHV-3 by immersion. These fish were then cohabitated with naïve common carp. Following this, the virus could not be detected in rudd or common carp by Gilad q-PCR.	(Fabian et al., 2016)
Cypriniformes	Tench	Cyprinidae	*Tinca tinca*	**Not-resistant**	None	CyHV-3 DNA was detected by Bercovier c-PCR, in fish collected from farms in the Oder River catchment, Poland. CyHV-3 could be transmitted to common carp by cohabitation, which was subsequently detected by Bergmann n-PCR and CEFAS n-PCR.	(J Kempter et al., 2012)
Cypriniformes	Tench	Cyprinidae	*Tinca tinca*	**Not-resistant**	None	Tench were cohabitated with asymptomatic common carp that had been exposed to CyHV-3. CyHV-3 was later detected in these tench by Bercovier c-PCR. These tench were then cohabitated with naïve common carp and the virus was later detected in these common carp by Bervcovier c-PCR.	(Radosavljević et al., 2012)
Cypriniformes	Tench	Cyprinidae	*Tinca tinca*	**Not-resistant**	None	Wild fish were collected from carp farms in Saxony, Germany and CyHV-3 was detected in their tissues by PCR (Adamek et al., 2012; Oren Gilad et al., 2004). When these fish were cohabitated with naïve common carp, CyHV-3 was detected in these carp by PCR (Adamek et al., 2012; Oren Gilad et al., 2004).	(Fabian et al., 2013)

Cypriniformes	Tench	Cyprinidae	*Tinca tinca*	**Detected/ Uncertain**	None	Tench were exposed to CyHV-3 by immersion. These fish were then cohabitated with naïve common carp. Following this, the virus was detected in a single tench but not in common carp by Gilad q-PCR. When tench were cohabitated with CyHV-3-infected common carp, the virus was subsequently detected in tench by Gilad q-PCR but not in carp cohabitated with these tench.	(Fabian et al., 2016)
Cypriniformes	Common nase	Cyprinidae	*Chondrostoma nasus*	**Resistant**	None	Common nase were exposed to CyHV-3 by immersion. These fish were then cohabitated with naïve common carp. Following this, the virus could not be detected in common nase or common carp by Gilad q-PCR.	(Fabian et al., 2016)
Acipenseriformes	Sterbel	Acipenseridae	*Acipenser ruthenus × Huso huso*	**Detected/ Uncertain**	None	Fish were exposed to CyHV-3-infected common carp and subsequently tested positive for the virus by CEFAS n-PCR. When naïve common carp were cohabitated with these fish, CEFAS n-PCR could not detect the virus in these common carp.	(Pospichal et al., 2016)
Cichliformes	Tilapia	Cichlidae	*Oreochromis niloticus*	**Resistant**	None	No mortality after exposure to infected common carp. Common carp not infected after cohabitation with exposed tilapia.	(Ayana Perelberg et al., 2003)
Esociformes	Northern pike	Esocidae	*Esox lucius*	**Uncertain**	None	Wild fish were collected from carp farms in Saxony, Germany and CyHV-3 was not detected in their tissues by PCR (Adamek et al., 2012; Oren Gilad et al., 2004). When these fish were cohabitated with naïve common carp, CyHV-3 was detected in these carp by PCR (Adamek et al., 2012; Oren Gilad et al., 2004).	(Fabian et al., 2013)
Gasterosteiformes	Three-spined stickleback	Gasterosteidae	*Gasterosteus aculeatus*	**Detected/ Uncertain**	None	Wild fish were collected from carp farms in Saxony, Germany and CyHV-3 was detected in their tissues by PCR (Adamek et al., 2012; Oren Gilad et al., 2004). When these fish were cohabitated with naïve common carp, CyHV-3 was not detected in these carp by PCR (Adamek et al., 2012; Oren Gilad et al., 2004).	(Fabian et al., 2013)
Gasterosteiformes	Three-spined stickleback	Gasterosteidae	*Gasterosteus aculeatus*	**Detected/ Unceratin**	None	Three-spined stickleback were exposed to CyHV-3 by immersion. These fish were then cohabitated with naïve common carp. Following this, the virus was detected in a single three-spined stickleback but not in common carp by Gilad q-PCR.	(Fabian et al., 2016)
Perciformes	American Silver Perch	Sciaenidae	*Bairdiella chrysoura*	**Resistant**	None	No mortality after exposure to infected common carp. Common carp not infected after cohabitation with exposed American silver perch.	(Ayana Perelberg et al., 2003)
Perciformes	Ruffe	Percidae	*Gymnocephalus cernua*	**Uncertain**	None	Wild fish were collected from carp farms in Saxony, Germany and CyHV-3 was not detected in their tissues by PCR (Adamek et al., 2012; Oren Gilad et al., 2004). When these fish were cohabitated with naïve common carp, CyHV-3 was detected in these carp by PCR (Adamek et al., 2012; Oren Gilad et al., 2004).	(Fabian et al., 2013)
Perciformes	Ruffe	Percidae	*Gymnocephalus cernua*	**Not-resistant**	None	CyHV-3 DNA was detected by Bercovier c-PCR, in fish collected from farms in the Oder River catchment, Poland. CyHV-3 could be transmitted to common carp by cohabitation, which was subsequently detected by Bergmann n-PCR and CEFAS n-PCR.	(J Kempter et al., 2012)
Perciformes	European perch	Percidae	*Perca fluviatilis*	**Detected/ Uncertain**	None	Wild fish were collected from carp farms in Saxony, Germany and CyHV-3 was detected in their tissues by PCR (Adamek et al., 2012; Oren Gilad et al., 2004). When these fish were cohabitated with naïve common carp, CyHV-3 was not detected in these carp by PCR (Adamek et al., 2012; Oren Gilad et al., 2004).	(Fabian et al., 2013)
Perciformes	European perch	Percidae	*Perca fluviatilis*	**Not-resistant**	None	CyHV-3 DNA was detected by Bercovier c-PCR, in fish collected from farms in the Oder River catchment, Poland. CyHV-3 could be transmitted to common carp by cohabitation, which was subsequently detected by Bergmann n-PCR and CEFAS n-PCR.	(J Kempter et al., 2012)

Perciformes	European perch	Percidae	*Perca fluviatilis*	**Detected/ Uncertain**	None	European perch were exposed to CyHV-3 by immersion. These fish were then cohabitated with naïve common carp. Following this, the virus was detected in a single European perch by Gilad q-PCR but not in common carp.	(Fabian et al., 2016)
Salmoniformes	Rainbow trout	Salmonidae	*Oncorhynchus mykiss*	**Not-resistant**	None	Rainbow trout were exposed to CyHV-3 by immersion. These fish were then cohabitated with naïve common carp. These common carp became sick and one died from CyHV-3. The virus was detected in rainbow trout and common carp by: Gilad q-PCR, as modified by S. M. Bergmann, M. Riechardt, et al. (2010); SNT; ELISA.	(Sven M Bergmann et al., 2016)
Siluriformes	Brown bullhead	Ictaluridae	*Ameiurus nebulosus*	**Detected/ Uncertain**	None	Wild fish were collected from carp farms in Saxony, Germany and CyHV-3 was detected in their tissues by PCR (Adamek et al., 2012; Oren Gilad et al., 2004). When these fish were cohabitated with naïve common carp, CyHV-3 was not detected in these carp by PCR (Adamek et al., 2012; Oren Gilad et al., 2004).	

1
2
3

Appendix 7. Evidence of the CyHV-3 carrier status fish species inhabiting the Odra River system in Poland (reproduced from Kempter. et al. (2008) cited in J Kempter et al. (2012).

Four PCR runs according to the methods described by O. Gilad et al. (2002), S. M. Bergmann et al. (2006), Bercovier et al. (2005) and (CEFAS, Unpublished), were used to detect CyHV-3 DNA in fish collected from fish farms, streams or brooks connected with the Oder River.

Order	Common name	Family	Genus species	Detected or Not-detected	Reference
Cypriniformes	Ide	Cyprinidae	*Leuciscus idus*	**Detected**	
Cypriniformes	Tench	Cyprinidae	*Tinca tinca*	**Detected**	
Cypriniformes	Common roach	Cyprinidae	*Rutilus rutilus*	**Detected**	
Cypriniformes	Common dace	Cyprinidae	*Leuciscus leuciscus*	**Detected**	
Cypriniformes	Gudgeon	Cyprinidae	*Gobio gobio*	**Detected**	
Cypriniformes	Common bream	Cyprinidae	*Abramis brama*	**Detected**	
Cypriniformes	Crucian carp	Cyprinidae	*Carassius carassius*	**Detected**	
Cypriniformes	European chub	Cyprinidae	*Squalius cephalus*	**Detected**	
Cypriniformes	Common barbel	Cyprinidae	*Barbus barbus*	**Detected**	
Cypriniformes	Vimba bream	Cyprinidae	*Vimba vimba*	**Detected**	
Cypriniformes	Spined loach	Cobitidae	*Cobitis taenia*	**Detected**	
Cypriniformes	Belica	Cyprinidae	*Leucaspius delineatus*	**Detected**	
Cypriniformes	Common nase	Cyprinidae	*Chondrostoma nasus*	**Detected**	
Cypriniformes	Common rudd	Cyprinidae	*Scardinius erythrophthalmus*	**Not-detected**	
Cypriniformes	Stone moroko	Cyprinidae	*Pseudorasbora parva*	**Not-detected**	
Cypriniformes	Common carp x crucian carp hybrid	Cyprinidae	*Cyprinus carpio × Carassius carassius*	**Not-detected**	
Cypriniformes	Common bleak	Cyprinidae	*Alburnus alburnus*	**Not-detected**	
Cypriniformes	Silver bream	Cyprinidae	*Blicca bjoerkna*	**Not-detected**	
Cypriniformes	Grass carp	Cyprinidae	*Ctenopharyngodon idella*	**Not-detected**	
Esociformes	Northern pike	Esocidae	*Esox lucius*	**Detected**	
Gasterosteiformes	Three-spined stickleback	Gasterosteidae	*Gasterosteus aculeatus*	**Not-detected**	

Perciformes	European perch	Percidae	*Perca fluviatilis*	**Detected**	
Perciformes	Zander	Percidae	*Sander lucioperca*	**Not-detected**	
Scorpaeniformes	European bullhead	Cottidae	*Cottus gobio*	**Detected**	**(Kempter. et al., 2008)**
Acipenseriformes	Russian sturgeon	Acipenseridae	*Acipenser gueldenstaedtii*	**Detected**	**(J Kempter et al., 2009)**
Acipenseriformes	Atlantic sturgeon	Acipenseridae	*Acipenser oxyrinchus*	**Detected**	**(J Kempter et al., 2009)**
Cypriniformes	Common bream	Cyprinidae	*Abramis brama*	**Not-detected**	**(J. Kempter & Bergmann, 2007)**
Cypriniformes	Common bream	Cyprinidae	*Abramis brama*	**Detected**	**(Fabian et al., 2013)**
Cypriniformes	Prussian carp	Cyprinidae	*Carassius gibelio*	**Not-detected**	**(J. Kempter & Bergmann, 2007)**
Cypriniformes	Prussian carp	Cyprinidae	*Carassius carassius gibelio*	**Uncertain**	**(Fabian et al., 2013)**
Cypriniformes	Common dace	Cyprinidae	*Leuciscus leuciscus*	**Detected**	**(J. Kempter & Bergmann, 2007)**
Cypriniformes	Common roach	Cyprinidae	*Rutilus rutilus*	**Not-detected**	**(J. Kempter & Bergmann, 2007)**
Cypriniformes	Tench	Cyprinidae	*Tinca tinca*	**Not-detected**	**(J. Kempter & Bergmann, 2007)**
Cypriniformes	Vimba bream	Cyprinidae	*Vimba vimba*	**Detected**	**(J. Kempter & Bergmann, 2007)**
Siluriformes	Wels catfish	Siluridae	*Silurus glanis*	**Not-detected**	**(J. Kempter & Bergmann, 2007)**
Cypriniformes	Goldfish	Cyprinidae	*Carassius auratus*	**Detected**	(S. M. Bergmann, Stumpf, Schütze, et al., 2007)
Cypriniformes	Grass carp	Cyprinidae	*Ctenopharyngodon idella*	**Not-resistant**	(S. M. Bergmann, Stumpf, Schütze, et al., 2007)
Cypriniformes	Golden ide	Cyprinidae	*Leuciscus idus*	**Not-resistant**	(S. M. Bergmann, Stumpf, Schütze, et al., 2007)
Siluriformes	Sheatfish	Ictaluridae	*Ictalurus melas*	**Not-resistant**	(S. M. Bergmann, Stumpf, Schütze, et al., 2007)
Cypriniformes	Goldfish	Cyprinidae	*Carassius auratus*	**Not-resistant**	(M. El-Matbouli, Saleh, & Soliman, 2007)
Cypriniformes	Goldfish	Cyprinidae	*Carassius auratus*	**Not-resistant**	(Sadler, Marecaux, & Goodwin, 2008)
Cypriniformes	Goldfish	Cyprinidae	*Carassius auratus*	**Not-resistant**	(S. Bergmann et al., 2009)
Cypriniformes	Grass carp	Cyprinidae	*Ctenopharyngodon idella*	**Not-resistant**	(S. Bergmann et al., 2009)
Cypriniformes	Blue back ide	Cyprinidae	*Leuciscus idus*	**Not-resistant**	(S. Bergmann et al., 2009)

Siluriformes	Bristlenose catfish	Loricariidae	*Ancistrus sp.*	**Not-resistant**	(S. Bergmann et al., 2009)
Cypriniformes	Sunbleak	Cyprinidae	*Leucaspius delineatus*	**Not-detected**	**(Fabian et al., 2013)**
Cypriniformes	European chub	Cyprinidae	*Squalius cephalus*	**Not-detected**	**(Fabian et al., 2013)**
Perciformes	Pike-perch	Percidae	*Sander lucioperca*	**Not-resistant**	**(Fabian et al., 2013)**

1

Order	**Common name**	**Family**	**Genus species**	Mortality suggests posible effect of virus	Estimated rate of positive Gilad q-PCR results in trial	Yuasa rt-PCR
Anguilliformes	Short-finned eel	**Anguillidae**	*Anguilla australis*	-	5.50%	-
Anura	Peron's tree frog, adult	**Hylidae**	*Litoria peronii*	-	15.10%	-
Anura	Peron's tree frog, tadpole	**Hylidae**	*Litoria peronii*	**+**	15.10%	-
Anura	Spotted Marsh frog, adult	**Myobatrachidae**	*Limnodynastes tasmaniensis*	-	15.10%	-
Atheriniformes	Crimson spotted rainbow fish	**Melanotaeniidae**	*Melanotaenia duboulayi*	-	5.50%	-
Clupeiformes	Bony bream	**Clupeidae**	Nematalosa erebi	-	**33.30%**	-
Decapoda	Common Yabby	**Parastacidae**	*Cherax destructor*	-	2.00%	-
Galliformes	Chicken	**Phasianidae**	Gallus gallus domesticus	-	6.10%	-
Gobiiformes	Carp gudgeon	**Eleotridae**	*Hypseleotris sp.*	-	2.00%	-
Mugiliformes	Sea mullet	**Mugilidae**	*Mugil cephalus*	**+**	3.20%	-
Osmeriformes	Australian smelt	**Retropinnidae**	*Retropinna semoni*	-	3.20%	-
Osmeriformes	Common galaxias (*G. maculatus*)	**Galaxiidae**	*Galaxias maculatus*	-	2.20%	-
Perciformes	Murray Cod	**Percichthyidae**	*Maccullochella peelii*	-	**29.40%**	-
Perciformes	Golden perch	**Percichthyidae**	*Macquaria ambigua*	-	**21.10%**	-
Perciformes	Olive perchlet	**Ambassidae**	*Ambassis agassizii*	-	2.00%	-
Perciformes	Silver perch	**Terapontidae**	*Bidyanus bidyanus*	**+**	**41.90%**	-
Petromyzontiformes	Short-headed lamprey	**Mordaciidae**	*Mordacia mordax*	-	2.00%	-
Rodentia	Mouse	**Muridae**	*Mus musculus*	-	6.10%	-
Salmoniformes	Rainbow trout	**Salmonidae**	*Oncorhynchus mykiss*	**+**	2.20%	-

Siluriformes	Salmon catfish	**Ariidae**	*Neoarius graefei*	-	5.50%	-
Siluriformes	Eel-tailed catfish	**Plotosidae**	*Tandanus tandanus*	-	3.20%	-
Squamata	Eastern water dragon	**Agamidae**	*Intellagama lesueurii*	-	15.10%	-
Testudines	Macquarie short necked turtle	**Chelidae**	*Emydura macquarii*	-	15.10%	-

1
2
3

NATIONAL CARP CONTROL PLAN

The National Carp Control Plan is managed by the
Fisheries Research and Development Corporation

Tel: 02 6285 0400
Post: Locked Bag 222, Deakin West ACT 2600

www.carp.gov.au