

Final report: Phase 3 of the carp herpesvirus project (CyHV-3)

Dr Ken McColl 2016







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Executive Summary

This report represents the culmination of work conducted in Phase 3 of a study to investigate the potential of Cyprinid herpesvirus 3 (CyHV-3; also known as koi herpesvirus, or the carp herpesvirus) as a biological control agent for carp in Australia. Following encouraging results from the initial studies, the aim of the current phase (Phase 3) was to extend the earlier results in order to be confident that use of CyHV-3 would be a safe and effective biocontrol agent for carp in Australia.

Perhaps the most significant results from Phase 3 relate to further susceptibility testing of non-target species. Following clinical, molecular and histological observations, we now know that CyHV-3 does not infect (and therefore cannot affect) a wide taxonomic range of non-target animals including: 14 species of fish (13 native species, and the introduced rainbow trout); yabbies; a species of lamprey; two amphibian species; two reptile species; chickens; and mice. These results strongly suggest that both spillover infections and species jumps are highly unlikely with CyHV-3, and, therefore, the results encourage further work on the use of CyHV-3 as a potential biocontrol agent for carp in Australia.

Very importantly, we have also made good progress on the development of a mathematical model for CyHV-3 to determine a release strategy for the virus in the Lachlan River catchment (LRC). In the course of developing the model, limitations in the availability of hydrological and carp demographic data became obvious, and while these deficiencies slowed progress, they were overcome in a variety of ways including the use of a Bayesian Belief Network workshop (to generate data on the distribution and abundance of carp in the waterways of the Murray-Darling Basin, MDB). The final mathematical model, which will be focused on the LRC, will allow the following questions to be addressed: (1) when will it be best to release the virus (what season and what part of the El Nino Southern Oscillation (ENSO) cycle)? (2) where will it be best to release the virus (upstream or downstream or in recruiting grounds)? (3) will supplementary regional controls - for example, fishing, trapping, netting - be needed? (4) if they are (which is probable), what will be the best way to apply these (before or after releasing the virus).

Transferring the Indonesian strain of CyHV-3 (the C07 strain) from the secure to the nonsecure area at AAHL has been an important achievement in order to allow future progress of the project. As an extension of this process, production of aliquots of freeze-dried (f-d) virus, and subsequent testing of the virulence of reconstituted f-d virus, was also an important achievement. It is likely that, if the virus is eventually used in the field in Australia, it will be delivered to the sites of distribution throughout the MDB in a f-d form. While concentrations of virus in the f-d preparations fell below useful levels, subsequent lessons learned from an experienced overseas organization should result in a better outcome. In the meantime, we have determined the decay rate of CyHV-3 in tissue culture supernatant fluid held at 4 °C as an alternative means of delivering virus to the field if necessary.

Sequencing and annotating the complete genome sequence of Indonesian CyHV-3 (C07 strain) has been a significant achievement in allowing ultimate recognition of the potential biocontrol virus in Australia. Additional studies, firstly, on the expression of immune-related genes of carp during CyHV-3 infection (assisting our understanding of the carp-virus interaction), and, secondly, in attempting to identify a marker of persistent/latent infection in carp, have also allowed further characterization of this potential future virus for biocontrol of carp in Australia.



Recommendations

The findings that are documented in this Report provide great encouragement for further work on the use of CyHV-3 as a potential biocontrol agent for carp in Australia. Particular recommendations for future work include:

- 1 Completion of the non-target species (NTS) testing (2 Western Australian and 3 Queensland species of fish).
- 2 Extension of the modelling work from the LRC to the MDB at large.
- 3 Development of tools (particularly a serological test) for monitoring the effect of the virus on carp populations to recognize when, and if, modifications are required to the virus or to control programs that will complement the effect of the virus.
- 4 Development of a quantitative measure of stress in carp in order to address animal ethics issue associated with the release of the virus.
- 5 Modification of the protocol for the production of freeze-dried virus.
- 6 Susceptibility testing of carp from throughout the entire MDB to the virus to ensure that CyHV-3 (C07 strain) will be effective.
- 7 Selection and development of a genetic strategy to complement the effect of CyHV-3 as a biocontrol agent.



Abbreviations

AAHL: Australian Animal Health Laboratory ASAG: AAHL Security Assessment Group BBN: Bayesian belief network Biocontrol: biological control BT request: biological transfer request C_T: cycle threshold CMI response: cell mediated immune response CPE: cytopathic effect CyHV-3: cyprinid herpesvirus 3 DAFF: Department of Agriculture, Fisheries and Forestry ENSO: El Nino Southern Oscillation Dpc: days post challenge Dpi: days post inoculation DR: direct repeat f-d: freeze-dried gDNA: genomic DNA HVR: hypervariable region I/N: intranuclear **IB:** inclusion bodies IFN: interferon IHNV: infectious haematopoietic necrosis virus IL: interleukin IN: intranasal IO: intra-ocular **IP: intraperitoneal** IPNV: infectious pancreatic necrosis virus ISAV: infectious salmon anaemia virus Kbp: kilobase pairs KHVD: koi herpesvirus disease LAF: Large Animal Facility LATs: latency associated transcripts



LRC: Lachlan River catchment mRNA: messenger RNA MDB: Murray-Darling Basin MHC: major histocompatibility complex ND: not done NTS: non-target species 0: oral OIE: World Organisation for Animal Health ORF: open reading frame PPM: population projection model qPCR: quantitative polymerase chain reaction rRNA: ribosomal RNA RSIV: red sea bream iridovirus RT-PCR: reverse transcriptase PCR SD: standard deviation SNP: single nucleotide polymorphism SVCV: spring viraemia of carp virus TCID₅₀: median tissue culture infective dose TK: thymidine kinase TNA: total nucleic acid TCSN: tissue culture supernatant fluid VHSV: viral haemorrhagic septicaemia virus VNTR: variable number of tandem repeats WSSV: white spot syndrome virus YHV: yellowhead virus



1. Introduction

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

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

In Phases 1 and 2 of this project, a number of fundamental studies were undertaken in order to allow a rational decision about the potential use of CyHV-3 as a biocontrol agent for carp in Australia (McColl and Crane, 2013). The main studies included: (1) an Indonesian strain of CyHV-3 was selected for all subsequent experimental work; (2) assays for the detection of CyHV-3 infections of carp were introduced to AAHL; (3) an understanding of the clinical course of the disease, and mortality, in different age-groups of Australian carp was obtained; (4) a preliminary study of the temporal pattern of excretion of virus from infected fish was conducted; (5) the susceptibility of a number of non-target species was examined; and (6) a preliminary study was undertaken to ensure that there were no cross-reactive cyprinid herpesviruses in the MDB that might compromise the efficacy of CyHV-3 were it to be released into the MDB.

Following encouraging results from the initial studies, the aim of the current phase (Phase 3) was to extend the earlier results in order to be confident that use of CyHV-3 would be a safe and effective biocontrol agent for carp in Australia. These further studies included: (1) additional susceptibility testing of non-target species; (2) development of an epidemiological model for CyHV-3 to determine a release strategy for the virus in the Lachlan River catchment (LRC); (3) transfer of the Indonesian strain of CyHV-3 (the C07 strain) from the secure to the non-secure area at AAHL; (4) production of freeze-dried (f-d) aliquots of the virus, and subsequent testing of the virulence of reconstituted f-d virus; (5) determination of the complete genome sequence of Indonesian CyHV-3 (C07 strain); and (6) preparation of a number of journal articles on the work conducted to this point. In addition, extra studies were conducted, firstly, on expression of immune-related genes of carp during CyHV-3 infection (in order to assist our understanding of the carp-virus interaction), secondly, in attempting to identify a marker of persistent/latent infection in carp, and, finally, on the 'shelf-life' of virus-infected tissue culture supernatant fluid held at 4 $^{\circ}$ C.



2. Methods

All methods are incorporated in the following section that provides Introduction, Methods, and Results and Discussion sections for each of the projects in Phase 3.



3. Results

3.1 Susceptibility testing of non-target species

3.1.1 Introduction

There are many important questions that must be considered prior to the use of a virus as a biocontrol agent (McColl et al, 2014), but perhaps the two most important criteria that had to be addressed before CyHV-3 could be considered as a potential biocontrol agent for carp in Australia were demonstrating that: (1) CyHV-3 is virulent in Australian carp; and (2) the virus has no adverse effects on non-target species (NTS). While results for the first criterion have already been reported (McColl and Crane, 2013), results for the second criterion are reported here.

CyHV-3 is considered to be specific for carp (Hedrick et al, 2000). The susceptibility of at least 22 other species of fish has been tested (Bretzinger et al, 1999; Perelberg et al, 2003; Kempter et al, 2009; Bergmann et al, 2010a; Fabian et al, 2013), and in all cases, there has been no evidence of disease in these other species. While CyHV-3 DNA was detected in 10 of the 15 species of fish that were exposed to acutely- or latently-infected carp, only a small proportion of each species was said to be infected, none showed clinical signs of disease, and only low copy numbers of CyHV-3 DNA were found (Fabian et al, 2013). Recently, it was suggested that CyHV-3 may be able to replicate in goldfish (*Carassius auratus*) without causing disease (El-Matbouli and Soliman, 2011). However, the results of Yuasa et al, (2012, 2013) cast considerable doubt on all studies that have claimed, directly or indirectly, to identify CyHV-3 replication in species other than carp. The work reported here involved not only testing the susceptibility of a range of native, and an important introduced, fish species, but representatives of other taxonomic groups were also examined.

3.1.2 Methods

Non-target species (NTS) included: introduced rainbow trout (*Oncorhynchus mykiss*), and 13 species of native Australian teleost fish comprising silver perch (*Bidyanus bidyanus*), Murray cod (*Maccullochella peelii*), golden perch (*Macquaria ambigua*), common galaxias (*Galaxias maculatus*), short-finned eel (*Anguilla australis*), salmon catfish (*Neoarius graeffei*), eel-tailed catfish (*Tandanus tandanus*), Australian smelt (*Retropinna semoni*), crimson-spotted rainbowfish (*Melanotaenia duboulayi*), sea mullet (*Mugil cephalus*), carp gudgeon (*Hypseleotris sp.*), olive perchlet (*Ambassis agassizii*), and bony bream (*Nematalosa erebi*) as well as short-headed lamprey (*Mordacia mordax*), 2 amphibian species comprising tadpoles and mature stages of Peron's tree frog (*Litoria peronii*) and mature spotted marsh frogs (*Lymnodynastes tasmaniensis*), 2 reptilian species comprising the Eastern water dragon (*Intellagama lesueurii*) and Macquarie short necked turtle (*Emydura macquarii*), a freshwater crustacean (*Cherax destructor*) and chickens (*Gallus gallus domesticus*) and laboratory mice (*Mus musculus*). All were challenged with CyHV-3, while carp were challenged as positive controls.

The *O. mykiss* were acclimated to 18 °C, while all other fish species were acclimated to 21-23 °C. Two to thirty fish were held per 80 L tank. *C. destructor* were held at 21-23 °C in similar tanks, and 9 lamprey ammocoetes (*M. mordax*) were held per 40 L tank. The lampreys burrowed into ~10 cm of washed sand in the bottom of each tank. All tanks incorporated an in-tank water filtration device. Immature chickens were held at an ambient temperature of ~25 °C in bio-secure animal rooms containing a deep litter of wood shavings and a communal



retreat in one corner to simulate free-range conditions. Elevated enclosed boxes each containing 3 mice were kept in the same room as the chickens. Amphibians were held at 21-23 °C in 2-5 L enclosed plastic boxes each with a water source in the same rooms as reptiles housed in ~2 m² enclosed areas each containing an open water source and a low-hanging heat lamp. Feed types and regimes, housing and environmental requirements and experimental protocols used for each species complied with AAHL Animal Ethics Committee regulations.

Animals were challenged by bath immersion $(10^{2.8} \text{ to } 10^{3.5} \text{ TCID}_{50} \text{ mL}^{-1} \text{ of CyHV-3-containing}$ cell culture supernatant for 2 hr), or by intraperitoneal (IP)-challenge (animals up to approximately 10 cm in length received 20 to 100 uL of tissue culture supernatant fluid (depending on size), delivering $10^{3.4}$ to $10^{4.5}$ TCID₅₀ of CyHV-3 per animal). Carp larger than 10 cm received 1 mL of supernatant, delivering $10^{4.5}$ TCID₅₀ per fish. Mice were challenged via IP and intranasal (IN) routes, each animal receiving $10^{4.1}$ TCID₅₀ of virus in 100 uL of supernatant IP and $10^{3.8}$ TCID₅₀ in 50 uL IN. Chickens were challenged via IP, intraocular (IO), IN and oral (O) routes, each animal receiving a dose of approximately $10^{4.1}$ TCID₅₀ of virus in 100 uL of supernatant IP and $10^{4.4}$ TCID₅₀ in 200 uL split across the remaining routes (IO, IN, O). Negative control animals were exposed identically to uninfected cell culture supernatant. Fish species that were physically fragile and/or difficult to handle were generally only challenged by bath immersion as it simulated a natural exposure route.

Following virus challenge, daily clinical observations were made on all NTS for up to 28 days post challenge (dpc). Samples for quantitative polymerase chain reaction tests (qPCR) and histopathology were collected from negative control, and from virus-challenged, animals at 0 dpc. Thereafter, dead animals were examined by qPCR, and moribund animals by histopathology and also, on occasion, by qPCR. At the end of each trial, samples of surviving NTS and controls were examined by histopathology and qPCR. Small numbers of apparently healthy animals were also sacrificed at various time-points in the post-challenge period. Measures were taken to minimise the risk of cross-contamination when processing animals. All samples from NTS yielding qPCR results with a C_T value <40 were tested by a RT-PCR designed to amplify CyHV-3 terminase gene mRNA as a means of detecting virus replication. Conditions for the RT-PCR were as described by Yuasa *et al.* (2012), and these allowed differentiation of viral mRNA from viral genomic DNA (gDNA).

Tissues sampled from recently-dead, moribund or healthy animals were preserved in RNAlater (Ambion) at -20 °C. Pieces of kidney and spleen (~5-10 mm³ in size depending on the size of the individual) were collected from all animals, and a gill arch was also collected from each fish. In very small fish and amphibia when specific organs could not be readily identified, pooled viscera were collected. Gills and 5-10 mm³ of hepatopancreas were sampled from crustaceans, and similar amounts of liver, spleen and kidney were collected from mice and chickens.

To extract total nucleic acid (TNA), tissue was homogenized in AVL buffer (QIAGEN) using MagNA Lyser Green Beads (Roche) and a Virus BioRobot MDx Kit (QIAmp). A qPCR using the 18S-F/R primers and an 18S-P probe for generic detection of the 18S ribosomal (r)DNA gene was used to confirm nucleic acid integrity and freedom from PCR inhibitors (Sunarto et al, 2014). A qPCR using the KHV-86f/163r primers and a KHV-109p probe was used to detect and quantify CyHV-3 DNA (Gilad et al, 2004). Duplicate reactions, each comprising 12.5 μ L of TaqMan Universal Master Mix, 1.25 μ L of each primer (18 μ M) and 5 μ M probe, 2 μ L of TNA, and 6.75 μ L of DNase-free water, were amplified using a 7500 Fast Real-time PCR System (Applied Biosystems) and the following thermal cycling conditions: one cycle at 50 °C/2 min, 95 °C/10 min; 45 cycles at 95 °C/15 s, 60 °C/1 min.



For pathology, all animals were examined for gross lesions, but tissues for histological examination were only collected into 10% neutral buffered formalin from live (healthy or moribund) animals. Following routine histological procedures, paraffin-embedded sections (3- $6 \mu m$) were stained with haematoxylin and eosin.

In total, 1782 animals were used in nine separate experimental trials to test the susceptibility of 22 NTS to CyHV-3 infection (Table 1). These included 945 NTS challenged with CyHV-3, and 550 NTS used as negative controls. To confirm the efficacy of the challenge procedures, 189 carp were also challenged with CyHV-3, while 98 were used as negative controls.

| Trial | Target species | es Size range (cm) | Source | % Mort tested) | ality (r | no. of | animals |
|-------|--|-------------------------------|--|---|--|------------------------------------|---|
| | | | | CyHV-3 challenged | | Negative controls | |
| | | | | IP | Bath | IP | Bath |
| 1 | <i>C. carpio B. bidyanus M. peelii</i> | 4-10 5-7 4-6 | K&C Fisheries Global, Sale, VIC Snobs Ck Hatchery, DPI, VIC Snobs Ck Hatchery, DPI, VIC | 63 (41) 46 (39) ND ^a | 54 (39) 27 (26) 23 (40) | 0 (20) 0 (20) ND | 0 (20) 0 (20) 35 (20) |
| 2 | <i>C. carpio M. ambigua M. peelii</i> | 6-12 3-6 6-11 | K&C Fisheries Global, Sale, VIC Snobs Ck Hatchery, DPI, VIC Snobs Ck Hatchery, DPI, VIC | 74 (43) 36 (28) 23 (31) | 78 (41) 42 (31) ND | 14 (21) 33 (9) 33 (15) | 0 (21) 33 (9) ND |
| 3 | <i>C. carpio G. maculatus O. mykiss</i> | 15-20 3-5 5-10 | Lake Brewster, DPI, NSW L Bullen Merri, VIC Snobs Ck Hatchery, DPI, VIC | 40 (5) ND 100 (22) | ND 83 (41) 45 (31) | 0 (3) ND 9 (23) | ND 91 (22) 6 (18) |
| 4 | <i>C. carpio A. australis N. graeffei M. duboulayi</i> | 19-28 9-15 10-13 3-7 | Queenscliff, DPI, VIC IFS, New Norfolk, TAS 'Aquagreen', NT Lake Minnie Waters, DPI, NSW | 100 (2) ND 0 (10) 12 (26) | ND 0 (44) 0 (10) 20 (30) | 0 (5) ND 0 (6) 35 (26) | ND 11 (44) 0 (6) 20 (21) |

Table 1: The size range, source, and percent mortality of carp and NTS tested in each
susceptibility trial with CyHP-3



| 5 | C. carpio T. tandanus M. cephalus R. semoni | 7-11 5-9 7-18 3-5 | Queenscliff, DPI, VIC Glenwaters Hatchery, Glenburn, VIC Nowra, DPI, NSW Narrandera, DPI, NSW | 100 (3) 0 (33) 33 (9) ND | ND 0 (33) 17 (12) 40 (30) | 0 (2) 0 (34) 0 (5) ND | ND 0 (31) 0 (4) 38 (45) |
|---|--|------------------------------------|---|--------------------------------------|---|--------------------------------|--|
| 6 | C. carpio G. g. Domesticus ^b M. musculus ^c | 31-51 Immature 31-37 gm | Queenscliff, DPI, VIC CSIRO-AAHL CSIRO-AAHL | 50 (2) 0 (12*) 0 (10*) | 100 (1) ND ND | 0 (1) 0 (4) 0 (4) | ND ND ND |
| 7 | C. carpio Hypseleotris sp. A. agassizii M. mordax C. destructor | 41-51 1-4 2-5 8-13 5-9 | Queenscliff, DPI, VIC Narrandera, DPI, NSW Narrandera, DPI, NSW IFS, New Norfolk, TAS Wagga Wagga, DPI, NSW | 100 (3) ND ND 9 (33) | ND 6 (141) 79 (63) 0 (9) 13 (38) | 0 (1) ND ND 9 (24) | ND 22 (36) 91 (33) 22 (9) 20 (25) |
| 8 | C. carpio I. lesueurii E. macquarii L. peronii | 13-15 15-20 5-6 | Narrandera, DPI, NSW Commercial supplier ^d Commercial supplier ^d | 100 (5) 0 (13) 0 (13) | ND ND ND | 0 (2) 0 (3) 33 (3) | ND ND ND |
| | Tadpole Adult L. tasmaniensis Adult | 3-7 2-5 3-5 | Wodonga, DPI, NSW Narrandera, DPI, NSW Narrandera, DPI, NSW | ND 0 (14) | 19 (16) 0 (15) | ND 0 (3) 0 (2) | 0 (3) 0 (3) ND |
| 9 | C. carpio N. erebi | 8-13 5-18 | Tumut, DPI, NSW Lake Brewster, DPI, NSW | 100 (2) ND | 100 (2) 0 (37) | ND ND | 0 (2) 0 (20) |
| Number of carp tested 106 83 55 4 Number of NTS tested 298 647 181 3 | | | | | 43 369 | | |
| Sub-totals 404 730 236 41 | | | | 412 | | | |
| TOTAL animals tested 1782 | | | | 1782 | | | |



^a ND: not done; ^{b,c} All chickens were challenged via IP, IN, IO and O routes, and mice were challenged via IN and IP routes; ^d Scales Aquarium and Reptile, Wodonga, VIC.

3.1.3 Results and Discussion

The percent mortality (and the number of animals used) in each trial are shown in Table 1. Among positive controls, in general, small (4 to 10 cm) carp responded to CyHV-3 challenge more uniformly than larger carp. Smaller carp became quiet, ceased to feed, and developed excess skin mucus followed by sandpaper-like focal skin lesions within 5 to 7 dpc. In larger carp, diffuse reddening, or a generalized increase in pigmentation, of the skin were frequently the earliest clinical signs of disease followed by excess skin mucus, pale patches and a sandpaper-like appearance of the skin. In the gills, excess mucus was often the only consistent sign of infection, regardless of the size of the carp. Moderate mortalities in carp were due to the inadvertent use of hybrid (less susceptible) carp, or to the use of small numbers of larger carp.

In NTS, clinical signs of disease were largely restricted to some fish species and the crustacean, *C. destructor*. However, in all except the fish species *B. bidyanus*, *O. mykiss* and *M. cephalus*, there was no difference in clinical signs exhibited by virus-challenged animals compared with the appropriate negative controls. Mortality among CyHV-3-challenged NTS ranged from 0% to 100%. While there were no mortalities in many NTS (*N. graeffei*, *T. tandanus*, *A. australis*, *N. erebi*, *M. mordax*, chickens, mice, amphibians and reptiles), low to high mortalities observed in virus-challenged *M. peelii*, *M. ambigua*, *G. maculatus*, *M. duboulayi*, *R. semoni*, *Hypseleotris* sp., *A. agassizii* and *C. destructor* were matched by similar, or higher, mortalities in the negative controls. The only species in which moderate to high mortalities occurred in virus-challenged, but not the negative control, groups were the fish *O. mykiss*, *M. cephalus* and *B. bidyanus*. In virus-challenged *O. mykiss*, mortalities occurred predominantly after 23 dpc, or prior to 5 dpc, in IP- or bath-challenged fish, respectively, while in *M. cephalus*, mortality occurred predominantly prior to 4 dpc, and after 23 dpc, in IP- and bath-challenged fish, respectively. For *B. bidyanus*, there was a steady accumulation of mortalities over the course of the trial in both IP- and bath-challenged fish.

TNA extracted from each sample of virus-challenged and negative control animals was screened with a CyHV-3-specific qPCR. Except for qPCR-positive carp, any samples generating a C_T value <40 were then retested using an RT-PCR to detect specifically CyHV-3 mRNA. Of 879 animals tested using the virus qPCR, 45 NTS animals in which TNA from kidney or viscera generated a C_T value >38 were then tested using the 18S rRNA gene qPCR. The results (mean C_T ± standard deviation [SD] = 18.56 ± 5.1) confirmed that TNA had indeed been extracted successfully.

CyHV-3 qPCR and RT-PCR data are documented in Table 2. In total, 975 samples (921 NTS, 54 carp) from CyHV-3-challenged animals, and 380 (353 NTS, 27 carp) from negative controls were tested. The mean C_T value \pm SD for samples collected from virus-challenged dead or moribund carp was 25.0 \pm 4.0. However, of 921 tissue samples tested from virus-challenged NTS, only 104 (11.3%) were qPCR-positive at a mean $C_T = 37.8 \pm 1.9$. Of 353 samples from negative-control NTS animals, only five (1.4%) were qPCR-positive at a mean $C_T = 35.3 \pm 2.9$. When all 109 of the qPCR-positive NTS samples (104 virus-challenged, and 5 negative control) were tested by a conventional RT-PCR, all were negative. As controls, 80% of virus-challenged carp were positive for viral mRNA, whereas no mock-infected carp were positive for viral mRNA (despite 30% being weakly-positive for viral DNA, presumably due to contamination).



Totals of 286 virus-challenged animals (234 NTS, and 52 carp), and 108 negative controls (98 NTS, and 10 carp) were examined by histopathology. There were no lesions consistent with a viral infection in any NTS, while CyHV-3-challenged carp displayed the most significant gross and/or histological lesions, most commonly in the gill-raker mucosa. Lesions in gill-rakers varied in severity among carp. In severe cases, the mucosa lining the scalloped gill-raker was thickened and ragged (compared with the relatively smooth mucosa on normal gill arches). There was prominent epithelial hyperplasia, almost polypoid in places, together with small multifocal areas of necrosis and diffuse infiltrations of mononuclear cells into the lamina propria. Moderate to high proportions of cells in affected areas displayed intranuclear (I/N) inclusion bodies (IB) with marginated nuclear chromatin.

| Trial | CyHV-3 challenged | | | Neg Ctl | | |
|----------------------|--------------------------|----------------------------------|-------------------------------|--------------------------|------------------------------|-----------------------------|
| | Total qPCRs ^a | qPCR+ ^b | mRNA+ ^c | Total qPCRs ^a | qPCR+ ^b | mRNA+ ^c |
| 1 | 86 (8) | 36 (8) | 0 (5) | 27 (8) | 0 (7) | NT ^d (0) |
| 2 | 128 (8) | 27 (8) | 0 (5) | 33 (4) | 0 (0) | NT (0) |
| 3 | 89 (6) | 2 (6) | 0 (4) | 15 (3) | 4 (0) | 0 (0) |
| 4 | 164 (6) | 9 (4) | 0 (3) | 92 (2) | 0 (1) | NT (0) |
| 5 | 158 (3) | 5 (3) | 0 (3) | 50 (2) | 0 (0) | NT (0) |
| 6 | 66 (6) | 4 (3) | 0 (2) | 24 (2) | 0 (0) | NT (0) |
| 7 | 147 (5) | 3 (5) | 0 (5) | 89 (2) | 1 (0) | 0 (0) |
| 8 | 53 (6) | 8 (6) | 0 (6) | 13 (2) | 0 (0) | NT (0) |
| 9 | 30 (6) | 10 (6) | 0 (6) | 10 (2) | 0 (0) | NT (0) |
| TOTALS PERCENTAGE | 921 (54) | 104 (49) 11 (91) ^e | 0 (39) 0 (80) ^f | 353 (27) | 5 (8) 1 (30) ^e | 0 (0) 0 (0) ^f |

Table 2: Number of qPCR and RT-PCR samples tested in CyHV-3 challenge trials on NTS

^a Number of samples tested by CyHV-3 qPCR (Gilad et al, 2004) from NTS and carp (parentheses) in challenge trials (in many animals more than one tissue was tested); ^b Number of NTS samples positive by qPCR for CyHV-3 DNA; ^c Number of qPCR-positive NTS samples that were positive when tested by a conventional RT-PCR for CyHV-3 mRNA (Yuasa et al, 2012); ^d NT: not tested; ^e Rounded percent qPCR+ samples from NTS and carp (parentheses); ^f Rounded percent RT-PCR+ samples from NTS and carp (parentheses).

The lamellae and filaments of gills of CyHV-3-challenged carp displayed only occasional multifocal fusion of lamellae, epithelial hyperplasia, and heavy infiltration of lamellae with mononuclear cells in virus-challenged carp. Kidney lesions were rare in virus-challenged carp



except for occasional widespread necrosis in the haematopoietic tissue particularly in the anterior kidney.

This study on CyHV-3 is the first full report on NTS testing of a potential biocontrol agent. The study was conducted to meet three major demands: (1) test the susceptibility of a representative range of animal taxa that could conceivably come in contact with CyHV-3 were it to be released into the freshwater systems of Australia. Such animals would include not only teleost fish, but also species representing invertebrates, lampreys, amphibians, reptiles, birds and mammals, any of which could drink water, or live, swim or bathe in water, containing CyHV-3. In particular, it was also important to test the susceptibility of representative native fish most closely related to the Order Cypriniformes, in this case *N. graeffei* and *T. tandanus*, from the Order Siluriformes; (2) meet a requirement of the World Organisation for Animal Health (OIE) which states that, when testing the susceptibility of aquatic animals to infectious agents, immersion (or bath) challenges are necessary (OIE 2015); and (3) NTS be deliberately exposed to 100 to 1000 times the amount of virus required to cause disease in carp.

Mortality, molecular (PCR and RT-PCR), and pathological data were used, in combination, to assess if CyHV-3 was likely to infect a range of NTS. In 11 cases where NTS were challenged with virus, no mortalities were recorded (Table 1). In a further 10 cases, mortalities in negative control groups matched, or exceeded, those in virus-challenged counterparts suggesting that CyHV-3 was not affecting these NTS. Only in *O. mykiss*, *M. cephalus* and *B. bidyanus* did mortality data alone suggest a possible effect by the virus.

The vast majority of samples from NTS (including the virus-challenged *B. bidyanus*, *O. mykiss* and *M. cephalus* with high mortalities) and from negative control carp were negative when tested by the CyHV-3 qPCR (Gilad et al, 2004; Table 2) indicating the likely absence of virus in these animals. However, 109 samples from virus-challenged and negative control NTS registered a C_T value <40 (Table 2). These positive results could possibly be explained either by low levels of replicating CyHV-3 in tissues from virus-challenged animals, or by inadvertent contamination of samples with viral DNA, possibly during the collection of samples, or during laboratory processing of the samples. To test for evidence of virus replication, all 109 samples were examined with an RT-PCR (Yuasa et al, 2012) capable of differentiating viral mRNA from any contaminating viral gDNA. RT-PCR testing provided no evidence for viral replication in any of the 109 qPCR-positive samples (including all 29 qPCR-positive samples from virus-challenged *B. bidyanus*) (Table 2). These results support the view that contamination from the virus inoculum was the likely cause of the positive results with the CyHV-3 qPCR.

This is the first time that the susceptibility of each of these NTS to CyHV-3 has been reported. Previous publications (Fabian et al, 2013; Perelberg et al, 2003) suggested that the susceptibility of *B. bidyanus* had been tested, but, in fact, it was American (*Bairdiella chrysoura*), not Australian, silver perch that were tested (Hedrick et al, 2006). In summary, clinical, molecular and histological observations, together with mortality data, strongly suggest that CyHV-3 is unable to replicate in, let alone affect, a wide taxonomic range of both native Australian animals and introduced species. These results strongly suggest that both spillover infections and species jumps are highly unlikely with CyHV-3 in NTS. As a result, one of the major arguments against the use of CyHV-3 as a biocontrol agent is weakened, thereby encouraging further work on the use of CyHV-3 as a potential biocontrol agent for carp in Australia.



3.2 Development of an epidemiological model for CyHV-3 in the Lachlan River Catchment

3.2.1 Introduction

While modelling of CyHV-3 has been used overseas as a component of programs to control outbreaks of koi herpesvirus disease (KHVD), in Australia, control of carp, particularly in the Murray-Darling Basin (MDB), is the important issue. The original aim of this project was to develop an agent-based epidemiological model for CyHV-3 in carp in the MDB. It quickly became apparent that there was a deficiency of data for the MDB at large, making an agent-based model unattainable. Furthermore, because of the paucity of data, it was also decided to focus on the Lachlan River Catchment (LRC) as an initial study on the overall MDB. More recently, as the project has made significant progress, we have been informed that, while there is a large amount of carp data for the LRC, there is possibly less hydrological data for this river system compared with any of the other major rivers in the MDB (Geoff Podger, CSIRO L&W, pers comm). This is because, as a largely endorheic system, the LRC was given less attention by hydrologists than other rivers in the MDB complex. Nevertheless, the LRC has been the focus of this study, and the project eventually evolved to a point where 4 inter-linked models were developed for the LRC.

3.2.2 Methods

An overall strategy, involving a 'hierarchy of inter-linked models', was devised to deliver the final model. Figure 1 shows that the final model is underpinned by hydrological and connectivity models for the LRC. Dr Peter Durr and Kerryne Graham (at AAHL) collected and analyzed data from a variety of sources for these models. Hydrological and connectivity data for the LRC were sourced on-line from both the NSW Office of Water, and from Water Observations from Space (Geoscience Australia).

In order to obtain data for the carp demographic model (abundance and distribution of carp), an initial niche model for carp in Victoria was developed using data supplied by Dr Paul Brown and the Victorian Fish Database. However, it became apparent that a Bayesian Belief Network (BBN) would be required to fill large gaps in the available data. BBN is an approach that utilizes available data combined with expert knowledge to compare a range of management options (Lethbridge and Harper, 2013). In this case, a BBN was used to model carp habitat suitability which then allowed development of the carp demographic model.

Finally, a viral infectious disease model was overlaid, allowing Dr Stephen Davis (RMIT University) to then develop the mathematical model that will use all the current data to assess how the virus might best be used - in particular, for the case of the Lachlan River catchment. It is anticipated that questions that will be able to be addressed include:

- 1. When will it be best to release the virus (what season and what part of the El Nino Southern Oscillation (ENSO) cycle)?
- 2. Where will it be best to release the virus (upstream or downstream or in recruiting grounds)?
- 3. Will supplementary regional controls for example, fishing, trapping, netting be needed? (Note that we will not be looking at 'daughterless' technology this has now been explored by previous studies).
- 4. If they are (which is probable), what will be the best way to apply these (before or after releasing the virus).





Figure 1: Strategy for modelling release of CyHV-3 to control populations of care in the Murray-Darling Basin

3.2.3 Results and Discussion

Initial attempts to develop a niche model for the distribution and abundance of carp in Victoria using data supplied by Dr Paul Brown and the Victorian Fish Database allowed the generation of a preliminary map (Figure 2). However, there were a number of limitations with this dataset, for example, the frequent absence of time-series data, and inconsistencies in the methods of collecting fish.

Therefore, the focus was shifted to the LRC as a case-study location. Furthermore, a BBN workshop was held to ultimately allow carp population values to be determined, and population density at varying spatio-temporal locations to be estimated. The LRC was chosen because: (1) it is an endorheic system in most years; (2) the adult carp population has been very stable over the past 15 yrs; (3) replenishment is generally expected to be driven by resident adults; and (4) trapping data are available for validation purposes. In October 2014, a two-day workshop was held at AAHL with 11 of Australia's carp experts from a number of States participating (Figure 3). These experts included: Martin Asmus (NSW

Department of Primary Industries-Fisheries, Narrandera, NSW 2700) Keith Bell (K & C Fisheries Global Pty Ltd, Cobains, VIC,3851), Paul Brown (Latrobe University, Mildura VIC 3502), Josh Fredberg (Primary Industries and Regions South Australia, Henley Beach SA 5022), Dean Gilligan (NSW Department of Primary Industries-Fisheries, Batemans Bay NSW 2536), Andrew Norris (Qld Department of Agriculture, Fisheries and Forestry, Bribie Island Research Centre, Woorim 4507), Rodney Price (NSW Department of Primary Industries-Fisheries, Dubbo NSW 2830), Stephen Ryan (Glenelg Hopkins CMA, Hamilton VIC 3300), Ivor Stuart (Kingfisher Research P/L, Diamond Creek, VIC 3089), Leigh Thwaites (Primary Industries and Regions South Australia, Henley Beach SA 5022), Chris Wisniewski (Inland Fisheries Service, New Norfolk TAS 7140). Agus Sunarto, Kerryne Graham and Ken McColl (all from AAHL) also attended, and the workshop was facilitated by Drs Rieks van Klinken (CSIRO Health and





Figure 2: A preliminary map of the distribution of carp in Victoria (based on data from Dr Paul Brown, and the Victorian Fish Database)

Biosecurity, Brisbane) and Peter Durr (AAHL). This workshop allowed essential carp demographic data to be determined for the LRC.

Next, connectivity and hydrological models were developed for the LRC. An overall map of the LRC is shown in Figure 4, and connectivity between water bodies in the LRC is shown in Figure 5 (for both dry and wet years in an ENSO cycle). Because the Lachlan River terminates in the Great Cumbung Swamp in most years (rather than flowing into the Murrumbidgee River), less hydrological data are available than for many of the other rivers in the MDB. Nevertheless, numerous Gauge Stations throughout the LRC provide a great deal of useful information. In particular, data were collected from nine Gauge Stations (Table 3) at the locations shown in Figure 6.

Water temperature is an important environmental factor to consider, not only in the distribution of carp, but eventually in the activity of CyHV-3. In order to be able to estimate water temperatures as a contiguous grid in the LRC, the relationship between surface temperature and water temperature (recorded at numerous Gauge Stations throughout the Lachlan system) had to be determined. A linear regression for the relationship between mean surface temperature and water temperature was determined at nine Gauge Stations over the course of 13 years. Figure 7 shows the data for two of these Stations. Note that, although the graphs show the relationship over the entire year, seasonal data can also be determined (data for winter, spring, summer and autumn are represented by blue, green, orange and red points, respectively). A similar approach could eventually be used for the MDB at large.





Figure 3: Participants in the BBN workshop held at AAHL, October 2014.

Standing (L->R): Ken McColl, Peter Durr, Agus Sunarto, Paul Brown, Rod Price, Kerryne Graham, Josh Fredberg, Dean Gilligan, Martin Asmus, Ivor Stuart, Rod Norris. Seated (L->R): Rieks van Klinken, Chris Wisniewski, Leigh Thwaites, Keith Bell, Stephen Ryan.



Figure 4: Main features in the Lachlan River Catchment (LRC)





Figure 5: Connectivity between water bodies in the LRC (for both dry and wet years in an ENSO cycle)



| Gauge Station | Identification |
|------------------------------------|----------------|
| Boorowa Prossers Crossing | 412029 |
| Abercrombie @ Abercrombie Crossing | 412028 |
| Lachlan @ Booligal | 412005 |
| Lachlan Forbes Cutting | 412004 |
| Lachlan Condobolin Bridge | 412006 |
| Lachlan @ Corrong | 412045 |
| Lachlan @ Nanami | 412057 |
| Lachlan @ Narrawa | 412065 |
| Lachlan Hillston Weir | 412039 |

Table 3: Gauge stations located in the LRC (see Figure 6 for geographical locations)



Figure 6: Location of Gauge Stations throughout the Lachlan River catchment





Figure 7: A linear regression for the relationship between mean surface temperature and water temperature at two Gauge Stations in the Lachlan River catchment over the course of a year (location of the Gauge Stations is shown in Figure 6)

The mathematical modelling undertaken by RMIT University initially built a theoretical timeseries of a carp population according to three age classes (adults, juveniles and young-of-theyear). This was then linked to the more realistic data arising from the BBN modelling using habitat suitability for spawning events to "drive" the system. The various scenarios of differing contact structure and duration of immunity following infection events were then able to be examined.

In summary, the model was used to ask the specific question: when is the best time to release CyHV-3 - before, during or after major recruitment events? The model strongly indicated that the best time was AFTER recruitment events since this allows the natural density-dependent mortality of young-of-the-year to reduce the overall population before virus is added. However, the exact time of release of the virus after recruitment has yet to be determined. It is also recognised that any aggregations of juveniles and adults would be a good time for release of CyHV-3. These aggregations could occur in dry periods, when water bodies are shrinking or fragmented, but also in good years when population explosions led to aggregations of carp below the many fish barriers distributed throughout the MDB.

It should be stressed that the current modelling work which is restricted to the LRC is clearly not the end. Some of the assumptions in the current model need to be addressed, and the model then modified. Inevitably further work will now be required, not only to extend these findings to the MDB at large, but also to determine the best way to monitor the effectiveness of the viral release - in terms of surveillance and tests. This might then allow modifications to virus distribution in more remote areas of the MDB. Ideally, further work would draw upon data from newly-developed fish serological tests, and it would also require a surveillance database system to be put in place.



3.3 Transfer of CyHP-3 (Indonesian strain C07) to non-secure area at AAHL

3.3.1 Introduction

The Invasive Animals Cooperative Research Centre (IA-CRC) has been funding a project at AAHL since 2006 to examine the use of CyHV-3 as a potential biological control agent for carp in Australia. During the first two Phases of the project, the virus was characterized *in vitro* and *in vivo* in carp, and there have also been a number of trials to examine the susceptibility of non-target species of fish. In Phase 3 of the project (which began in July 2012), further non-target species testing was conducted, and an epidemiological model for KHV in carp in Australia was also under development. It was also necessary to transfer CyHV-3 across the barrier at AAHL in anticipation that the Federal Government will authorize the release of virus into the waterways of the Murray-Darling Basin in Australia by 2018.

Cyprinid herpesvirus-3 (CyHV-3, also known as koi herpesvirus, KHV) is an exotic virus in Australia, and the virus that has been used in work at AAHL is an Indonesian isolate (KHV C07, AQIS Import Permit: IP06014396) obtained from the Fish Health Research Laboratory, Central Research Institute of Aquaculture, JI. Ragunan 20, Pasar Minggu, Jakarta 12540, Indonesia. On arrival at AAHL, it was PCR-tested to demonstrate the absence of other adventitious viruses including infectious pancreatic necrosis virus (IPNV), infectious salmon anaemia virus (ISAV), viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), and spring viraemia of carp virus (SVCV). This virus was then grown in KF-1 cells, and stocks of the tissue culture supernatant fluid were stored at -80 °C, and logged in Microstores (KHV C07, Microstores number: 0906-09-1201). KF-1 cells had been obtained from Professor Ron Hedrick's lab (University of California, Davis, USA) in 2005, and they also had been innocuity-tested on arrival, and shown to be free of CyHV-3, IPNV, ISAV, VHSV, IHNV and SVCV. In all *in vivo* work that has already been undertaken with stocks of KHV C07 there has been no evidence (eg, clinical signs of disease, or histological lesions) of other adventitious agents in the stock virus.

3.3.2 Methods

The two aims of a protocol for the transfer of CyHV-3 to the non-secure lab at AAHL are to (1) confirm the presence of CyHV-3, and (2) demonstrate the absence of other exotic viruses of aquatic animals in the sample to be transferred. The latter could include: IPNV, ISAV, VHSV, IHNV, SVCV, Red Sea Bream Iridovirus (RSIV), Yellowhead Virus (YHV) and White Spot Syndrome Virus (WSSV).

An ampoule containing 1 mL of CyHV-3 (KHV C07, Microstores number: 0906-09-1201; original Import Permit: IP06014396) tissue culture supernatant (TCSN) was transferred to a clean isolation room where aquatic viruses had not been routinely handled. Work was conducted in a Class II Biological Safety Cabinet (BSC II).

The sample was thawed, and 140 uL removed for extraction of nucleic acid. The remaining sample was placed in a screw-top 2 mL Sarstedt tube that had not previously been opened in the secure area, and this sample was re-frozen at -80 $^{\circ}$ C.

Nucleic acid was extracted in the PCR Suite (in the South Suite), and the extracted nucleic acid was then tested by specific PCRs for the agents shown in Table 4 (qPCRs were used where available, but, if any of these were positive, then conventional PCRs, using the primers in Table 3, were used to generate a PCR product. Any PCR products of the expected specific size were purified and sequenced to confirm the presence of a particular virus).



Once the original sample was shown to contain KHV C07, while other viruses were absent, the Sarstedt was recovered from -80 °C storage, and returned to the BSC II in the isolation room. There, the surface of the tube was saturated with Virkon, and left to dry in the BSC II.

The cap of the Sarstedt was then sealed with parafilm, and placed in a small plastic container (a 15 mL Falcon tube, 352096, or equivalent) containing 10% neutral buffered formalin.

The outer plastic container was clearly labeled 'CyHV-3', and the designation 'Exotic' to signify that it was an imported AQIS-controlled item. The container was then transferred to the non-secure laboratory (Lab 019) via the dunk tank after completion of a BT request form. This non-secure laboratory has always been free from potential contamination with all of the exotic agents stored at AAHL.

| Virus | PCR Primer set | Reference |
|-------|--|----------------------|
| KHV | 5'-GACGACGCCGGAGACCTTGTG-3' 5'-CACAAGTTCAGTCTGTTCCTCAAC-3' | Gilad et al, 2002 |
| IPNV | 5'-ACGAACCCTCAGGACAA-3' 5'-CACAGGATCATCTTGGCATAGG-3' | Davies et al, 2010 |
| ISAV | 5'-GGCTATCTACCATGAACGAATC-3' 5'-GCCAAGTGTAAGTAGCACTCC-3' | Mjaaland et al. 1997 |
| VHSV | 5'-gtccccagggatgatgncc-3' 5'-AGTCCCCAGGGATGATGNCC-3' Nested PCR set: 5'-cacgagtacccgttcttccc-3' 5'-AGTCCCCAGGGATGATGNCC-3' | OIE (2015) |
| IHNV | 5'-ATGATCACCACTCCGCTCATT-3' 5'-CTCTGGACAAGCTCTCCAAGG-3' Nested PCR set: 5'-gattggagattttatcaaca-3' 5'-ctctggacaagctctccaagg-3' | OIE (2015) |
| SVCV | 5'-TCTTGGAGCCAAATAGCTCARRTC-3' 5'-AGATGGTATGGACCCCAATACATHACNCAY-3' Nested PCR set: 5'-TCTTGGAGCCAAATAGCTCARRTC-3' | OIE (2015) |

Table 4: Specific PCRs used to test KHV C07 for the presence of CyHV-3 and other exotic viruses of aquatic animals



| | 5'-CTGGGGTTTCCNCCTCAAAGYTGY-3' | |
|------|---|------------|
| RSIV | 5'-CTCAAACACTCTGGCTCATC-3' 5'-GCACCAACACATCTCCTATC-3' | OIE (2015) |
| YHV | 5'-CCGCTAATTTCAAAAACTACG-3' 5'-AAGGTGTTATGTCGAGGAAGT-3' | OIE (2015) |
| WSSV | 5'-ACTACTAACTTCAGCCTATCTAG-3' 5'-TAATGCGGGTGTAATGTTCTTACGA-3' Nested PCR set: 5'-GTAACTGCCCCTTCCATCTCCA-3' 5'-TACGGCAGCTGCTGCACCTTGT-3' | OIE (2015) |

In non-secure Lab 019, the plastic container was placed in a BSC II. The Sarstedt was removed from the formalin, and the parafilm discarded. An aliquot of the TCSN was added to a monolayer of KF-1 cells in three T75 flasks, and allowed to adsorb for 1 hr. Tissue culture medium was added to each flask, and virus was cultured using an incubator in Lab 019. Any leftover TCSN in the original Sarstedt was frozen at -80 $^{\circ}$ C, and stored in microstores. From experience, we expected only low titres of virus to grow on the KF-1 cells after two freeze-thaws of KHV C07; this is why we proposed inoculating three T75 flasks.

When moderate cytopathic effect (CPE) was observed in the cultures (between 4-6 days post inoculation), the TCSN from the culture(s) was passaged onto fresh monolayers of KF-1 cells in a further three T75 flasks. Experience has shown that this allows significant amplification of CyHV-3 (usually to about 10^5 TCID₅₀ per mL at 4-6 days post inoculation). The TCSN from one (or all) cultures was stored under lock and key in microstores in the non-secure area as 1 mL aliquots in screw-top Sarstedt tubes at -80 °C. The stock of virus prepared in this way will become the Australian master seed of CyHV-3 (strain KHV C07). It represents a completely identified and characterized stock of CyHV-3, and will be used to produce any subsequent working stocks of the virus.

Note that this proposed procedure varies from that used for the preparation of stocks of RHDV. For the latter, Row D of the LAF was effectively separated from the rest of the secure area of AAHL, and managed as a separate biocontainment zone. Approved staff accessed the row from non-secure areas of AAHL to avoid any potential contamination of Row D with agents stored in the secure area of the laboratory. These arrangements were necessary because there were no known cell-culture systems for the preparation of stocks of RHDV. Therefore, a sizable decontaminated laboratory and equipment were required for: maintenance of rabbits, homogenization of infected rabbit tissues, ultracentrifugation of supernatants from these tissues, and preparation of virus stocks (summarized in: the Minutes of ASAG 29, Agenda Item 13; and, the strategy for establishment of Row D is found in File Note: DC06/0005/8 [or possibly changed to 2]). By comparison with RHDV, once a small amount of CyHV-3 is removed from the secure area at AAHL, it can be amplified relatively easily and securely in KF-1 cell cultures.



3.3.3 Results and Discussion

Following consultation with the Microbiological Security Manager (MSM) at AAHL, a proposed protocol for the transfer of the C07 strain of KHV across the secure barrier at AAHL was developed. This protocol was then presented to the AAHL Security Assessment Group (ASAG) which endorsed the proposal on 25 February, 2013. The Australian Government Department of Agriculture, Fisheries and Forestry (DAFF), the ultimate decision-maker, then informally endorsed the proposal at a meeting at AAHL on 23 April, 2013.

CyHV-3 was removed from the secure area at AAHL (BT-2014-00225), cultured in KF-1 cells, and then 47 x 1 mL aliquots of 4th passage virus were stored at -80 °C. An aliquot of this stock was cultured in KF-1 cells (5th passage), and, when cytopathic effect was well-advanced, 1 mL samples of tissue-culture supernatant fluid (TCSN) were collected, and freeze-dried.



3.4 Preparation and testing of freeze-dried CyHV-3 aliquots

3.4.1 Introduction

Without pre-judging the results of the modelling work (which will be important in devising a strategy for release of CyHV-3 in the field), it is likely that virus will be distributed at selected sites in the Murray-Darling Basin (MDB) by on-site intraperitoneal (IP) inoculation, and then release, of a number of carp. Distribution of virus in this manner will allow natural transmission of CyHV-3 among carp, thereby overcoming the problem that virus can only be grown to low titre in tissue culture (in which case, adding virus cultures directly to large water bodies would rapidly dilute virus, probably to ineffectual levels).

If carp are to be inoculated at various sites in the MDB, then it will be necessary to deliver live virus to these sites. It is likely that the best way to achieve this will be to prepare freezedried (f-d) aliquots of virus for distribution throughout the MDB. Therefore, the following preliminary work was undertaken as part of a process to determine whether freeze-drying of CyHV-3 was a practical approach.

3.4.2 Methods

CyHV-3 was removed from the secure area at AAHL (BT-2014-00225), cultured in KF-1 cells, and then 47 x 1 mL aliquots of 4th passage virus were stored at -80 °C. An aliquot of this stock was cultured in KF-1 cells (5th passage), and, when cytopathic effect was well-advanced, 1 mL samples of tissue-culture supernatant fluid (TCSN) were collected, and freeze-dried.

Two ampoules of f-d virus were each reconstituted with 1 mL of water, and four carp were inoculated IP (100 uL) with each ampoule. Negative control carp were inoculated IP with 100 uL of virus-free tissue culture medium. All carp were examined daily. Moribund fish were euthanized with an overdose of Aqui-S (Aqui-S New Zealand Ltd), and samples collected for histopathological and PCR tests. Dead fish were examined by PCR alone. Animal Ethics permission was obtained for the *in vivo* testing of the reconstituted virus.

3.4.3 Results and Discussion

The results of the challenge trials with f-d CyHV-3 are shown in Table 5. For f-d aliquot #1, two fish developed reddened skin by 15 and 20 days post inoculation (dpi), and both were sacrificed. Each had low Ct values in gill, spleen and kidney (means of 17 and 22.3), indicative of high levels of viral DNA (which, in turn, suggests virus replication had occurred in these fish). Histological lesions in these fish were not particularly remarkable, although one fish had gill lesions consistent with infection by CyHV-3. The other two fish had mean Ct values of 27.1 and 23.6, indicating slightly lower levels of virus in these fish. Again, histological findings were unremarkable.

For f-d aliquot #2, one fish was found dead at 9 dpi, and it had very low Ct values for spleen, kidney and gills (mean 14.6). Two other fish had low mean Ct values when they were euthanized at 11 dpi (19.3 and 25), and these fish had gill lesions that would be consistent with infection by CyHV-3. The remaining fish was euthanized at 9 dpi due to reddening of the skin, but PCR results revealed only moderate Ct values (34) in gills, spleen and kidney, and no significant lesions were noted on histological examination.



Table 5: Challenge trials with aliquots of reconstituted freeze-dried (f-d) CyHV-3 in carp.The qPCR data are the mean and standard deviation (parentheses) of four samples

| Inoculum | n | qPCR | Histopathology |
|---------------------------|---|------------|---|
| Virus-free culture medium | 2 | 40 | No significant lesions |
| F-d aliquot #1 | 4 | 22.5 (4.4) | Probable gill lesion in one fish; others unremarkable |
| FD aliquot #2 | 4 | 24.5 (7.6) | Probable gill lesions in two fish; other unremarkable |

These results would be consistent with viral infection of all fish inoculated with f-d virus. However, it also suggests that the titre of virus in the reconstituted f-d aliquots was low, and that virus replication following infection of each fish was variable (and possibly slow to reach high titres). There were technical difficulties in determining the actual titre of the f-d aliquots *in vitro*, and this was shown to be due to low titres of virus.

These preliminary trials showed that it is certainly possible to freeze-dry CyHV-3, and to reconstitute it as virulent virus. However, the results also suggested that the titre of reconstituted virus was low. This might be because the titre of the TCSN that was freeze-dried as 1 mL aliquots was low, or because reconstitution was associated with a drop in titre. This needs to be examined further. In addition, once f-d aliquots of high titre are produced, the stability of virus needs to be determined over the weeks and months following production.

At the time this work on f-d virus was being completed, there was an urgency that developed with respect to the availability of data on shelf-life (for the purposes of registration). As a result, a decision was made to examine the possibility of infected TCSN being used to deliver the virus to field sites for inoculation of fish. This alternative possibility thus required data on the shelf-life of virus in TCSN held at 4 $^{\circ}$ C (see Section 7).



3.5 Complete genome sequencing of CyHV-3 (C07 strain)

3.5.1 Introduction

The genetic sequence of an organism provides a definitive 'fingerprint' of that organism, and, in addition, it has the potential to reveal important features about the biology of the organism. Next-generation sequencing has made it feasible to obtain complete viral genome sequences relatively quickly, even from viruses with a large genome such as CyHV-3 (at approximately 295 Kb, it is one of the largest known genomes for a herpesvirus).

Complete sequencing and annotation of the Indonesian strain of CyHV-3 (C07 strain) will not only provide an unequivocal fingerprint of the virus, but it will allow comparison of the sequence of this isolate with those of other related isolates of CyHV-3. In particular, a coldtemperature mutant of CyHV-3 that has been developed at AAHL will also be sequenced for no extra cost, but with potentially great benefits in understanding the activity of CyHV-3.

3.5.2 Methods

3.5.2.1 Growth of viruses

The Indonesian CyHV-3 C07 isolate used in this project was obtained from common carp suffering mass mortality in West Java, Indonesia in 2007 (Sunarto et al., 2011). The virus was grown in cultures of koi fin cell-line (KF-1) maintained in Leibovitz L-15 medium (Life Technologies). Bulk virus was cultured in T150 flasks and incubated at 25 $^{\circ}$ C.

3.5.2.2 Virus purification and DNS extraction

The virus was harvested at 21 days post infection (dpi) when cytopathic effect (CPE) was complete. Tissue culture supernatant of CyHV-3-infected KF-1 cells was cleared of cell debris by centrifugation at 3,000 x g for 30 minutes at 4 °C. The virus was then pelleted by centrifugation at 100,000 xg for 3 hours at 15 °C in an Avanti J-30l high speed centrifuge using JS24.38 rotor (Beckman). The viral genomic DNA was extracted using a QIAamp mini elute virus spin kit (QIAGEN) according to the manufacturer's protocols with minor modification. The viral DNA was eluted in 30 μ L RNase-free water, and stored at -20 °C.

3.5.2.3 Estimation of DNA concentration

The purity and concentration of the DNA was quantified by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.). A TaqMan qPCR targeting the thymidine kinase (TK) gene was also used for detection and quantification of CyHV-3 DNA with primers and probe being: TK F95 (5'-GCTGCATCGCCGTCAAG-3'), TK R162 (5'-GCTGTGCAT GGCCACCTT-3') and TK P113 (5'-ACGCCATAGACCAGCGCTACACCG-3'). A 7500 Fast Real-time PCR System (Applied Biosystems) was used. A plasmid bearing the TK gene was diluted in a 10-fold dilution series and used to generate a standard curve. Absolute quantification of viral DNA (viral copy number per ng total DNA) was determined using 7500 Fast SDS software version 2.0.6.

3.5.2.4 Next-generation sequencing protocol

The genomic DNA was fragmented, tagged, and adapters added by the Nextera XT transposome kit (Illumina). Index libraries were prepared using a Nextera XT DNA sample preparation kit and Nextera XT index kit. The libraries were sequenced using MiSeq reagent kit v2 chemistry by the next-generation sequencing (NGS) Illumina MiSeq platform.



3.5.2.5 PCR and Sanger sequencing

Conventional PCR and Sanger sequencing were used to fill the gaps and to amplify the highly variable regions (HVRs) and variable number tandem repeats (VNTRs) of the CyHV-3 genome. Primers flanking the gaps, HVRs or VNTRs were designed to generate PCR products from genomic viral DNA using HotStarTaq[®] Master Mix (Qiagen) with the following amplification profile: one cycle at 95 °C for 15 min; 40 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C 30 s; and 72 °C for 7 min. Amplified products were analysed by electrophoresis in 2% agarose gel (Bio-Rad), and DNAs were purified from the gel using QIAquick gel extraction kit (Qiagen). The DNAs were directly sequenced using Big Dye[®] terminator v3.1 (Applied Biosystems) by ABI PRISM[™] 377 DNA Sequencer (Applied Biosystems).

3.5.2.6 Bioinformatics and phylogenetic analysis

Raw reads that passed the quality control were trimmed and mapped to the reference genome using Geneious 8 and the CLC Genomics Workbench 8 software. The genome was annotated using the genome utility transfer unit (GATU). The gaps were filled using PCR and Sanger sequencing. Clone Manager 9 was used for routine sequence analyses including primer design, sequence alignments of HVRs and VNTRs and filling the gaps. The genome was assembled using CLC Genomics, annotated using Genious 8 and visualized using OmicCircos (Hu et al, 2014). Single nucleotide polymorphisms (SNPs) between the genome and the NCBI reference genome were calculated using the CLC Genomic and Genome Analysis Toolkit (GATK). Phylogenetic analysis was conducted using Molecular Evolutionary Genetic Analysis (MEGA 6; Tamura et al, 2007) software based on currently available WGS of CyHV-3 in Genbank (CyHV-3-U, CyHV-3-I, CyHV-3-J and CyHV-3-C) and the newly completed CO7 genome. Two CyHV-3 genomes from Belgium (GenBank accession numbers KP343683 and KP343684) were not included in this analysis because they have been modified for other purposes.

3.5.3 Results and Discussion

A bulk culture of wt-CyHV-3 was prepared from passage 3, and 120 mL of virus was obtained and purified. Viral genomic DNA was then extracted from purified virus. As shown in Table 6, the viral DNA concentration was 120 ng/µL for wt-CyHV-3. The concentration was well above the minimum requirement for whole genome sequencing (WGS) using the MiSeq platform, which requires 1 ng of input DNA. The results not only show that the virus could be cultured in bulk quantity and the DNA extracted in high concentration, but also show that the DNA was of high quality as shown by the A_{260}/A_{280} ratio of 1.98. For optimal results of genome sequencing, high quality of DNA with an A_{260}/A_{280} ratio of 1.8 to 2.0 is required. The TaqMan qPCR results confirmed that the DNA originated from CyHV-3 with 2 x 10⁷ viral copy number per ng DNA.

In summary, the viral genomic DNA obtained from the virus in this experiment passed the quality control requirements for genome sequencing. Following DNA tagmentation (tagging and fragmentation) and library preparation, CyHV-3 DNA was then sequenced using MiSeq, an Illumina next-generation sequencing platform.

The deep sequencing yielded 6,176,466 raw reads from both Read1 and Read2 with an average read of 150 bp. Most of the filtered reads (99.7%) were mapped to the reference genome of CyHV-3 (GenBank accession no NC_009127), generating a 295 kbp consensus sequence. Following annotation, the 295 kbp genome was predicted to have a Unique Long (UL) region flanked by 22 kbp Direct Repeat (DR) regions at each terminus. A total of 156 ORFs were predicted in the CyHV-3 genome: 8 within each DR and 148 within the UL region.



| Parameter | Measurements | Tools | CyHV-3 |
|-------------------|--|-------------------|---------------------|
| DNA concentration | ng/uL | Spectrophotometer | 120.42 |
| DNA purity | A ₂₆₀ /A ₂₈₀ ratio | Spectrophotometer | 1.98 |
| DNA quantity | Viral copy number/ng DNA | TaqMan qPCR | 2 x 10 ⁷ |

Table 6: Quality and quantity of CyHV-3 DNA for whole genome sequencing

The tentative gene layout was very similar to CyHV-3-U, the reference genome of CyHV-3 (Figure 8). We identified 310 genetic variations between the C07 strain and the reference genome, which include 172 single nucleotide variations, 4 multiple variations, 70 insertions, 62 deletions and 2 replacements within the CyHV-3 C07 genome. Phylogenetic analysis inferred from whole-genome sequence revealed CyHV-3 clustered into two major lineages: European and Asian lineages (Figure 9). As expected, the Indonesian isolate is more closely related to the Japanese isolate within the Asian lineage cluster. This 'fingerprint' provides the basis for tracking the evolution of the host-virus relationship following release of CyHV-3 in the Murray-Darling Basin.

When closing the gaps using PCR and Sanger sequencing, we found variable number tandem repeats (VNTRs): VNTR 21 bp (tcctcatcctcgaccgtaccc) and VNTR 24 bp (tcctcaccggtaacctctgtaccc). All Indonesian, UK and Japanese isolates have one copy of both VNTR 21 bp and VNTR 24 bp. Both the US isolates have 4 and 5 copies of VNTR 21 bp and VNTR 24 bp, respectively. All isolates from Israel have 4 copies of VNTR 21 bp and 3 copies of VNTR 24 bp (Figures 10 & 11).

PCR assays were developed targeting these VNTRs, allowing the Asian and European lineages to be distinguished. VNTR may evolve rapidly, and therefore they may be a useful tool for epidemiological studies. We also identified highly variable regions (HVRs) within the CyHV-3 genome, and developed PCR tests amplifying the regions (Figure 12). Based on an examination of 11 different isolates of CyHV-3 from around the world, the assay can distinguish CyHV-3 isolates from Indonesia (3 isolates examined), US (2), UK (1), Israel (4) and Japan (1) (Figure 13). Both PCR assays targeting VNTRs and HVRs would be useful tools for epidemiological studies and for monitoring the virus, respectively.





Figure 8: Genome organization of the Indonesian CyHV-3 C07 isolate

Circular tracks from outside to inside: nucleotide positions in kilobase pairs (kbps), gene positions in positive strand (blue lines) and negative strand (green lines), SNP distributions (red dots), SNP density in positive and negative strands (brown lines) - note ORFs with high proportion of SNPs, coverage (grey lines), GC content (red and green lines indicate GC content above and below 50%, respectively). Note eight duplicated genes in 22 kbp Direct Repeat at each terminus of the genome.





Figure 9: Phylogenetic tree of CyHV-3 inferred from the whole-genome sequences

Nucleotide sequences were aligned in ClustalW, and the tree was constructed by the Neighbour-Joining methods using the MEGA 6 program.





Figure 10: PCR assays targeting variable number tandem repeats of VNTR 21 bp (upper panel) and VNTR 24 bp (lower panel) of CyHV3

Lanes 1: Indo K05; 2: Indo C06; 3: Indo C07; 4: USA F9821; 5: USA F9850; 6: UK G406; 7: Israel 3112; 8: Israel 10612; 9: Israel 6114; 10: Israel 14114; 11: Japan NRIA0301; 12: no template control; M: 100 bp molecular ladder.





Figure 11: Copy number of VNTR 21 bp (upper panel) and VNTR 24 bp (lower panel)

All Indonesian, UK and Japan isolates have one copy of both VNTR 21 bp and VNTR 24 bp. All US isolates have 4 and 5 copies of VNTR 21 bp and VNTR 24 bp, respectively. All isolates from Israel have 4 copies of VNTR 21 bp and 3 copies of VNTR 24 bp. The motifs are underlined in red.





Figure 12: PCR assays targeting highly variable regions of CyHV-3 genome

HVR1 (upper panel), HVR2 (middle panel) and HVR3 (lower panel). Lanes 1: Indo K05; 2: Indo C06; 3: Indo C07; 4: USA F9821; 5: USA F9850; 6: UK G406; 7: Israel 3112; 8: Israel 10612; 9: Israel 6114; 10: Israel 14114; 11: Japan NRIA0301; 12: no template control; M: 100 bp molecular ladder.



| Indo KOS HVR1 | 1 | |
|------------------|------|---|
| Indo CO6 HVR1 | 1 | aggagotgaagaagcaagaagcaagaagcagcogaagcagcogaagcagcogaagcagcogaagcagcogaagcagacgaagcagacgaagacgacgacgacgacgaccaacaa |
| Indo CO7 HVR1 | 1 | |
| US F9821 HVR1 | 1 | aggagetgaagcaagaagcaagaagcagcagcagcagcegaagcagcegaagcagcegaagcagcegacgacgacgacgacgacgacgacgacgacgacgacgacg |
| US F9850 HVR1 | 1 | aggagetgaagcaagaagcaagcaagcagecaagaagcagecgaegcagecgaegcegaegcegaegcegaegcegaegaegaegaegaegaegaeccaeceaaacceceegaegaegaegaegaegaegaegaegaegaegaegaega |
| UK G406 HVR1 | 1 | aggagotgaagcaagaagcaagagcogaagcagoogaagcagcogaagcagcogaagcagacgaagcagacgaagcagacgaagcagacgacg |
| Israel 3112 HVR1 | 1 | aggagetgaageageageageageageageageageageageageag |
| Israel 10612 HVR | 1 | aggagetgaageageageageageageageageageageageageag |
| Israel 6114 HVR1 | 1 | aggagetgaagcaagaagcaagcagcogacgacgacgacgacgacgacgacgacgacgacgacgacg |
| Israel 14114 HVR | 1 | aggagotgaagcaagcaagcaagcaagcagcogaagcagcogaagcagcogaagcagcogaagcagacgaagcagacgaagcagacgacgacgacgacg |
| Japan NRIA0301 H | 3 | |
| nosmassyuamy | 1000 | |
| Indo KOS HVR2 | 171 | dtaacctaaccctaaccctaaccctaaccctaaccctaaccctaaccctaaccctaccctaccctaccctaccctaccctaccctaacccctaacccctaacc |
| Indo CO6 HVR2 | 170 | ctaaccctaaccctaaccctaaccctaaccctaaccctaacctaaccctagccctagccctaaccctatccgtcgtccaagacggcgtgaascagtcgcccaagtagggtgtccgcccacacgac |
| Indo C07 HVR2 | 171 | ctaaccctaaccctaaccctaaccctaaccctaaccctaaccctaaccctaaccctaaccctaccctaccctatcogtcgtccaagacggcgtgaaacagccccaagtagggtgtccgcaccacacgac |
| US F9821 HVR2 | 171 | dtaacctagoootagoo |
| US F9850 HVR2 | 171 | ctascctagecctagecctagecctagecctagecctagecctagecctagecctagecctagecctagecctasecctatecgtegteccasgaggggggaaacagecgaggggggaaacagecgagggggggaaacagecgagggggggaaacagecgagggggggg |
| UK G406 EVR2 | 171 | ctaaccctaaccctaaccctaaccctaaccctaaccctaacctaaccctagccctagccctaaccctatccgtcgtgtgtgt |
| Israel 3112 HVR2 | 171 | dtaacctagoootagoootagoootagoootagoootagoootagoootagoootagoootagoootaacootaacootaacootatoogtogtocaagaoggogtgaaacagooocaagtagggtgtoogcacacaogao |
| Israel 10612 MVR | 171 | ctaacctagocctagocctagocctagocctagocctagocctagocctagocctagocctagocctagocctaacoctatocgtogtogtogtogtogtogtogtogtogtogtogtogtog |
| Israel 6114 HVR2 | 171 | ctascctagcctagcctagcctagcctagcctagccctagccctagccctagccctagccctagccctaccctasccctatcogtcgtccaagacggcgtgaaacagccccaagtagggtgtccgcacacacgac |
| Israel 14114 HVR | 171 | ctaacctagecetagecetagecetagecetagecetagecetagecetagecetacecetaaceetaaceetaaceetateegtegtecaagaeggegtgaaacagececaagtagggtgtcogcaccacacgae |
| Japan NRIA0301 | 171 | ctasccctasccctasccctasccctasccctascctasccctagccctagccctasccctatccgtcgtccasgacggogtgasscagccccasgtagggtgtcogcacacacgac |
| Indo KO5 HVR3 | 171 | deadtatttdtdtaadttaatdtdtaadtattdtotaadtataatdtattodtdaadtataatdttaadtatadtatadttaatdttaatdttaatdttaatdttaaddattaadaatttatadoottottdtdoottattad |
| Indo CO6 HVR3 | 171 | ctaagtatttgtctsagtataatgtctaagtattatgtctsagtataatgtctssgtatt |
| Indo CO7 HVR3 | 171 | ctaagtatttgtctaagtataatgtctaagtataatgtctaagtataatgtctaagtataatgtctaagtataatgtctaagtataatgtctaagtataagaactttatggcctcttgtgcctcattag |
| US F9821 HVR3 | 171 | ctaagtatttgtctaagtttaatgtctaagtattt |
| US F9850 HVR3 | 171 | cteagtatttgtctaagtttaatgtctaagtattt |
| UK G406 HVR3 | 171 | classisticiciasofilasisticicasofallociasofalasisticasofallociasofalasistasofasofilasisticasofilasisticasofilasofalasofalasofactilatoccicilotoccicallas |
| Israel 3112 HVR3 | 171 | ctaagtatttgtctaagtttaagtsttaagtattt |
| Israel 10612 HVR | 171 | ctsagtatttgtctaagtttaatgtctaagtatttgtctaagtatasgtatagtaagtttaatgtctaagtttaatgtctaagtatasgaactttatggoctcttgtgcgctcatag |
| Israel 6114 HVR3 | 171 | cteagtatttotctaagtttaatgtctaagtattt |
| Israel 14114 HVR | 171 | ctaagtatttgtotaagtttaatgtotaagtattt |
| Japan NRIA 0301 | 171 | cta a gtatttgtcta a gtttattgtcta a gtattattgtcta a gtattattgtcta a gtattattgtcta a gtata a tgtcta a gtttatgtcta a gtttattgtcta a gtattatggcctca t g g g g g g g g g g g g g g g g g g |
| | | |

Figure 13: Genetic diversity of CyHV-3 isolates based on three highly variable regions (HVRs)

HVR1 (upper panel), HVR2 (middle panel) and HVR3 (lower panel). PCR and DNA sequencing targeting these HVRs are able to distinguish CyHV-3 C07 isolate from other Indonesian isolates as well as those from other countries.



3.6 Studies on expression of immune-related genes of carp during CyHV-3 infection

3.6.1 Introduction

The success of a virus infection in a host depends to a large extent on the host immune response to the infection. Hosts have equipped themselves with an elaborate immune system to defend themselves from invading viruses, whereas viruses have developed strategies to evade host immunity, including the expression of cytokine genes that have been captured from the host. Understanding the host-virus interaction may contribute to the effective use of the virus as a biocontrol agent in the MDB. Taking advantage of our experimental model for persistent/latent infection of CyHV-3 in carp (Sunarto et al, 2014), we investigated the expression of immune-related genes of carp during CyHV-3 infection, and, as a result, we propose a possible interaction between carp IL-12, carp IL-10 and viral-encoded IL-10 (khvIL-10) during the course of viral infection.

3.6.2 Methods

Samples of spleen were collected from carp during acute, persistent and reactivation phases of CyHV-3 infection (Sunarto et al, 2014). All samples, along with samples from mock-infected fish, were preserved in RNAlater, and total RNA was extracted from each sample using an AIIPrep DNA/RNA extraction kit (Qiagen). Contaminating DNA in total RNA extracts was removed by on-column digestion with RNase-free DNase I (Qiagen). The DNA-free RNAs were adjusted to the same concentration and the expression of host and viral genes during each phase of infection was quantified by SYBR Green real-time reverse transcription-PCR (qRT-PCR) and normalised against the expression of 18S rRNA. Eight viral and host genes including interferons (IFN γ -1 and -2), interleukins (IL-1B, -10 and -12), khv-IL-10, major histocompatibility complex class II (MHC II) molecules, and tumour necrosis factor (TNF α -1) were analysed.

3.6.3 Results and Discussion

Carp IL-12 was expressed at significantly higher levels (p < 0.05) during all phases of CyHV-3 infection compared with mock-infected fish. However, expression during the persistent and reactivation phases was significantly lower (p < 0.05) than during the acute phase. Host IL-10 was significantly up-regulated (p < 0.05) during the acute phase of CyHV-3 infection, but not during the persistent and reactivation phases. khvIL-10 was expressed at significantly higher levels during all phases of infection compared with mock-infected fish. In addition, expression of khvIL-10 was significantly higher in the acute and reactivation phases compared with the persistent phase (p < 0.05) of infected fish. Based on these data, we propose a possible interaction between carp IL-12, carp IL-10 and khvIL-10 following infection of carp by CyHV-3. We suggest that viral khvIL-10 is produced early in the productive (acute and reactivation) phases of infection, and that it may delay and blunt the host cell-mediated immune (CMI) response by suppressing expression of host IL-12. This would allow the virus to establish an intracellular infection that could potentially lead to a persistent/latent infection.

In addition, IL-1B was significantly up-regulated (p < 0.05) in the acute phase of infection. There was little difference in expression levels of IL-1B between productive and non-productive phases.

IFN γ -1 and -2 were expressed variably during different course of infection. There was no statistical difference in the expression of TNF α -1 and MHC-II genes in the spleen of carp



during different phases of CyHV-3 infection, nor between infected and mock-infected fish (Sunarto and McCoII, 2015).

As mentioned in the Introduction, understanding the host-virus interaction may contribute not only to the effective use of the virus as a biocontrol agent in the MDB, but also to developing markers of persistently infected carp. Understanding these issues will be important for future use of the virus in Australia.



3.7 Decay ("Shelf-life") of CyHV-3-infected tissue culture supernatant fluid at 4 °C

3.7.1 Introduction

It is likely that virus will be distributed at selected sites in the Murray-Darling Basin (MDB) by on-site intraperitoneal (IP) inoculation, and then release, of a number of carp. If carp are to be inoculated at various sites in the MDB, then it will be necessary to deliver live virus to these sites, and it is likely that use of freeze-dried (f-d) virus will be the approach of choice. However, while some work has already been completed on the use of f-d virus (see Section 4), problems arose with that work, and there was a need to examine alternative strategies for release of virus. As a result, the studies reported here investigated the shelf-life of CyHV-3 in infected tissue culture supernatant fluid stored at 4 $^{\circ}$ C.

3.7.2 Methods

Cultures of three different strains of stock CyHV-3 stored at -80 $^{\circ}\text{C}$ were set up in T75 flasks. The strains were:

- 1. Expt #1. Microstores 1408-11-1150, virus stored in non-secure lab
- 2. Expt #2. Microstores 1505-22-1506, virus stored in secure lab
- 3. Expt #3. Microstores 1509-14-1100, virus stored in secure lab

After 4 days in culture, 1 mL of tissue culture supernatant (TCSN) from each strain of virus was titrated, in triplicate, on KF-1 cells (Day 0), and 9 tubes @ 1 mL/tube of TCSN were aliquoted from each culture. The nine tubes from each strain were held at 4 °C. At Days 1, 2, 3, 4, 5, 6, 7, 10 and 14, a tube was removed from 4 °C storage, and titrated in triplicate.

A statistical analysis (Pairwise Anovas) was performed on all titrations, comparing virus titres each day with the original D0 titre for each strain of virus.

3.7.3 Results and Discussion

The results in Figure 14 show that there appears to be a decrease of virus titre by approximately one log over the two week experiment. However, a statistical analysis (Table 7) revealed that, due to high standard deviations, the only significant difference from the control (virus titrated at D0) was in Experiment 2 for virus stored at 4 $^{\circ}$ C for 5 or more days.





Figure 14: The decay of CyHV-3 in tissue culture supernatant at 4 oC (showing the mean and standard deviation at each time-point)



Table 7: Raw data from titrations of three replicates for each strain of CyHV-3 held at 4 oCfor 14 days (*: statistically significant at the 95% confidence level)

| TCID ₅₀ /mL | | KHV decay at 4 °C (days) | | | | | | | | |
|------------------------|----------|--------------------------|----------|----------|----------|----------|----------|----------|----------|----------|
| Expt #1 | Virus #0 | Virus #1 | Virus #2 | Virus #3 | Virus #4 | Virus #5 | Virus #6 | Virus #7 | Virus #8 | Virus #9 |
| Day at 4 °C | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 10 | 14 |
| Replicate1 | 7.96E+03 | 1.44E+04 | 2.00E+04 | 7.47E+03 | 5.02E+03 | 3.22E+03 | 3.56E+03 | 1.70E+03 | 8.66E+02 | 6.32E+02 |
| Replicate2 | 2.40E+04 | 3.74E+04 | 1.12E+04 | 5.60E+03 | 4.83E+03 | 4.04E+03 | 6.12E+03 | 2.00E+03 | 4.04E+03 | 2.00E+03 |
| Replicate3 | 4.57E+04 | 1.22E+05 | 5.30E+04 | 9.14E+03 | 4.31E+04 | 7.19E+03 | 4.83E+03 | 1.40E+04 | 4.31E+03 | 8.93E+02 |
| p-value compared to D0 | | 0.405739 | 0.903278 | 0.167578 | 0.648444 | 0.127928 | 0.127124 | 0.161694 | 0.106489 | 0.086841 |
| | | | | | | | | | | |
| | | | | | | | | | | |
| Expt #2 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 10 | 14 |
| Replicate1 | 7.80E+02 | 1.44E+03 | 3.22E+02 | 2.86E+02 | 4.62E+02 | 2.00E+02 | 3.22E+02 | 1.30E+02 | 3.22E+02 | 4.31E+01 |
| Replicate2 | 6.32E+02 | 8.27E+02 | 3.56E+02 | 5.02E+02 | 1.05E+02 | 3.74E+02 | 2.00E+02 | 2.86E+02 | 1.44E+02 | 4.04E+01 |
| Replicate3 | 9.28E+02 | 7.96E+02 | 6.32E+02 | 6.32E+02 | 5.48E+02 | 2.78E+02 | 9.28E+01 | 3.74E+02 | 9.28E+01 | 1.24E+02 |
| p-value compared to D0 | | 0.347298 | 0.057773 | 0.081415 | 0.063408 | 0.007469 | 0.006011 | 0.009715 | 0.005729 | 0.001369 |
| | | | | | | * | * | * | * | * |
| | | | | | | | | | | |
| Expt #3 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 10 | 14 |
| Replicate1 | 1.06E+04 | 4.04E+03 | 5.48E+03 | 1.06E+03 | 1.12E+03 | 1.31E+03 | 1.14E+03 | 1.00E+03 | 1.45E+03 | 4.04E+02 |
| Replicate2 | 1.70E+03 | 3.56E+03 | 1.07E+03 | 1.70E+03 | 6.32E+02 | 6.32E+02 | 6.32E+02 | 9.38E+02 | 2.00E+02 | 4.57E+02 |
| Replicate3 | 5.48E+03 | 6.05E+03 | 8.93E+02 | 1.44E+03 | 9.79E+02 | 4.31E+02 | 5.70E+02 | 7.15E+02 | 4.04E+02 | 3.74E+02 |
| p-value compared to D0 | | 0.634188 | 0.312575 | 0.155486 | 0.124799 | 0.119299 | 0.117967 | 0.123044 | 0.115465 | 0.099863 |
| | | | | | | | | | | |

The data generated in this experiment suggest that there would be no statistically significant loss of titre in TCSN for at least 4 days if the TCSN were held at 4 $^{\circ}$ C. Depending on the initial titre of the TCSN at D0, this may allow sufficient time for the TCSN to be transported from the laboratory of origin to the site of inoculation of fish in the field.



3.8 Developing methods to identify carp persistentlyinfected with CyHV-3

3.8.1 Introduction

One of the characteristic features of all herpesviruses is the inevitability of persistent infections (usually a latent infection) occurring in hosts that survive the initial acute infection. This is also likely to occur with CyHV-3 infection in carp and koi. Understanding the dynamics of persistent CyHV-3 infections in carp, and being able to identify persistently-infected carp, may be critical in the use of the virus as a biocontrol agent in the MDB. Taking advantage of our experimental model for persistent/latent infection of CyHV-3 in carp (Sunarto et al, 2014), we conducted additional activities with the main aim of identifying a 'marker' of fish harbouring a persistent/latent infection with CyHV-3. In particular, we investigated messenger RNA (mRNA) of CyHV-3 infected carp in the search for a potential marker for CyHV-3 latency/persistence.

3.8.2 Methods

Samples of kidney were collected from carp during acute, persistent and reactivation phases of CyHV-3 infection (Sunarto et al, 2014). All samples, along with samples of mock-infected fish, were preserved in RNAIater and total RNA was extracted using AllPrep DNA/RNA extraction kit (Qiagen). RNA concentration and integrity was assessed by NanoDrop and Bioanalyzer. For each group, equal amounts of RNA from three individual carp were used to provide template for RNAseq library construction. RNAseq library preparation and sequencing for mRNA was performed by the Australian Genome and Research Facility Ltd using an Illumina HiSeq next-generation sequencing platform.

For validation purposes, the expression of several open reading frames from the same samples was determined using quantitative reverse transcription-PCR (qRT-PCR; for primers see Table 1 in Sunarto et al, 2014). Expression from the open reading frames was normalised to expression of carp 18S rRNA to enable sample comparison.

Raw RNA-Seq reads were trimmed for quality and Illumina adapters were removed. Residual ribosomal RNA was removed from the cleaned reads. The resulting mRNA was mapped to the *Cyprinus carpio* genome and the CyHV-3 genome. Mapped reads were assembled into 11 separate transcriptomes (3 acute, 3 persistent, 3 reactivated, 2 mock), then merged into a master assembly. Reads from individual samples were mapped to the master assembly and differential expression testing was conducted. Reads per kilobase of transcript per million mapped reads (RPKM) values were calculated. The correlation between RNA-Seq and qRT-PCR values was calculated. A heatmap of CyHV-3 RPKM values over the sample treatments and pairwise comparisons of RPKM values were plotted.

3.8.3 Results and Discussion

We used RNA-Seq in one of the first studies to examine the transcriptome of *Cyprinid herpesvirus 3* (CyHV-3) in its host, the common carp (*Cyprinus carpio*). RNA-Seq expression levels were validated for six genes using qRT-PCR, which confirmed that RNA-Seq is a reliable technique for gene expression studies. The goals of this experiment were, firstly, to determine if the CyHV-3 gene expression profile was different during acute, persistent and reactivated infections, and secondly, to examine gene expression patterns during the CyHV-3 persistent phase.



Fish in the acute and reactivated stages of infection displayed similar CyHV-3 gene expression profiles. However, absolute levels of gene expression were highly variable in both acute and reactivated replicates. Samples taken from fish in the persistent phase of infection only contained a few scattered CyHV-3 transcripts. At these low expression levels, no genes could be identified as "latency associated transcripts" (LATs). This may be because the experimental model used here (Sunarto et al, 2014), where fish were placed at the non-permissive temperature of 11°C for 28 days, may simply induce a low-level persistent infection rather than latency. Another possibility for the absence of apparent LATs in the current study is that the sequencing of CyHV-3 transcripts was not deep enough for detection of the lowly expressed LATs during the latent phase. Overall, the current data suggest that the Sunarto et al (2014) experimental model produces a persistent infection rather than latency, although further work on the site and mechanism of CyHV-3 latency are required. Further studies are in progress to investigate the potential of microRNA (of both host and virus origin) as a potential biomarker of latency/persistent infection.



3.9 Preparation of journal articles

Over the course of this project, the following papers and book chapter have either been published, submitted as manuscripts for publication, or are in an advanced state of preparation (prior to submission):

3.9.1 Published

Sunarto A, Liongue C, McColl KA, Adams MM, Bulach D, Crane MStJ, Schat KA, Slobedman B, Barnes AC, Ward AC, Walker PJ (2012) Koi herpesvirus encodes and expresses a functional interleukin-10. *Journal of Virology* 86:11512-11520.

McColl KA, Cooke BD, 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* 72:109-117.

Sunarto A, McColl KA, Crane MStJ, Schat KA, Slobedman B, Barnes AC, Walker PJ (2014). Characteristics of cyprinid herpesvirus 3 in different phases of infection: implications for disease transmission and control. *Virus Research* 188:45-53.

Sunarto A, McColl KA (2015). Expression of immune-related genes of common carp during cyprinid herpesvirus 3 infection. Diseases of Aquatic Organisms 113:127-135.

3.9.2 Submitted for publication

McColl KA, Sunarto A, Slater J, Bell K, Asmus M, Fulton W, Hall K, Brown P, Gilligan DM, Hoad J, Williams LM, Crane MStJ. Cyprinid herpesvirus 3 as a potential biological control agent for carp (*Cyprinus carpio*) in Australia: non-target species testing. *Journal of Fish Diseases* (now accepted).

Neave MJ, Sunarto A, McColl KA. Transcription of the *Cyprinid herpesvirus 3* genome during acute, persistent and reactivated phases of infection in common carp, *Cyprinus carpio. Journal of General Virology.*

McColl KA, Sunarto A, Li L (accepted) *Cypriniviruses* in *Molecular detection of animal viral pathogens*. D Liu (ed). CRC Press, Taylor & Francis, London.

3.9.3 Advanced state of preparation

Durr P, Davis S, Graham K, Brown P, Gilligan DM, Sunarto A, McColl KA. Development of an epidemiological model for the strategic release of Cyprinid herpesvirus 3 as a potential biological control agent for carp (Cyprinus carpio) in the Lachlan River catchment in New South Wales, Australia.

McColl KA, Sunarto A, Holmes EC. Cyprinid herpesvirus 3 and its evolutionary future as a potential biological control agent for carp (*Cyprinus carpio*) in Australia. *Virology Journal* (now submitted).

Sunarto A, Neave MJ, Yuasa K, Dishon A, McColl KA. Cyprinid herpesvirus 3 as a potential biological control agent for carp (*Cyprinus carpio*) in Australia: Whole-genome analysis of the Indonesian C07 isolate. *Archives of Virology*.



4. Conclusions and recommendations

The most important conclusion from Phase 3 work on CyHV-3 is that, in summary, clinical, molecular and histological observations, together with mortality data, strongly suggest that CyHV-3 is unable to replicate in, let alone affect, a wide taxonomic range of both native Australian animals and introduced species. Although mortality in virus-challenged *O. mykiss*, *M. cephalus* and *B. bidyanus* was greater than in their negative control counterparts, the vast majority of samples from NTS (including the virus-challenged *B. bidyanus*, *O. mykiss* and *M. cephalus* with high mortalities) were negative when tested by the CyHV-3 qPCR (Gilad et al, 2004) indicating the likely absence of virus in these animals.

However, 109 samples from virus-challenged and negative control NTS registered a C_T value <40 (Table 2). These positive results could possibly be explained either by low levels of replicating CyHV-3 in tissues from virus-challenged animals, or by inadvertent contamination of samples with viral DNA, possibly during the collection of samples, or during laboratory processing of the samples. To test for evidence of virus replication, all 109 samples were examined with an RT-PCR (Yuasa et al, 2012) capable of differentiating viral mRNA from any contaminating viral gDNA. RT-PCR testing provided no evidence for viral replication in any of the 109 qPCR-positive samples (including all 29 qPCR-positive samples from virus-challenged *B. bidyanus*) (Table 2).

These results strongly suggest that both spillover infections and species jumps are highly unlikely with CyHV-3 in NTS. As a result, one of the major arguments against the use of CyHV-3 as a biocontrol agent is weakened, thereby encouraging further work on the use of CyHV-3 as a potential biocontrol agent for carp in Australia.

A further important conclusion from the work has been the development of a mathematical model that will allow assessment of how CyHV-3 might best be used - in particular, for the case of the Lachlan River catchment. It is anticipated that questions that will be able to be addressed include:

- 1. When will it be best to release the virus (what season and what part of the El Nino Southern Oscillation (ENSO) cycle)?
- 2. Where will it be best to release the virus (upstream or downstream or in recruiting grounds)?
- 3. Will supplementary regional controls for example, fishing, trapping, netting be needed? (Note that we will not be looking at 'daughterless' technology this has now been explored by previous studies).
- 4. If they are (which is probable), what will be the best way to apply these (before or after releasing the virus).

Transferring CyHV-3 to the non-secure laboratory at AAHL has also been an important achievement in allowing the future use of the virus in the field, as has gathering data on how best to deliver the virus to field locations. The ultimate characterisation of CyHV-3 (C07 strain) has been achieved by completely sequencing and annotating the virus.

The findings that are documented in this Report provide great encouragement for further work on the use of CyHV-3 as a potential biocontrol agent for carp in Australia. Particular recommendations for future work include:

1. Completion of the non-target species (NTS) testing (2 Western Australian and 3 Queensland species of fish).



- 2. Extension of the modelling work from the LRC to the MDB at large.
- 3. Development of tools (particularly a serological test) for monitoring the effect of the virus on carp populations to recognize when, and if, modifications are required to the virus or to control programs that will complement the effect of the virus.
- 4. Development of a quantitative measure of stress in carp in order to address animal ethics issue associated with the release of the virus.
- 5. Modification of the protocol for the production of freeze-dried virus.
- 6. Susceptibility testing of carp from throughout the entire MDB to the virus to ensure that CyHV-3 (C07 strain) will be effective.
- 7. Selection and development of a genetic strategy to complement the effect of CyHV-3 as a biocontrol agent.



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Appendix

There has always been low-level media interest in the carp herpesvirus project, but media interest intensified enormously following a Media Release on 13 January, 2016 by a coalition of industry groups calling on State and Federal governments to take action against European carp. The coalition consisted of: Australian Conservation Foundation, Australian Recreational Fishing Foundation, Invasive Species Council, National Farmers Federation, and the National Irrigators' Council.

In the week after the announcement, Ken McColl was interviewed by 19 media outlets (reaching approximately 30 interviews by mid-June, 2016) across print, radio and television. Further highlights from that week included:

- In Australia we captured more than 100 media clips including prominent features in The Australian (two separate features), The Age, 3AW and ABC TV News
- The story featured on important industry sites including the Fish site and made it into the Shanghai times
- An issue of the First Dog on the Moon cartoon was devoted entirely to the carp herpesvirus, and it reached an international audience
- On twitter, the story reached 6M+ accounts with 11M+ impressions
- 113 items reaching a cumulative audience of 3,554,263 & an advertising space rate of AUD 510,092
- Online News had the highest volume of coverage (34 items or 30% of the total volume of coverage)
- AM Radio reached the highest cumulative audience (1,757,800 or 49% of the cumulative audience)
- Overall media tone rating: Positive

In May 2016, there was a further burst of media activity following another Media Release by the same coalition of industry groups. During this time, media interviews included those by Canadian television (for a show called "Découverte"), The Wall Street Journal, The New York Times, and New Scientist.



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