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Incursions of *Cyprinid herpes virus 2* in goldfish populations in Australia despite quarantine practices

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ABSTRACT

The international trade in ornamental fish is considered a major factor for the transboundary spread of aquatic pathogens that can affect both wild and farmed fish populations. Nearly 18 million ornamental fish were imported into Australia in 2007, including approximately 3.9 million goldfish. Despite quarantine regulations during importation, there have been several incidents in Australia where exotic pathogens from ornamental fish have become established in farmed or free-living fish species. The exotic virus *Cyprinid herpesvirus 2* (CyHV2) was first found in Australia in 2003 in goldfish, suggesting that sub-clinically infected goldfish were passing through quarantine regardless of health certification and three weeks of quarantine. Repeated cross sectional surveys were conducted to determine whether CyHV2 has already established in farmed or wild ornamental fish in Australia. Goldfish populations were tested to OIE standard to detect 2% prevalence with 95% confidence assuming a test of 100% sensitivity and specificity. CyHV2 was found at retail outlets, farms and in several populations of wild goldfish in the ACT and Victoria. The prevalence and moderate to high viral loads in sub-clinically infected goldfish from different domestic populations suggested the introduction was not a recent event. This study demonstrated that CyHV2 has established in Australia and informed quarantine policy to revoke the requirement for goldfish exported to Australia to be certified free of CyHV2. The results provided clear evidence that an aquatic pathogen from imported ornamental fish can become established in farmed and wild populations. This is of particular significance to Australia as there are many endemic and ecologically sensitive populations of fish that may be severely affected by exotic pathogens. The incursion of CyHV2 in Australia should be considered a case study to inform pathway analysis for pathogen establishment.

Keywords: herpes, virus, goldfish, biosecurity, quarantine policy, wild fish

1. Introduction

The international trade in ornamental pet fish is an efficient system for the translocation of aquatic pathogens (Whittington and Chong, 2007; Peeler and Feist, 2011). In Australia, around 18 million ornamental fish are imported each year, including approximately 4 million goldfish (*Carassius auratus*) (O'Sullivan et al., 2008). A further 7 million goldfish were sold from domestic breeders to supply the pet fish industry (O'Sullivan et al., 2008). Australia is one of a handful of countries in the world with stringent import controls for ornamental fish (Whittington and Chong, 2007). Controls include health certification at export premises and upon arrival, fish are subject to a visual health inspection and a quarantine period of 7 to 21 days depending on species. Despite quarantine regulations during importation, there have been several incidents in Australia where exotic pathogens from ornamental fish have affected wild and farmed populations (Whittington et al., 1987; Lancaster et al., 2003; Stephens et al., 2004). In particular, the international trade in goldfish has led to the establishment of significant pathogens with potential to affect aquaculture including atypical *Aeromonas salmonicida* responsible for goldfish ulcer disease (Trust et al., 1980) and *Cyprinid herpesvirus 2* (CyHV2) (Stephens et al., 2004), which was the focus of this study.

In general, herpesviruses are characterized by a high level of host specificity and infections are often persistent with few or no clinical signs (Hanson et al., 2011). The three species, *Cyprinid herpesvirus 1*, *Cyprinid herpesvirus 2*, and *Cyprinid herpesvirus 3* belonging to the genus *Cyprinivirus* are associated with both chronic infections and mass mortality events in common carp (*Cyprinus carpio*) or goldfish (Davison et al., 2013). Historically, the host species for CyHV1 and CyHV3 were considered to be common carp and koi carp (a variety of *Cyprinus carpio*), while CyHV2 was thought to be a pathogen of goldfish. It is now known that the three cyprinid herpesviruses are able to infect a much wider range of cyprinid species than previously assumed (Hedrick et al., 2006; Bergmann et al. 2010; Fichi et al., 2013). Crucian carp (*Carassius carassius*) (Fichi et al., 2013) and Prussian carp (*C. gibelio*) are susceptible to infection with CyHV2 with the latter species showing haematopoietic necrosis resulting in

mortality (Danek et al., 2012; Luo et al., 2013). CyHV3 can infect goldfish (Sadler et al., 2008), grass carp (*Ctenopharyngodon idella*), ide (*Leuciscus idus*) and ornamental catfish (*Ancistrus* sp.) (OIE, 2012). This is of importance to cyprinid aquaculture, which represents 40% of global production of aquatic animals with 24.2 million tonnes produced in 2010 (Food and Agriculture Organization of the United Nations, 2012). The international trade in ornamental koi carp and goldfish may facilitate the spread of these viruses without detection potentially placing local food fish production systems at risk.

First identified in Japan in 1992, CyHV2 has caused mortality events in farmed goldfish in Taiwan (Chang et al., 1999), USA (Goodwin et al., 2006b), UK (Jeffrey et al., 2007) and Australia (Stephens et al., 2004). Outbreaks of CyHV2 are often associated with high mortality in all age classes and typically occur in spring and autumn or during handling events with sudden drops in temperature, such as shipping and holding of goldfish at wholesalers (Goodwin et al., 2009; Davison et al., 2013). The virus is difficult to isolate in cell culture (Jung and Miyazaki, 1995) and laboratory methods demonstrating continual cultivation have only recently been published (Ito et al., 2013). Several molecular-based assays have been developed to identify infected fish (Goodwin et al., 2006a; Waltzek et al., 2009). The intensity of CyHV2 infection in apparently healthy goldfish can be as high as 10^7 to 10^9 copies μg^{-1} host DNA and was commonly found to be in the range of 10^3 to 10^5 copies (Goodwin et al., 2009).

In Australia, CyHV2 was first identified in 2003 at a goldfish farm in Western Australia (Stephens et al., 2004). It was suggested that sub-clinically infected goldfish were passing through quarantine and coming into contact with domestic stocks through live fish trading (Stephens et al., 2004). An opportunistic survey of sick or moribund fish collected from retail outlets in Sydney revealed that CyHV2 was present in 17% of goldfish (Whittington et al., 2009). However, since retail outlets contained an assortment of imported and domestic fish sharing the same environment, the source of the virus could not be identified. Therefore, the objective was to ascertain if CyHV2 was indeed passing through quarantine undetected and if so, to determine whether the virus has become established in domestic stocks.

2. Materials and methods

2.1 Selection of the goldfish populations and animals

The study involved repeated cross sectional surveys of farmed and wild goldfish populations in Australia. Also, we conducted opportunistic surveys of retail outlets located in New South Wales (NSW) and wild goldfish from Victoria (VIC). For the cross sectional surveys, a sample size was set at 150 individuals to detect 2% prevalence with 95% confidence assuming a test of 100% sensitivity and specificity. For all surveys, populations were defined as groups of goldfish that were in close contact (e.g. sharing water, same farm location) and were collected at the same time or within the same season. Where possible, samples were collected four times each year to account for possible seasonal variations in prevalence of infection in order to increase the likelihood of detecting infections.

Four goldfish farms were involved in the survey, all of which used outdoor pond or tank-based rearing facilities (Table 2). These farms supply over 90% of the domestic production (by quantity) of goldfish to wholesale and retail outlets in Australia. For each collection at Farm 1, 30 ponds were selected using a table of random numbers and then five fish were collected using a dip net at the edge of the pond. This sampling strategy was used for two collections at Farm 2. The other two collections at Farm 2 consisted of between four and seven fish collected from 37 and 21 outdoor tanks chosen from a table of random numbers. Farm 3 consisted of many small cages held within three large ponds (Table 2). Three to five fish were collected by dip net from all cages in each pond to reach the sample size of at least 150. At Farm 4, five out of 12 ponds were selected using a table of random numbers and approximately 25 fish were collected by dip net from each one.

Wild fish were collected opportunistically by means of electrofishing and netting techniques in areas of known feral goldfish populations in the Australian Capital Territory (ACT) for the cross sectional survey. Wild goldfish from Victoria were opportunistically collected during annual native fish monitoring programs by fisheries biologists.

Retail outlets located in NSW with a prior relationship with Future Fisheries Veterinary Service Pty Ltd were visited on two occasions and asked to participate in the opportunistic survey. Three of eight retail outlets agreed to participate and periodically placed dead or moribund goldfish in a freezer for collection and testing for CyHV2.

2.2 Detection and Confirmation of CyHV2

Upon death, fish were kept on ice or immediately frozen, transported to the laboratory and stored at -80°C until the time of processing. Kidney, liver and spleen were dissected from each fish using aseptic techniques. The tissue was homogenized and clarified by bead beating followed by centrifugation as described by Rimmer et al. (2012). Nucleic acids were extracted from a 50 µl aliquot of the clarified tissue homogenate (1:10 w/v) of individual fish using a MagMax-96 viral isolation kit (Ambion, USA) according to manufacturer's instructions.

A quantitative polymerase chain reaction (qPCR) assay targeting a specific sequence of the DNA polymerase (DNApol) gene of CyHV2 was used to detect the virus (Rimmer et al., 2012). Briefly, amplification was performed in 25 µl reactions containing 5 µl of template DNA, 0.125 µl of forward primer and reverse primer (100 pmol/µl), 12.5 µl of Quantitect SYBR Green Master Mix (Qiagen) and 7.25 µl of molecular grade water. The primers C1153 and C1154 were used for the initial screening assay and were designed to exclude detection of CyHV3 (Table 1). Thermocycling was performed on a MX3000 Multiplex Quantitative PCR System (Stratagene) with reaction conditions: hotstart activation and denaturation at 95°C for 15 minutes, followed by 40 cycles of 95°C denaturation for 30 seconds, 62°C annealing for 30 seconds and 72°C extension for 30 seconds. Reactions were completed in duplicate with a positive and no template controls included in each test. A final melt curve analysis was completed to ensure reaction specificity. The melting temperature of standards was between 81.1 and 82.1°C. A sample was considered positive when a threshold cycle value was achieved for at least one of the duplicates and the melting temperature of the product was within the range of those recorded for

the standards. The analytical specificity of the assay was evaluated using a panel of DNA virus reference samples including CyHV3 and four iridoviruses (Epizootic haematopoietic necrosis virus, Dwarf gourami iridovirus, Bohle iridovirus and Red sea bream iridovirus) (Rimmer et al., 2012). No products were amplified in the reference samples using the primers C1153 and C1154. Known positive and negative goldfish (based on the assay described in Goodwin et al. 2006a) were used to test the primers C1153 and C1154. PCR products were confirmed to be specific for CyHV2 by sequence analysis.

Quantification of viral DNA was interpolated from the plasmid standard curve, with a reaction efficiency of 90 to 110%. For each consignment, extracted nucleic acids from a maximum of five fish were pooled for testing and any positive pools were re-tested individually. Standard curves were prepared by amplification of a dilution series of known quantities of plasmid DNA (see below for description). Based on the highest dilution of the plasmid that was detected at least 50% of the time (OIE, 2009), the analytical sensitivity of the qPCR assay was 100 copies. Figure 1 represented an indicative standard curve. The plasmid control provided an appropriate external standard for comparison of qPCR assay results obtained at different times and on different PCR machines.

Confirmation of the initial detection of CyHV2 in domestic populations at Farm 1 and in the wild-caught goldfish was completed using conventional PCR assays, followed by purification of PCR products and sequencing. Specifically, a conventional PCR assay was performed using the primers C1109 and C1158 (Table 1), which targets a 401 base pair segment of the DNAPol gene and results were assessed by electrophoresis in 2% w/v agarose gels stained with ethidium bromide. Next, PCR products were purified and sent to a commercial laboratory for sequencing. BLAST analyses confirmed the presence of CyHV2 sequence with 100% homology to the partial DNA polymerase gene sequence available in GenBank (Accession no: DQ085628.1, data not shown). All other positive results were confirmed using a conventional PCR for the helicase gene (Waltzek et al., 2009) with the primers C1281 and C1282 (Table 1).

2.3 Preparation of cloned CyHV2 DNA

The plasmid DNA control used with the CyHV2 qPCR assay (pCYHV 2DNApol) was prepared by cloning a sequence specific insert into plasmid pCR2.1 (Invitrogen). The sequence specific insert was produced from conventional PCR using primers C1109 and C1158 (Table 1) and was 401 nucleotides in length and corresponded to start position 29 of the DNA polymerase gene of *Cyprinid Herpesvirus 2* (Genbank accession no. AY939863.1) (Whittington et al., 2009). Nucleic acid sequence alignments were compared with known CyHV2 positive control DNA obtained from a clinically affected goldfish that was supplied by Professor Andrew Goodwin (University of Arkansas). Large quantities of the plasmid were obtained by culture of transformed TOP10 *E.coli* (Invitrogen). When linearised by restriction enzyme digestion, pCYHV 2DNApol was amplified efficiently by the CyHV2 qPCR assay (Figure 1.).

2.4 Descriptive statistics

Prevalence was defined as the proportion fish with a positive qPCR test in each population. Exact binomial confidence intervals were calculated using Stata, version 10 (Stata Corporation, College Station, TX, USA). A one-sided 97.5% confidence interval was presented in cases where either 0% or 100% were found to be positive in a given population. If no positive samples were detected in a population, it was defined as not infected (within the assumptions specified). The presence or absence of virus in retail premises was reported. Estimates of true prevalence with Wilson 95% confidence intervals were calculated using EpiTools (Sergeant, 2013) with sensitivity and specificity equal to 90% and 100%, respectively.

3. Results

CyHV2 was detected in populations of goldfish collected at farms, retail outlets and from the wild. CyHV2 was initially detected at Farm 1 located in Victoria with true prevalence equal to 3.0% (95% CI: 1.2-7.4%) (Table 2). This confirmed that the virus was endemic in the largest goldfish farm in Australia. The virus was also found at Farm 3, which is located on the NSW Central Coast with true prevalence at

the first sample point estimated to be 7%. However the prevalence was much higher (37-43%) from the subsequent samplings. The intensity of infection for the positive goldfish collected from Farms 1 and 2 ranged from a median of 1×10^3 to 4.7×10^3 copies/mg of fish tissue (Table 2). Farm 2 located in northern NSW was considered to be free of CyHV2 as the virus was not detected in any of the samples collected over four seasons during 2010-2011. Also, CyHV2 was not detected in one sample of goldfish collected from Farm 4 (Table 2). Several attempts were later made to collect further samples at Farm 4. However, the farm declined further involvement with the study.

CyHV2 was detected in wild goldfish collected from peri-urban and rural water bodies during 2010 and 2011. Approximately 6-7% of the goldfish from Cotter Reservoir, ACT in October 2010 and March 2011 were qPCR positive for CyHV2 (Table 3). True prevalence estimates for these two populations were 6.8% (95% CI: 3.6-12.6) and 7.9% (95% CI: 4.7-12.8), respectively. Also, CyHV2 was detected in goldfish caught in Victoria from the Ovens River and the Murray River with true prevalence ranging from 7 to 41% (Table 3; Figure 2). The intensity of infection for the wild caught goldfish ranged from a median of 1.1×10^2 to 1.5×10^6 copies/mg of fish tissue (Table 3). Ten consignments of sick or dead goldfish were opportunistically collected from three retail outlets during the study. CyHV2 was detected in six of these consignments with prevalence ranging from 50% to 100% (Table 4). These moribund or dead goldfish had median copy numbers ranging from 3.9×10^6 to 2.8×10^7 per mg of fish tissue (Table 4).

4. Discussion

The findings of the study provided evidence that CyHV2 has established in Australia and were used to inform quarantine policy. Within nine months of the notification that CyHV2 was endemic at domestic farms the Australian Government Department of Agriculture, Fisheries and Forestry revoked the requirement for goldfish exported to Australia to be certified free of CyHV2 (Biosecurity Australia, 2011). The example of CyHV2 provides clear demonstration that an aquatic pathogen from the ornamental fish

industry can pass through Australian quarantine and become established in farmed and wild populations. This is of particular relevance to the current import risk analysis for megalocytiviruses in live ornamental fish imported to Australia (Biosecurity Australia, 2012). Similar to CyHV2, megalocytiviruses are known to cause large mortality events in aquaculture affecting both food and ornamental fish production (Kurita and Nakajima, 2012).

The CyHV2 surveys revealed that the virus was present at farms, retail outlets and in several populations of wild goldfish. CyHV2 is presumed to have originated from infected stocks that were imported to Australia. The evidence suggested that the introduction was not a recent event given the observed prevalence and moderate to high viral loads in sub-clinically infected goldfish of various sizes (i.e. age classes) from this study and the previous report of CyHV2 in Australia (Stephens et al., 2004). This was supported by previous research showing that the virus can be detected in broodfish several years after infection (Goodwin et al., 2006a). However, the hypothesis that older goldfish exhibit a true latent infection that is capable of being re-activated remains untested.

Interestingly, there are no goldfish farms upstream of Cotter Reservoir (or anywhere near the ACT) and there is no mechanism for natural dispersal of fish from downstream areas as the dam wall is a complete barrier to upstream fish passage. Given the prevalence and viral load in the wild-caught goldfish population, the most likely explanations for the presence of CyHV2 is that (i) unwanted goldfish with sub-clinical infections were released by pet owners into the natural environment, or (ii) illegal use and release of goldfish as live bait. Human-assisted dispersal of ornamental fish via both these pathways has been identified as a major pathway for the establishment of alien freshwater fish in Australia (Lintermans, 2004) and any pathogens they may be carrying. CyHV2 infections in other wild goldfish populations may be due to unintentional release of infected fish from aquaculture ponds following flooding events.

CyHV2 was detected at Farm 1, which widely distributes goldfish across Australia and at a smaller farm (Farm 3) that mostly sells fish directly to retail outlets in Sydney. Farm 1 has traded for several

decades selling goldfish across Australia and has an open system with mixing of multiple year classes and frequent acquisition of new stock for breeding. Farm 3 is also an open system and annually receives breeding stock from Farm 1. The owner of Farm 3 reported that goldfish from Farm 1 were brought to the farm between the first and second sampling period which was presumably responsible for the six fold increase in the prevalence of CyHV2. The owner from Farm 2 reported that the facility was closed to the importation of goldfish and was the only farm to use a tank-based rearing system that is protected with bird netting and uses only bore water. There was no information available regarding the breeding stock used at Farm 4. Maintaining a closed population in tanks supplied with water from an underground aquifer appeared to be important to ensure CyHV2 freedom within Australia.

Evidence suggests that CyHV2 can infect another cyprinid, crucian carp (Fichi et al., 2013). Native to Europe and Asia, crucian carp ranks ninth among all the fish species used in aquaculture and is well established in China and several eastern European countries with 2.2 million tonnes produced in 2010 (Food and Agriculture Organization of the United Nations, 2010). If CyHV2 infection results in disease in crucian carp, there is a potentially significant risk to food production if the virus becomes established in farmed goldfish sharing environments with crucian carp aquaculture. Moreover, CyHV2 was responsible for several mortality events with associated haematopoietic necrosis in farmed (Luo et al., 2013) and wild populations (Danek et al., 2012) of Prussian carp. It would be prudent to assume that all members of the genus *Carassius* are potential carriers for CyHV2 and this virus should be considered to be a multi-host pathogen. The example of CyHV2 in cyprinids highlights the need to consider the production and trade of pet fish in the trans-boundary spread of pathogens affecting food fish production.

The goldfish is a known host and carrier for several viral pathogens that are of international concern due to their socio-economic importance and their documented trans-boundary spread (Sano et al., 2011). Goldfish are capable of carrying CyHV3 (koi herpesvirus) (Sadler et al., 2008; El-Matbouli and Soliman, 2011) and are susceptible to infections with spring viremia of carp virus (SVCV) (OIE, 2012).

Both viruses are considered exotic to Australia and infection with either pathogen is reportable in Australia and to the OIE (OIE, 2012). These viruses are highly contagious and have caused serious sporadic mortality events at carp farms in Europe, North America and Asia (Sano et al., 2011), including a loss of 80 tonnes of carp from a farm in Germany following a single outbreak of CyHV3 (Bocklisch et al., 2006 reported in Fabian et al., 2013). Moreover, both of these viruses cause significant disease events in populations of the highly traded ornamental koi carp (Sano et al., 2011; Fabian et al., 2013). Current import conditions for goldfish to Australia require additional veterinary certification to be free of SVCV but not CyHV3 (Department of Agriculture, Fisheries and Forestry, 2013). Given the structure of the ornamental fish industry with large distribution centres in Asia (Ling and Lim, 2005), it is possible that goldfish destined for export may also harbour these viruses.

5. Conclusion

As a result of this study, CyHV2 was declared an established pathogen in Australia. The virus was identified at domestic farms and in wild populations. The findings were used to inform quarantine policy to revoke the requirement for goldfish exported to Australia to be certified free of CyHV2. The occurrence of the formerly exotic pathogen, CyHV2 in post-quarantine populations of goldfish contributed to the conclusion that current quarantine measures provide an unacceptably low level of protection. This was despite Australia being considered to have some of the most stringent standards for the importation of ornamental fish in the world (Whittington and Chong, 2007). The results of the CyHV2 surveys can be used to inform risk assessment for the importation of other ornamental fish species. This study supports the recommendation that in addition to quarantine at the border, pre-border laboratory testing be carried out to detect exotic viruses in imported ornamental fish (Whittington and Chong, 2007).

Conflict of Interest Statement

None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

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References

- Bergmann, S. M., Sadowski, J., Kiełpiński, M., Bartłomiejczyk, M., Fichtner, D., Riebe, R., Lenk, M., Kempter, J., 2010. Susceptibility of koi x crucian carp and koi x goldfish hybrids to koi herpesvirus (KHV) and the development of KHV disease (KHVD). *J. Fish Dis.* 33, 267-272.
- Biosecurity Australia 2012. Import risk analysis for freshwater ornamental finfish with respect to gourami iridovirus and related viruses. *Biosecurity Australia Advice 2012/01*, Canberra, http://www.daff.gov.au/ba/ira/current-animal/ornamental_finfish/ba-2012-01_gourami_iridovirus_ira.
- Biosecurity Australia 2011. Conditions for the importation of live freshwater ornamental finfish into Australia: amended health certification requirements for goldfish. *Biosecurity Australia Advice 2011/16*, Canberra, <http://www.daff.gov.au/ba/reviews/final-animal/goldfish/baa2011-16-goldfish-amended-requirements>.

- Chang, P.H., Lee, S.H., Chiang, H.C., Jong, M.H., 1999. Epizootic of herpes-like virus infection in goldfish, *Carassius auratus* in Taiwan. *Fish Pathol.* 34, 209-210.
- Danek, T., Kalous, L., Vesely, T., Krasova, E., Reschova, S., Rylkova, K., Kulich, P., Petrtyl, M., Pokorova, D., Knytl, M., 2012. Massive mortality of Prussian carp *Carassius gibelio* in the upper Elbe basin associated with herpesviral hematopoietic necrosis (CyHV2). *Dis. Aquat. Org.* 102, 87-95.
- Davison, A.J., Kurobe, T., Gatherer, D., Cunningham, C., Korf, I., Fukuda, H., Hedrick, R.P., Waltzek, T.B., 2013. Comparative genomics of carp herpesviruses. *J. Virol.* 87, 2908-2922.
- Department of Agriculture, Fisheries and Forestry, 2013. Import Conditions (ICON) database – Freshwater fish (other than Salmonidae), <http://www.daff.gov.au/biosecurity/import/icon-icd> (accessed on 27 November 2013).
- El-Matbouli, M., Soliman, H., 2011. Transmission of *Cyprinid herpesvirus-3* (CyHV-3) from goldfish to naive common carp by cohabitation. *Res. Vet. Sci.* 90, 536-539.
- Fabian, M., Baumer, A., Steinhagen, D., 2013. Do wild fish species contribute to the transmission of koi herpesvirus to carp in hatchery ponds? *J. Fish Dis.* 36, 505-514.
- Fichi, G., Cardeti, G., Cocumelli, C., Vendramin, N., Toffan, A., Eleni, C., Siemoni, N., Fischetti, R., Susini, F., 2013. Detection of Cyprinid herpesvirus 2 in association with an *Aeromonas sobria* infection of *Carassius carassius* (L.), in Italy. *J. Fish Dis.* 36, 823–830.
- Food and Agriculture Organization of the United Nations, 2012. The State of World Fisheries and Aquaculture. FAO, Rome, pp. 36-37.
- Food and Agriculture Organization of the United Nations, 2010. Aquaculture production. In: FAO Yearbook, Rome, p. 46.
- Goodwin, A.E., Khoo, L., LaPatra, S.E., Bonar, A., Key, D.W., Garner, M., Lee, M.V., Hanson, L., 2006b. Goldfish hematopoietic necrosis herpesvirus (cyprinid herpesvirus 2) in the USA: molecular confirmation of isolates from diseased fish. *J. Aquat. Anim. Health* 18, 11-18.

- Goodwin, A.E., Merry, G.E., Sadler, J., 2006a. Detection of the herpesviral hematopoietic necrosis disease agent (*Cyprinid herpesvirus 2*) in moribund and healthy goldfish: validation of a quantitative PCR diagnostic method. *Dis. Aquat. Organ.* 69, 137-143.
- Goodwin, A.E., Sadler, J., Merry, G.E., Marecaux, E.N., 2009. Herpesviral haematopoietic necrosis virus (CyHV2) infection: case studies from commercial goldfish farms. *J. Fish Dis.* 32, 271-278.
- Hanson, L., Dishon, A., Kotler, M., 2011. Herpesviruses that infect fish. *Viruses* 3, 2160-2191.
- Hedrick, R.P., Waltzek, T.B., McDowell, T.S., 2006. Susceptibility of koi carp, common carp, goldfish, and goldfish x common carp hybrids to cyprinid herpesvirus-2 and herpesvirus-3. *J. Aquat. Anim. Health* 18, 26-34.
- Ito, T., Kurita, J., Ozaki, A., Sano, M., Fukuda, H., Ototake, M., 2013. Growth of cyprinid herpesvirus 2 (CyHV2) in cell culture and experimental infection of goldfish *Carassius auratus*. *Dis. Aquat. Org.* 105, 193-202.
- Jeffery, K.R., Bateman, K., Bayley, A., Feist, S.W., Hlland, J., Longshaw, C., Stone, D., Woolford, G., Way, K., 2007. Isolation of a cyprinid herpesvirus 2 from goldfish, *Carassius auratus* (L.), in the UK. *J. Fish Dis.* 30:649-656.
- Jung, S.J., Miyazaki, T., 1995. Herpesviral haematopoietic necrosis of goldfish, *Carassius auratus* (L.). *J. Fish Dis.* 18, 211-220.
- Kurita, J., Nakajima, K., 2012. Megalocytiviruses. *Viruses*. 4, 521-538.
- Lancaster, M.J., Williamson, M.M., Schroen, C.J., 2003. Iridovirus-associated mortality in farmed Murray cod (*Maccullochella peelii peelii*). *Aust. Vet. J.* 81, 633-634.
- Ling, K.H., Lim, L.Y., 2005. The status of ornamental fish industry in Singapore. *Singap. J. Prim. Ind.* 32, 59-69.
- Lintermans, M., 2004. Human-assisted dispersal of alien freshwater fish in Australia. *N.Z. J. Mar. Freshw. Res.* 38, 481-501.
- Luo, Y.Z., Lin, L., Liu, Y., Wu, Z.X., Gu, Z.M., Li, L.J., Yuan, J.F., 2013. Haematopoietic necrosis of

- cultured Prussian carp, *Carassius gibelio* (Bloch), associated with *Cyprinid herpesvirus 2*. J. Fish Dis. DOI: 10.1111/jfd.12110
- OIE, 2009. Manual of diagnostic tests for aquatic animals. Office international des epizooties, Paris France, p 17.
- OIE, 2012. Manual of diagnostic tests for aquatic animals. (Online) www.oie.int, Paris, France.
- O'Sullivan, D., Clark, E., Morison, J., 2008. The Australian ornamental fish industry in 2006/07. Fisheries Research and Development Corporation, Department of Agriculture, Fisheries & Forestry, Dosaqua Pty Ltd and EconSearch Pty Ltd.
- Peeler, E.J., Feist, S.W., 2011. Human intervention in freshwater ecosystems drives disease emergence. *Freshw. Biol.* 56, 705-716.
- Rimmer, A.E., Becker, J.A., Tweedie, A., Whittington, R.J., 2012. Development of a quantitative polymerase chain reaction (qPCR) assay for the detection of dwarf gourami iridovirus (DGIV) and other megalocytiviruses and comparison with the Office International des Epizooties (OIE) reference PCR protocol. *Aquaculture*. 358, 155-163.
- Sadler, J., Marecaux, E., Goodwin, A.E., 2008. Detection of koi herpes virus (CyHV-3) in goldfish, *Carassius auratus* (L.), exposed to infected koi. *J. Fish Dis.* 31, 71-72.
- Sano, M., Nakai, T., Fijan, N., 2011. Viral diseases and agents of warmwater fish. In: Woo, P.T.K., Bruno, D.W. (Eds.), *Fish Diseases and Disorders*, Volume 3, 2nd edition. CABI, Wallingford, pp. 166-244.
- Sergeant, E.S.G., 2013. Epitools epidemiological calculators. AusVet Animal Health Services and Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease. Available at: <http://epitools.ausvet.com.au>.
- Stephens, F.J., Raidal, S.R., Jones, B., 2004. Haematopoietic necrosis in a goldfish (*Carassius auratus*) associated with an agent morphologically similar to herpesvirus. *Aust. Vet. J.* 82, 167-169.
- Trust, T.J., Khouri, A.G., Austen, R.A., Ashburner, L.D., 1980. First isolation in Australia of atypical

Aeromonas salmonicida. FEMS Microbiol. Lett. 9, 39-42.

Waltzek, T.B., Kurobe, T., Goodwin, A.E., Hedrick, R. P. 2009. Development of a polymerase chain reaction assay to detect cyprinid herpesvirus 2 in goldfish. J. Aquat. Anim. Health 21:60-67.

Whittington, R.J., Chong, R., 2007. Global trade in ornamental fish from an Australian perspective: the case for revised import risk analysis and management strategies. Prev. Vet. Med. 81, 92-116.

Whittington, R.J., Gudkovs, N., Carrigan, M.J., Ashburner, L.D., Thurstan, S.J., 1987. Clinical, microbiological and epidemiological findings in recent outbreaks of goldfish ulcer disease due to atypical *Aeromonas salmonicida* in south-eastern Australia. J. Fish Dis. 10, 353-362.

Whittington, R., Tweedie, A., Dennis, M., Becker, J., Landos, M., 2009. Optimisation of PCR tests for diagnosis of megalocytivirus (gourami iridovirus) and cyprinid herpesvirus 2 (goldfish herpesvirus), FRDC final report; no. 2007/007. University of Sydney and Fisheries Research Development Corporation.

Table 1. Description of the primers used in this study to detect CyHV2.

Oligonucleotide		Product size (base pairs)	Gene	Reference
Name	Sequence (5' – 3')			
C1153	GTCGGTTGGACTCGGTTTGT	121	DNAPol ¹	Whittington, et al., 2009
C1154	CATAGTACGCCTTGAGACTC			
C1109	CCCAGCAACATGTGCGACGG	401	DNAPol ¹	Whittington, et al., 2009
C1158	GACCAAGTAGTCAGATGCTG			
C1281	GGA CTTGCGAAGAGTTTGATTTCTAC	366	Helicase	Waltzek, et al., 2009
C1282	CCATAGTCACCATCGTCTCATC			

¹DNAPol = DNA polymerase

Table 2. CyHV2 prevalence in goldfish (*Carassius auratus*) collected from domestic aquaculture producers.

Farm ID and Location	Farm Description	Date Collected	Mean weight \pm SE (g)	Mean total length \pm SE (mm)	Proportion of qPCR positive fish	True prevalence (95% confidence interval)	qPCR copy number ^a
1 Victoria	150 (ca) earthen ponds; each pond 0.25 to 0.3 ha	June 2010	8.3 \pm 0.8	69.2 \pm 2.1	4/150	3.0 (1.2-7.4)	1.02 x 10 ³ (2.31 x 10 ³)
		May 2011	10.2 \pm 1.2	68.7 \pm 2.7	4/150	3.0 (1.2-7.4)	4.10 x 10 ³ (3.68 x 10 ³)
2 Northern New South Wales	60 outdoor concrete tanks; each tank 20 kL	May 2010	6.6 \pm 1.0	62.3 \pm 1.9	0/151	0 (0-2.8)	
		August 2010	4.1 \pm 0.7 ^a	67.5 \pm 4.6 ^b	0/158	0 (0-2.6)	
		January 2011	1.6 \pm 0.1	48.7 \pm 0.9	0/150	0 (0-2.8)	
		June 2011	5.0 \pm 0.7	63.3 \pm 1.5	0/150	0 (0-2.8)	
3 Central Coast New South Wales	3 earthen ponds; each pond 0.2 ha; fish held in cages in ponds	October 2010	3.4 \pm 0.4	51.8 \pm 2.1	10/167	6.7 (3.6-11.9)	4.68 x 10 ³ (3.12 x 10 ⁵)
		April 2011	3.0 \pm 0.1	53.2 \pm 0.8	86/224	42.7 (35.9-50)	1.14 x 10 ³ (1.21 x 10 ⁴)
		February 2012	4.7 \pm 0.2	63.9 \pm 1.0	50/150	37 (29.2-45.8)	1.85 x 10 ³ (1.25 x 10 ⁴)
4 South East Queensland	12 earthen ponds; each pond 0.1 ha	June 2011	3.7 \pm 0.3	50.3 \pm 1.7	0/124	0 (0-3.3)	

^a for positive test results, represents median copy number per mg of fish tissue (interquartile range)

^b weight and length measurements were recorded for first 15 fish sampled

Table 3. CyHV2 prevalence in goldfish (*Carassius auratus*) collected from wild populations.

Location	Date collected	Mean weight ± SE (g)	Mean total length ± SE (mm)	Proportion of qPCR positive fish	True prevalence (95% confidence interval)	qPCR copy number ^a
Cotter Reservoir, ACT	27-28 October 2010	29.1 ± 3.1	79.8 ± 3.2	9/146	6.8 (3.6-12.6)	1.16 × 10 ² (1.04 × 10 ³)
	1-2 March 2011	26.4 ± 2.4	105.3 ± 2.8	14/198	7.9 (4.7-12.8)	1.58 × 10 ² (2.24 × 10 ²)
Ovens River, VIC	January 2011	148 ± 19.8	207.3 ± 15.0	0/8	0 (0-36.0)	
	April 2011	38.9 ± 9.7	110.9 ± 9.1	15/41	40.7 (26.2-57.6)	1.53 × 10 ³ (1.49 × 10 ⁴)
	October 2011	65.2 ± 8.0	147.8 ± 7.5	1/15	7.4 (0.4-33.1)	1.45 × 10 ²
Murray River, VIC	June 2011	26.7 ± 5.4	87.9 ± 3.0	8/75	11.9 (6.1-21.8)	1.53 × 10 ⁶ (3.41 × 10 ⁶)
Dartmouth, 8 Mile Creek, VIC	Feb 2011	17.5 ± 3.0	100.3 ± 4.8	0/4	0 (0-54.4)	
Nagambie/Murchison Region, VIC	Feb 2011	9.9 ± 3.2	67.8 ± 7.0	0/19	0 (0-18.7)	
Hughes Creek, VIC	March 2011	33.4 ± 10.4	121.0 ± 10.3	0/5	0 (0-48.3)	
Lance Creek, VIC	April 2011	24.0 ± 3.4	110.3 ± 5.1	0/7	0 (0-39.4)	
Buffalo River, VIC	April 2011	22.9 ± 1.9	105.0 ± 5.0	0/2	0 (0-73.1)	
VIC	unknown	120.0 ± 13.5	182.0 ± 8.4	0/13	0 (0-25.3)	

^a for positive test results, represents median copy number per mg of fish tissue (interquartile range)

Table 4. CyHV2 prevalence for sick or dead goldfish (*Carassius auratus*) collected from retail outlets.

Retail Outlet ID	Date Collected	Location of retailer	Origin of fish ^a	Proportion of PCR positive fish	True prevalence (95% confidence interval)	qPCR copy number ^b
1	30 July 2010	Northern NSW	Melbourne	30/30	100 (98.8-100)	2.09 x 10 ⁷ (4.66 x 10 ⁷)
1	13 August 2010	Northern NSW	Melbourne	29/29	100 (98.1-100)	2.01 x 10 ⁷ (3.13 x 10 ⁷)
1	30 July 2010	Northern NSW	Melbourne	29/30	100 (92.6-100)	3.94 x 10 ⁶ (1.39 x 10 ⁷)
1	5 July 2010	Northern NSW	Unknown	3/5	66.7 (25.6-98.0)	1.19 x 10 ⁷ (3.84 x 10 ⁷)
2	16 July 2010	Northern NSW	Melbourne and Brisbane	1/2	55.6 (2.8-100)	2.78 x 10 ⁷
3	4 November 2010	Northern NSW	Domestic	1/2	55.6 (2.8-100)	not quantifiable ^c
3	30 July 2010	Northern NSW	Unknown	0/9	0 (0-33.2)	
3	13 August 2010	Northern NSW	Unknown	0/1	0 (0-100)	
3	4 November 2010	Northern NSW	Melbourne	0/1	0 (0-100)	
3	4 November 2010	Northern NSW	Melbourne	0/1	0 (0-100)	

^a origin was provided by the retail outlet owner or manager and could not be verified

^b for positive test results, represents median copy number per mg of fish tissue (interquartile range)

^c both replicates tested positive in the qPCR with Ct values outside the standard curve

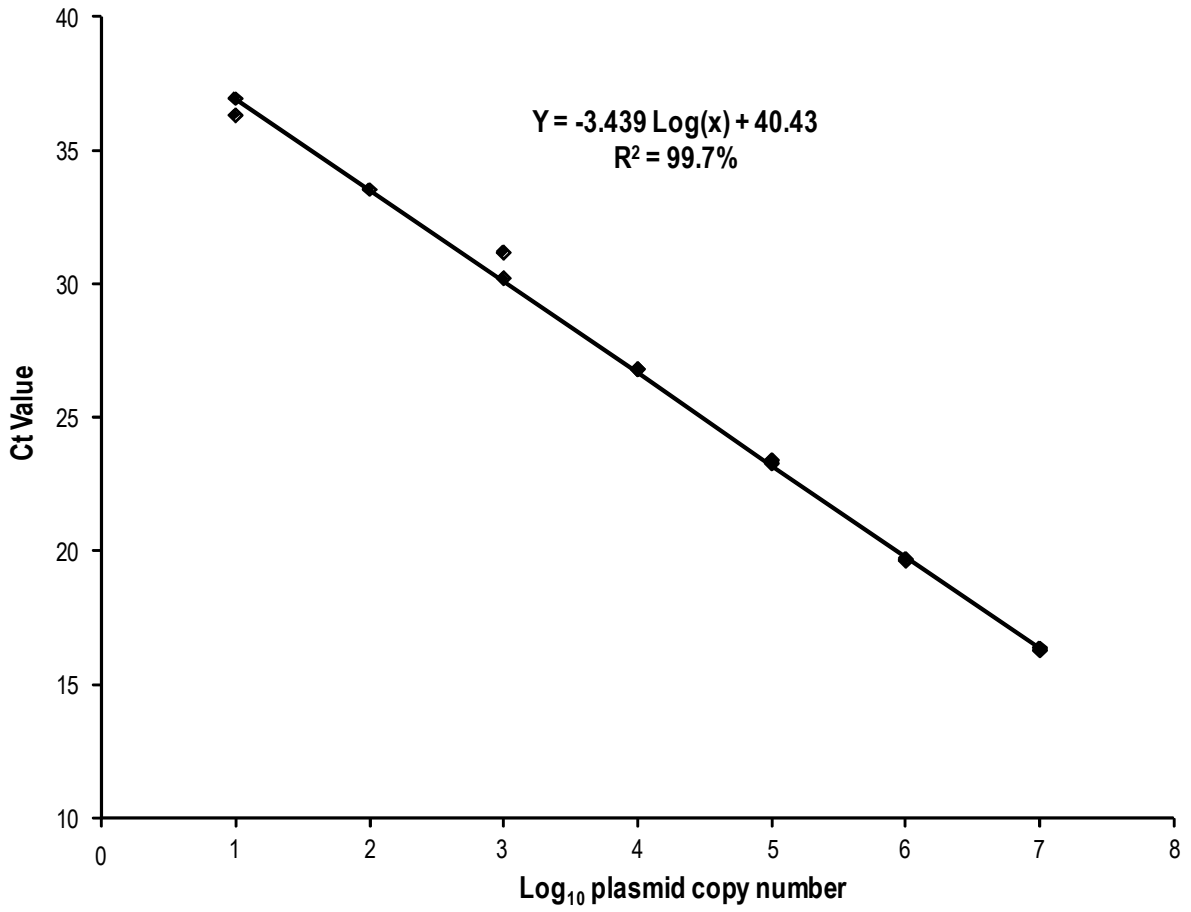


Figure 1. Indicative standard curve showing the threshold cycle value (Ct) for a serial dilution of the plasmid control *pCyHV-2-DNApol* that was used for viral load quantification.

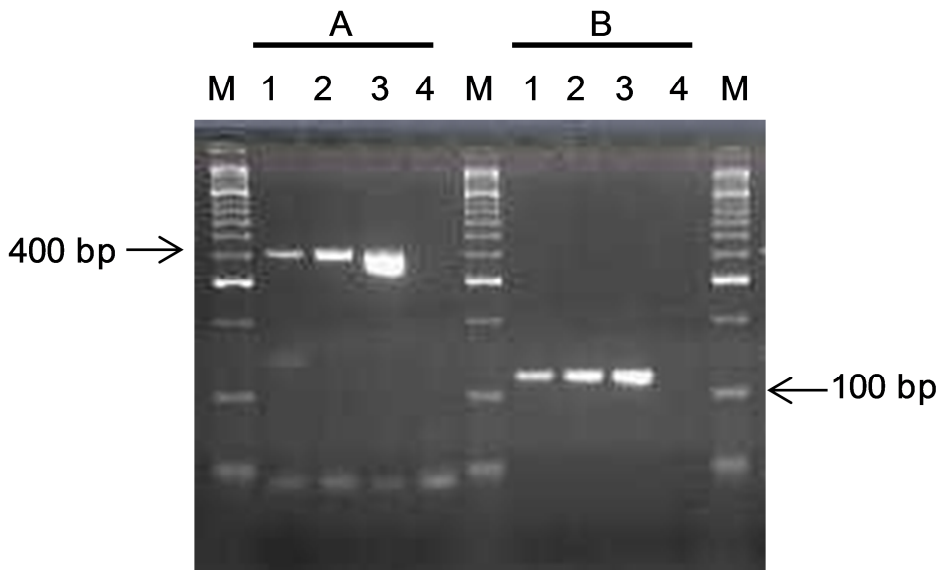


Figure 2. Agarose gel electrophoresis of CyHV2 DNA in tissues of a positive goldfish collected from Farm 1 (1), molecular weight marker (M), positive control DNA (2), *pCyHV-2-DNApol* plasmid DNA (3) and no template control (4). Amplicons are of (A) 401 base pairs (bp) and (B) 121 bp for the DNApol gene (See Table 1 for primer details).

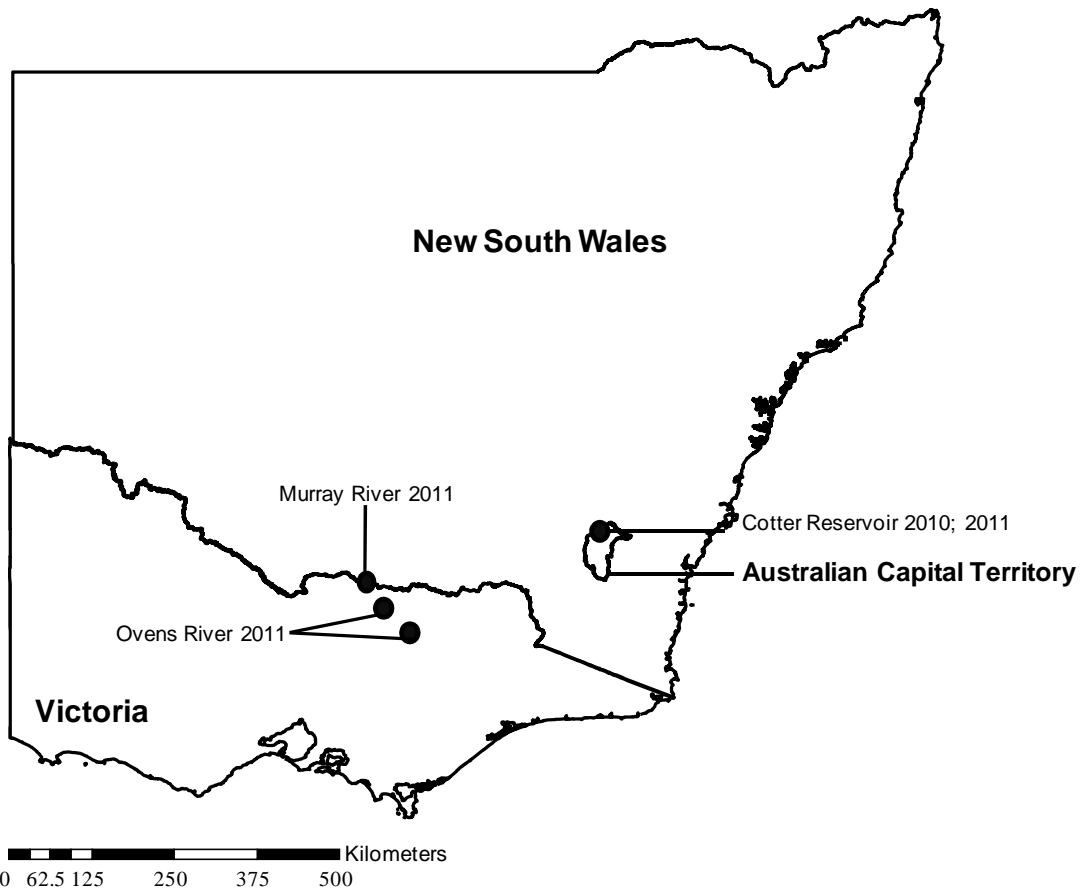


Figure 3. Collection sites of wild goldfish (*Carassius auratus*) in 2010 and 2011 with positive detection of CyHV2.