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2014/001 Aquatic Animal Health Subprogram: Strategic approaches to identifying pathogens of quarantine concern associated with the importation of ornamental fish

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

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Executive Summary

This project was developed by The University of Sydney in collaboration with James Cook University and the Centre of Excellence for Biosecurity Risk Analysis. Prior research showed that despite the biosecurity measures in place since 2000, there have been several incidents of exotic pathogens from ornamental fish affecting wild and farmed fish populations in Australia. There is a need to acquire new knowledge to support policy reform as the ornamental fish industry advances and new pathogens emerge. During 2015, a cross sectional survey was completed for the nationally and internationally significant pathogens carried by ornamental fish entering Australia. The report contains the first comprehensive survey of the parasite assemblages affecting imported ornamental fish under quarantine. The research findings will be used to refine biosecurity policy to support safe trade and protect Australia's natural resources and its fisheries and aquaculture industries, including the developing local ornamental fish breeding sector. The project was funded by the Australian Government Department of Agriculture and Water Resources, through the Fisheries Research and Development Corporation.

Background

Imported ornamental fish are known to be subclinical carriers of exotic pathogens of biosecurity concern. Nearly 18 million ornamental fish are imported annually under a policy based on an Import Risk Analysis published in 1999. The potential for these aquatic animals to be released into the environment presents additional risk to Australia's ecosystems. Determination of the range of infectious agents carried by imported fish is required so that appropriate regulations can be put in place to manage the risk. In recent years, there has been particular interest in the risk associated with imported ornamental fish infected with iridoviruses, specifically those belonging to the genus *Megalocytivirus*.

This project was developed from the need to acquire new knowledge to support policy reform as the ornamental fish industry advances and new pathogens emerge. There is little information available on viral and bacterial disease agents carried by imported ornamental fish. Additionally, there is a paucity of information available on parasites of imported fish and their potential risk to Australian industries and ecosystems. There is a need to determine whether pathogens of biosecurity significance associated with ornamental fish are entering Australia despite the import conditions.

Objectives

1. Determine if pathogens of potential biosecurity concern on the national list are entering QAPs in Australia through the trade in ornamental fish

2. Determine if current import conditions for goldfish requiring freedom from specific pathogens are being met

3. Document parasites of potential biosecurity concern that are associated with imported ornamental fish

4. Develop efficient pooled sample strategies for testing imported fish

Methodology

A repeated cross sectional survey was conducted to examine imported ornamental fish under quarantine for the presence of nationally listed aquatic pathogens that are associated with at least one ornamental fish host. All fish collected were considered pre-import and under quarantine at the time of testing. We assessed whether or not the existing import conditions for goldfish requiring sourcing from populations free from specific diseases were being met, and identified parasites of potential biosecurity significance. To support surveillance testing for freedom from infection with megalocytiviruses (MCV), diagnostic sensitivity was examined using clinically-relevant fish tissue homogenates.

Results

Australia's biosecurity policy framework for the importation of ornamental fish was being met for the OIE-listed pathogens koi herpesvirus (KHV), spring viraemia of carp virus (SVCV) and viral haemorrhagic septicaemia virus (VHS) as there was no evidence of subclinically infected fish being imported to Australia. Similarly, there was no evidence of the bacteria, *Edwardsiella ictaluri* and *Aeromonas salmonicida* which are listed on national list of aquatic pathogens.

Australia's biosecurity policy framework for the importation of ornamental fish was not being met for the nationally listed MCV and Nervous necrosis virus (NNV). MCV was detected in more than half of the ornamental fish populations tested during this project. This included five of nine marine species and all freshwater species except Siamese fighting fish that were tested. MCV-positive fish were detected in fish received from eight different exporters from Indonesia, Malaysia, Singapore, Sri Lanka and Thailand. Sequence analysis determined that all populations were infected with the megalocytivirus identified as ISKNV-like. NNV was detected in 3 populations (13%) of marine ornamental fish which were all exported from Indonesia.

No parasites (e.g. *Gyrodactylus salaris and Myxobolus cerebralis*) were detected that are listed on Australia's National List of Reportable Diseases of Aquatic Animals. Current import conditions for Goldfish, which had been treated with an effective parasiticide to eliminate infestation by the gill flukes *Dactylogyrus vastator* and *D. extensus* are not being met. *Dactylogyrus vastator* was detected in three populations of Goldfish received from the same

exporter in Thailand on two separate occasions. Further, six other dactylogyrid monogenean species (but not *D. extensus*) and one *Gyrodactylus* were detected in imported Goldfish.

Protozoan or metazoan parasites were detected in all populations of fish (33/33) examined. We found that significant parasitic disease agents including ciliates (e.g. *Trichodina*), myxozoans (e.g. *Ceratomyxa*, *Kudoa* and *Myxobolus* spp.), helminths (primarily larval trematode metacercariae of the family Heterophyidae) and a crustacean (e.g. *Argulus japonicus*, a known exotic parasite) infected freshwater and marine imported ornamental fish.

The diagnostic sensitivity of the qPCR assay to detect MCV was substantially reduced when testing fish tissues in pools of five or 10 compared to individual testing. The sensitivity for a single positive sample in a pool of five was 68% (95% CI, 55-80%). The sensitivity was further reduced for one positive in a pool of 10, being equal to 63% (95% CI, 51-74). In addition to prevalence of infection in the source population, pooled sensitivity was strongly influenced by the pathobiology of the infection. For example, the nominal surveillance sensitivity was achieved by testing 40 pools of five (200 fish, 40 tests) or 26 pools of ten (260 fish, 26 tests) instead of 150 tests on individuals for a population of Dwarf gourami (*Trichogaster lalius*) with subclinical infection. However, this surveillance sensitivity (95% confident of detecting 2% prevalence assuming a perfect test) could not be achieved when using pooled samples for a population of Kissing gourami (*H. temminckii*) also with subclinical infection.

Implications for relevant stakeholders

All of the freshwater species that were positive for MCV fall under the revised biosecurity policy framework requiring batch testing for freedom from infection. However, none of the marine fish species that were MCV-positive require batch testing under existing policy.

The risk imported ornamental fish present to Australian aquatic animal industries and natural resources is high with respect to parasitic agents. The recovery from Goldfish of numerous *Dactylogyrus* species indicates the pre-export health requirement for a parasiticide treatment was not effective at eliminating monogeneans. The project results show that freshwater and marine ornamental fish arrive with a diversity of external and internal parasite fauna. Host specificity is a significant component of parasite ecology and determining if the parasite can become established in a new environment and on novel fish hosts.

There was a reduction in diagnostic sensitivity for MCV when fish tissue homogenates were pooled for testing. However, pooling strategies were effective for maintaining surveillance sensitivity when the prevalence of MCV infection exceeded 10%. In some cases the lower pooled diagnostic sensitivity was compatible with the desired surveillance sensitivity if the sample size was increased above 150 yet fewer assays were required. This study demonstrated that the pathobiology of MCV infection influenced pooled diagnostic sensitivity, thus the applicability of pooled testing must be considered on a case-by-case basis.

Recommendations

1. Revision of biosecurity policy to prevent incursions of *Megalocytivirus* virus from marine ornamental fish
2. Revision of biosecurity policy to require the application of an effective parasite treatment of all ornamental fish being exported to Australia.
3. Revision of biosecurity policy to require health certification to be free of the parasitic agents, *Argulus japonicus* and *Lernaea cyprinacea*.
4. From the project findings, visual inspection of consignments was not effective at detecting misidentified fish species and did not prevent importation of live parasite fauna. Revision of biosecurity policy to include import conditions that state a maximum number of fish that can be transported in a bag based on fish species and size.
5. Pooled testing cannot be applied empirically for surveillance using PCR assays. Factors additional to prevalence and viral load affect the pooled diagnostic sensitivity and need to be better understood.

Keywords

disease freedom, biosecurity, exotic pathogens, infectious spleen, kidney and necrosis virus, Megalocytivirus, nervous necrosis virus, Koi herpesvirus, spring viraemia of carp virus, viral haemorrhagic septicaemia virus, *Aeromonas salmonicida, Edwardsiella ictaluri*, *Dactylogyrus vastator, Argulus japonicas, Gyrodactylus, Myxobolus, Centrocestus*, ornamental fish, Goldfish, *Carassius auratus*, Blue gourami, *Trichopodus trichopterus*, Dwarf gourami, *Trichogaster lalius*, Rosy barb, *Pethia conchonius*, Guppy, Banggai cardinalfish, *Pterapogon kauderni*, Clownfish, *Amphiprion* spp.

Introduction

Imported ornamental fish are known to be subclinical carriers of exotic pathogens of biosecurity concern. Nearly 18 million ornamental fish are imported annually under a policy based on an Import Risk Analysis published in 1999. Despite the biosecurity measures in place since 2000, there have been several incidents of exotic pathogens from ornamental fish affecting wild and farmed fish populations. These include atypical *Aeromonas salmonicida* (Whittington et al., 1987), *Cyprinid herpesvirus 2* (CyHV2) (Becker et al., 2014), *Edwardsiella ictaluri* (Lymbery et al., 2016) and dwarf gourami iridovirues (DGIV) (Lancaster et al., 2003). This project was developed from the need to acquire new knowledge to support policy reform as the ornamental fish industry advances and new pathogens emerge.

In recent years, there has been a particular interest in the risk associated with imported ornamental fish infected with iridoviruses, specifically those belonging to the genus *Megalocytivirus* (MCV). Prior research funded by the FRDC (Project FRDC 2009/044: AAH: surveys of ornamental fish for pathogens of quarantine significance) showed that infections with MCV were detected in ornamental fish at all steps in the supply chain. This included imported fish prior to and following quarantine release, moribund fish from retail outlets and subclinical platy collected from a domestic farm with recirculating systems (Rimmer et al., 2015). The major outcome from Project FRDC 2009/044 was that the pre-export biosecurity measures (and associated health certification provided by exporting country authorities) and post-arrival quarantining of fish in Australia were insufficient to detect and prevent fish with subclinical infections of exotic pathogens from entering Australia.

In 2014, the Australian Department of Agriculture and Water Resources (ADA&WR) (2014b) published a risk assessment stating the current import controls for the importation of freshwater fish belonging to the cichlid, gourami and poeciliid families did not meet Australia's appropriate level of protection. From 1 March 2016, health certification demonstrating freedom from infection has been required to import fish belonging to the families mentioned above (Australian Department of Agriculture, 2014a). Furthermore, ADA&WR is completing a trial for the proposed changes to the way it manages the disease risks associated with imported ornamental fish. The proposed changes include the removal of quarantine and the introduction of on-arrival health surveillance (Australian Department of Agriculture, 2012).

There is little information available on other viral, bacterial and parasitic disease agents carried by imported ornamental fish. Specifically, there is a paucity of information available on parasites of imported fish and their potential risk to Australian industries and ecosystems. There is a need to determine whether pathogens of biosecurity significance associated with ornamental fish are entering Australia despite the import conditions.

This project addressed a priority identified by the FRDC through the annual competitive round for the Aquatic Animal Health Subprogram. The priority was to conduct research to underpin disease risk minimization for imported ornamental fish and associated risks. There is little information available on disease agents carried by imported live ornamental fish. Further, the potential for these aquatic animals to be released into the environment, presents additional risk. Determination of the range of infectious agents carried by imported fish was required so that appropriate regulations can be put in place to manage the risk. The current project is the based on previous projects funded by the FRDC (FRDC 2007/007 and FRDC 2009/044), all aimed at identifying exotic aquatic pathogens of potential biosecurity concern from imported ornamental fish. This project will provide new knowledge to support improved regulation of imported commodities leading to facilitation of safe trade and enhanced biosecurity for Australia's aquatic animal industries, the developing domestic ornamental fish breeding industry and Australia's natural resources.

Objectives

1. Determine if pathogens of potential biosecurity concern on the national list are entering QAPs in Australia through the trade in ornamental fish

2. Determine if current import conditions for goldfish requiring freedom from specific pathogens are being met

3. Document parasites of potential biosecurity concern that are associated with imported ornamental fish

4. Develop efficient pooled sample strategies for testing imported fish

Method

The main research strategy was to conduct a repeated cross sectional survey examining imported ornamental fish under quarantine for the presence of nationally listed aquatic pathogens that are associated with at least one ornamental fish host (Objective 1). The summary of the notable aquatic pathogens investigated in this project is presented in Table 1. Further, we assessed if the existing import conditions for goldfish requiring sourcing from populations free from specific diseases were being met (Objective 2), and identified parasites of potential biosecurity significance (Objective 3). To support surveillance testing for freedom from infection with megalocytiviruses, diagnostic sensitivity was examined using clinically-relevant fish tissue homogenates (Objective 4). The results provide new information that can be used to support the proposed imported ornamental fish health monitoring system that is central to the Department of Agriculture and Water Resources' planned reforms.

Fish collection

Fish hosts were prioritized based on prior knowledge of potential for infection with the listed pathogens, volumes/regularity of importation to Australia and current import conditions, in consultation with Department of Agriculture and Water Resources. For budgetary efficiency, individual fish were tested for pathogens of potential biosecurity concern for which the selected fish species is a potential host (Table 2).

Sample collection was purposive, in that whole consignments were specifically acquired from overseas exporters for the project, and the samples were a census of that consignment. The sources of fish were those suppliers that agreed to lower their minimum order size, in keeping with methods for sample acquisition developed in FRDC project 2009/044." At present, an importer with an Approved Arrangements (AA, formerly a quarantine approved premise, QAP) can import from many possible sources, many of which aggregate fish from a range of non-disclosed primary sources, and these sources may change over time. Therefore, it was not possible to predict whether any imported consignments into any AA are representative of all possible sources. However, the sources of consignments were reported to country level to provide some geographic reference (Table 2). On arrival to Australia, all fish were subjected to standard quarantine practices which involved clearance from customs and visual inspection and approval from quarantine services. Further, as part of the import conditions required by

the Department of Agriculture and Water Resources, all fish were euthanized within 12 hours of arriving at the AA laboratory at the University of Sydney. This time restriction impacted the species available for parasitology as the fish needed be processed as freshly killed specimens.

From the project application, it was intended that a maximum of three consignments from each of six species of ornamental fish would be imported directly to the AA laboratory for testing (Appendix 3 Table 1). However, there were considerable difficulties in meeting the minimum order size from overseas exporters, especially for marine species. Exporters would not agree to sell us fish unless we met their minimum number of boxes.

To accommodate the minimum size order and to maximize the diversity of fish species available for parasitology within the 12 hour time limit, we completed three large sampling events in January, May and October 2015. Each sampling event was approximately two weeks in duration (Appendix 3 Table 2).

More than sixty ornamental fish exporters were contacted by email. We were able to meet the minimum order size for our project needs from nine exporters representing five different countries. Also, there was a difficulty in securing consignments of clownfish that were allowed to be exported to Australia. As mandated in the import permit, all exported marine fish are required to be certified as wild collected and not to be bred or hatched on a farm or other premises. Thus, we tested other species of fish within the *Amphiprion* genus that were allowed to be exported.

Marine fish are transported individually in approximately 350 mL of water, whereas freshwater fish are sent in larger bags of 40 to 200 individuals depending species and size. Fish speciation based on visual inspection by an expert fish biologist (JCU) was completed on two consignments of marine fish received from the exporter identified as Indonesia-2. Fish were purchased and labelled for export as *Apogon pillionatus*, *Apogon* spp. and *A. leptacanthus.* However, they were all identified as *Cheilodipterus quinquelineatus* (Table 2).

Sample size

A consignment was defined as a shipment of ornamental fish received from an exporter on a specific day. A population was defined as a single fish species received from an exporter on a

specific day. Where possible, freshwater species were tested for viral pathogens to detect a minimum of 2% prevalence with 95% confidence assuming a test of 100% sensitivity and specificity. In general, this required testing at least 150 individual fish from each population. Marine species were tested for viral pathogens to detect a minimum of 10%, which required a sample size of 30. The minimum detection prevalence for bacterial and parasitic agents was set at 10% with a sample size of at least 30. For a species ordered in excess of the required sample size, a simple random selection was used to choose the individual fish for testing. As there is uncertainty as to the diagnostic sensitivity and specificity of the assays, each was assumed to be 100%. The proportion of test positive fish was calculated with 95% confidence limits using the exact binomial approximation. When there were no positive tests, the proportion was calculated with a one-sided upper 97.5% confidence limit.

Objective 1. Determining if pathogens of potential biosecurity concern on the national list are entering QAPs in Australia through the trade in ornamental fish

Tissue sample collection for viral pathogens

After euthanasia, fish were kept frozen at -80°C until time of processing. Fish were processed in batches to maximize efficiency of automated laboratory processes. The target tissue was dissected from each fish using sterile techniques (Table 1). The tissue was homogenized and clarified by the Fastprep (bead beating) technique followed by centrifugation as described by Rimmer et al. (2012). For each sample, 1/10 clarified homogenates and excess fish tissue (where available) were placed in the -80°C archive for future retrospective testing.

Nucleic acid purification

Total nucleic acids were purified from 50 µl of the clarified tissue homogenates using the MagMax-96 Viral RNA Isolation Kit (ThermoFisher). The procedure was conducted according to directions for the AM1836 standard deep-well program on a MagMax Express magnetic particle processor (ThermoFisher). The positive and negative control samples described below were included on each extraction plate. Nucleic acids were eluted in a 75 µl volume and used immediately or stored at -80°C.

Molecular tests for viral pathogens

Real-time quantitative PCR (qPCR) assays for DNA viruses and combined reverse transcriptase quantitative PCR (RT-qPCR) for RNA viruses were used as sensitive and specific screening tests. Previously described assays were selected according to the available validation data and applicability for the range of species and genotypes of the relevant pathogens (Appendix 3 Table 3). The methods described in the OIE Manual of Diagnostic Tests were reserved for confirmatory testing if a suitable high throughput real-time PCR was not described.

Real-time quantitative PCR assays

An Mx3000P real-time PCR machine (Stratagene) was used with several common aspects in the methods for the five assays described below. Tests were initially conducted on pools of 4 DNA samples and a negative result for the pool was interpreted as negative result for each sample. Samples that contributed to a positive pool were tested individually with a quantitative standard to identify which samples were positive and to estimate viral load. All samples were tested in duplicate reactions prepared with 5 µl of undiluted nucleic acid template in a total reaction volume of 25 µl.

Fluorescence data was analyzed with the MxPro Software (Version 4, Stratagene). Signals from the FAM or SYBR reporter dyes were normalized to the ROX passive reference dye. A fluorescence threshold was assigned using the amplification-based threshold algorithm for the positive control and quantitative standard samples. Threshold cycle (Ct) values were assigned as the fractional cycle number during which the fluorescence first exceeded the threshold. A post-amplification dissociation curve was performed for the SYBR assays and a melting temperature (Tm) was assigned if there was a single distinct peak when the negative first derivative of fluorescence was plotted against the temperature. The absence of a Ct value for experimental samples was interpreted as a negative result. Amplification curves were visualized and a positive result was assigned if a Ct value was associated with an exponential increase in fluorescence signal, and if there was an appropriate Tm for the SYBR assays. A positive result for the screening assays was assigned for a sample if either replicate produced a positive result; Ct cut-off values were not applied.

For each PCR run (plate of samples), the following negative control samples were generated and tested: (i) homogenization control, homogenizing medium without tissue processed

concurrently with the tissue samples; (ii) extraction control, nuclease free water used as the sample in nucleic acid purification; (iii) no template control, nucleic acid free water used as template in the qPCR. Assay specific positive control samples were used at two dilutions, close to the limit of detection of the assays. Generally, a qPCR run was considered valid if a negative result was obtained for all negative control samples and if the Ct values for the positive control dilutions was within 2 standard deviations of previous results.

Quantitative plasmid standards were used on runs used to identify individual positive samples. A 10-fold dilution series containing $1 \times 10^1 - 1 \times 10^7$ copies of the plasmid standard was tested in duplicate and a standard curve was generated by plotting the Ct value against the template quantity. The number of copies of the viral genome in experimental samples was estimated by interpolation within the range of the standard curve with $r^2>0.95$ and efficiency between 90-110%.

Specific details of the assays for five pathogens of interest were as follows:

Megalocytivirus

Tests for megalocytiviruses were performed according to the protocol described by Rimmer, et al. (2012). This assay uses SYBR Green detection chemistry to target a conserved region of the major capsid protein (MCP) gene, and detected multiple genotypes of ISKNV and RSIV. Reactions were prepared with the QuantiTect SYBR Green PCR Kit (Qiagen) with the primers described in Appendix 3 Table 3. Thermocycling was according to the following protocol: 95°C for 15min (hot start activation); 40 cycles of 95°C for 30s (denaturation), 62°C for 30s (annealing), 72°C for 30s (extension); followed by a dissociation curve. Samples with a Ct value were considered positive only if the Tm was in the range 84 – 86°C. The positive control for this assay was a dilution of total nucleic acids from a fish infected with DGIV genotype of ISKNV. The plasmid standard pDGIV-MCP1, which contained a 694 nucleotide portion of MCP gene sequence, was used for quantification. As this assay could detect a wide range of megalocytiviruses, the nucleotide sequence analysis described below was used to further characterize positive samples.

Koi herpesvirus

A qPCR assay previously developed for detection of KHV with SYBR Green detection chemistry was used (Whittington et al., 2009). This assay amplified a portion of the DNA polymerase gene and was specific for Cyprinid herpesvirus 3 (KHV). Reactions were prepared with the Quantitect SYBR Green PCR kit (Qiagen) and the primers described in Appendix 3 Table 3. Thermocycling was according to the following protocol: 95°C for 15min (hot start activation); 40 cycles of 95°C for 30s (denaturation), 62°C for 30s (annealing), 72°C for 30s (extension); followed by a dissociation curve. Positive control material was a purified viral DNA preparation provided by Professor Ron Hedrick (USA).

Spring viraemia of carp virus

Tests for SVCV used the RT-qPCR assay described by Zhang, et al. (2009). This assay was specific for Genogroup I of SVCV with hydrolysis probe detection chemistry for a target in the G gene. Reactions were prepared with AgPath-ID One-Step RT-PCR kit (Applied Biosystems) with the primers and probe described in Appendix 3 Table 3. Thermocycling was according to the following protocol: 50°C for 10min (reverse transcription); 95°C for 10min (hotstart activation); 40 cycles of 95°C for 15s and 60°C for 45s (annealing and extension). Positive control material for this assay was supplied as cell culture supernatant infected with Genogroup 1 SVCV in RNA later by Professor Andrew Goodwin (US Fish and Wildlife Service, Pacific Region, Portland Oregon).

Viral hemorrhagic septicaemia virus

Tests for VHSV used the RT-qPCR assay described by Jonstrup, et al. (2013). This assay used hydrolysis probe detection chemistry to detect a target in the N gene and was able to detect all four genotypes of VHSV. Reactions were prepared with AgPath-ID One-Step RT-PCR kit (Applied Biosystems) with the primers and probe described in Appendix 3 Table 3. Thermocycling was according to the following protocol: 50 °C for 10min (reverse transcription); 95°C for 10min (hotstart activation); 40 cycles of 95°C for 15s and 60°C for 45s (annealing and extension). Positive control material was supplied as an ethanol fixed cell culture supernatant infected with VHSV by AFDL (AAHL, CSIRO).

Nervous necrosis virus

A protocol validated for detection of NNV with hydrolysis probe detection chemistry was used according to the method described by Hick and Whittington (2010). This assay targets a conserved region the capsid protein gene (RNA2) and is suitable for four genotypes of NNV. Reactions were prepared using the AgPath-ID One-Step RT-PCR kit (Applied Biosystems) with the primers and probe described in Appendix 3 Table 3. The thermocycling protocol was: 45°C for 10min (reverse transcription); 95°C for 10min (hotstart activation); 40 cycles of 95°C for 15s and 60°C for 45s (annealing, extension and fluorescent data acquisition). The positive control for this assay was viral RNA extracted from a cell culture supernatant infected with the *Red spotted grouper nervous necrosis virus* (RGNNV) genotype. The plasmid standard qR2Tp3.8 which contained the full length capsid protein gene sequence was used for quantification.

Virus sequence analysis

Sequence analysis was used to confirm positive screening test results for MCV and to determine the genotypic identity of the viruses that were detected. Where there were several positive samples within a consignment, the viral sequence was determined for representative samples. Conventional PCR assays were used to amplify the key regions of the MCV genome that are used for genotyping this genus. In some cases where the viral load was very low, the conventional PCR tests were negative and sequencing was not possible. Conventional PCR assays were prepared with the Expand High Fidelity PCR System (Roche) according to directions with a reaction volume 50 µl.

Three regions of sequence were targeted for megalocytivirus: (i) the assay described by the OIE for detection of ISKNV and RSIV which used the primers OIE1-F (CTCAAACACTCTGGCTCATC) and OIE1-R (GCACCAACACATCTCCTATC) to amplify 524 nucleotides within the IRB6 gene (OIE, 2012); (ii) a 520 nucleotide region of sequence from the ATPase gene was generated using the primers ATPase-C84 and ATPase-C85 (Go, et al., 2006); and, (iii) the major capsid protein (MCP) gene product from the qPCR provided 166 base pairs of sequence.

Amplification products were visualized using SYBR Safe gel stain (ThermoFisher) after agarose gel electrophoresis. DNA bands of the appropriate size were purified using the QIAquick Gel Extraction Kit (Qiagen). Conventional sanger sequencing with BigDye Terminator v3.1 Cycle

Sequencing chemistry was performed at Australian Genome Research Facility (AGRF with a reaction primed by each of the primers used in PCR for the respective amplicons. Chromatograms were analyzed and primer sequence was removed, using FinchTV (Geospiza).

Multiple sequence alignment was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/ msa/clustalo/). Phylogenetic analyses were conducted in Mega6 (Tamura, et al., 2013) with representative nucleotide sequences available on Genbank (http://www.ncbi.nlm.nih.gov/genbank/).

Bacteriology

The posterior kidney was sampled using a sterile swab and streaked for isolation on plates as described in Aquavetplan (2014c) and the OIE manual (2009) for *Aeromonas salmonicida* and *Edwardsiella ictaluri*, respectively (Table 1). Tissues were plated onto SBA (blood agar base containing 5–7% (v/v) defibrinated sheep's blood, BD Difco™). Cultures were incubated in air at 22°C and examined every two days for up to 7 days. Two presumptive colonies per fish were sub-cultured onto an agar plate of the same medium used for primary isolation. After incubation at 22°C for 3 days, colonies were harvested into nutrient broth with 10% glycerol and stored at –80 C. Representative colonies were also preserved in 70% ethanol.

Objective 2. Determining if the import conditions for goldfish are being met

Current health certification for freshwater ornamental finfish imported into Australia require that '*all goldfish in the consignment have been treated with an effective parasiticide (e.g. trichlorfon, formaldehyde, sodium chloride) during the 7 days prior to export to Australia to eliminate infestation by the gill flukes Dactylogyrus vastator and D. extensus*.' To determine whether important conditions are being met for parasites, seven populations of Goldfish (fantail and calico varieties) were examined for the monogenean gill flukes *Dactylogyrus vastator* and *D. extensus*. Goldfish were purchased from Singapore (two populations), Thailand (three populations) and Malaysia (two populations) in 2015. Thirty fish were examined per consignment for a total of 210 fish.

Monogenean gill flukes in the genus *Dactylogyrus* are most easily identified by the morphology of their posterior attachment organ, the haptor. The haptor has sclerotized structures called sclerites, hamuli and hooklets which tend to exhibit unique morphology at species level. Gill parasites collected from Goldfish (see collection and sedation methods below) were preserved in molecular grade ethanol and stored in Eppendorf tubes until examination. Parasite specimens were collected from tubes using a micropipette, and individually placed on a microscope slide for dissection. The body of each parasite was carefully separated from the haptor using a 30 G gauge needle and placed in a 1 mL Eppendorf tube for molecular analysis. The haptor was prepared for proteolytic digestion to liberate haptoral armature as per Vaughan and Christison (2012). In brief, all haptors were digested using 5 µL of Proteinase-K (1 mg/L) with ATL buffer added directly to the haptor on a microscope slide using a micro-pipette. The digestion process was monitored and controlled by adding additional Proteinase-K solution heated to 55°C or cool distilled water to inhibit the process and re-hydrate crystals during the procedure. Excess crystals were re-hydrated and removed using paper towelling until only the sclerites and hamuli remained. Thereafter, a small drop of molten glycerine jelly was placed quickly onto an inverted coverslip and slowly lowered onto the liberated sclerites and hamuli. Once the glycerine jelly had hardened the edges of the coverslips were sealed with clear nail varnish.

Hamuli from each individual monogenean were measured to facilitate species identification. Measurements for *Dactylogyrus* spp. hamuli are shown in Figure 1. Monogenean marginal hooklets display different sizes and measurement variations preventing accurate identification, and were not considered (Shinn et al., 2004). Copulatory organs were not measured because the main body was used for molecular analysis. Separate measurements were performed for only one of the two hamuli per specimen to avoid pseudo-replication. Prior to performing individual measurements, each hamulus was photographed and orientated into a superimposed rectangle to eliminate excessive measurement error by using a quadrangular grid reference to repeatedly return the same measurement points of origin between specimens as per Vaughan and Christison (2012).

Objective 3. Documenting parasite assemblages of imported ornamental fish

Imported fish species were examined for protozoan and metazoan parasites via external macroscopic examination and using wet mount microscopy preparations of gill filaments, skin

scrapes and internal tissues. On arrival to Australia, fish were subjected to standard quarantine practices which involved clearance from customs and visual inspection and approval from quarantine services. At this point fish were considered pre-quarantine once released (Khan et al., 1999). Pre-quarantine fish were taken to the University of Sydney, Camden, Australia, where they were delivered to the AA for necropsy. Comprehensive parasitological examinations were completed on thirty individuals per population with the minimum detection prevalence set at 10%.

Fish were euthanized with a benzocaine overdose. Immediately following euthanasia, individual fish were measured, weighed, photographed, and examined using a dissecting microscope (Olympus SZ 61, Germany) for external parasites or physical abnormalities. Skin scrapes were taken from each individual fish with the blunt edge of a scalpel, the gill basket was removed and gill arches were separated individually onto a slide with saline solution and a cover slip. Following dissection of the gill basket, the head was separated from the main body and the brain was collected for examination, followed by the heart, liver, spleen, kidneys, gall bladder and digestive tract. Each organ was carefully dissected and placed onto separate slide, immersed in physiological saline and sealed with a coverslip. This dissecting order was followed to avoid cross contamination between organs.

A total of 10 slide preparations were examined for each fish under the microscope (gills, skin/fins, heart, liver, spleen, posterior kidney, gall bladder, gastrointestinal tract, brain and muscle). If parasites were observed, the slide was preserved for further taxonomic analysis. Negative control slides from the same population were also preserved for comparison. Parasite identification, using a combined morphological and molecular diagnostic approach, was conducted at JCU.

Objective 4: Developing efficient pooled sample strategies for testing imported fish

Pools of 5 and 10 tissue homogenates were prepared to determine the sensitivity of qPCR tests for MCV on pooled samples compared to tests of individual fish. For this exercise, a number of populations of apparently healthy imported gourami were sourced and the infection status of individual fish was determined as negative or subclinically infected with MCV.

Briefly, a tissue homogenate was prepared from each individual fish using 0.1 g of kidney, liver and spleen (Table 3). Tissues were homogenized as described above and stored at -80°C. Nucleic acids were purified from a 50 µL aliquot of the clarified tissue homogenate using the MagMax-96 Viral Isolation Kit (Ambion) according to directions of the manufacturer. Extracted nucleic acids were eluted in 75µL of elution buffer and stored at -80°C. A qPCR amplification of individual nucleic acid extract was performed according to the method described by Rimmer et al. (2012) for the detection of megalocytiviruses. As described above, this amplified a 167 bp section of the MCP gene using C1073 forward (5'-AATGCCGTGACCTACTTTGC- 3') and C1074 reverse (5'- GATCTTAACACGCAGCCACA -3') primers (Rimmer et al., 2012).

The preparation of pools was undertaken after homogenization of individual fish tissues as this is a practical and cost effective stage of the laboratory workflow for pooling in practice. Separately by fish species, a list of the positive or negative fish was generated and used to randomly allocate samples for preparation of 630 unique pools of 5 or 10 tissue homogenates. The ratio of positive and negative samples in each pool is indicated in Table 10. The rationale for the pattern of positive fish was based on probability theory for prevalence in the range 2% to 20% (Table 10); at 2% prevalence the probability of having more than 3 positive samples in a 5-fish pool or more than 5 positives in a 10-fish pool is negligible. There were 462 pools prepared using Dwarf gourami (*Trichogaster lalius*) and 168 pools prepared using Kissing gourami (*Helostoma Temminckii*). Pools were prepared in 1.5 ml laboratory tubes using 15 - 20 µl of each homogenate. These were vortexed and centrifuged at 855 x *g* for 10 minutes at room temperature. Nucleic acid purification and the qPCR test for MCV was conducted as described above.

Statistical analyses

The outcome of the experiment was a positive or negative qPCR test result for MCV for each of the 630 pooled tissue homogenates (experimental units). The data were then analyzed using a generalized mixed-effects model. Briefly, a unique model was fit for each species, with the following characteristics, the PCR plate was used as a random effect and a three-knot penalized spline of the proportion of positive samples for each of the two pool sizes as a fixed effect. The response variable was assumed to be binomial with a probit link function. This model form was selected after an extensive exploratory analysis phase. Next, the fitted model was used to estimate the probability of successful detection as a function of pool size and the

number of positive samples contributing to the pool. These predictions were used as a basis of a simulation model to determine the confidence for testing for freedom from MCV in a population of fish at a minimum expected prevalence of 2%. Briefly, we simulated the process of selecting N units from a population , and then: (i) testing the units individually, assuming 100% sensitivity and 100% specificity; (ii) pooling the units into 30 pools of size 5, then predicting the test outcome for each pool as a function of predicted ratio of positive samples using the model, and (iii) pooling the units into 15 pools of size 10, then predicting the test outcome for each pool as a function of its contamination rate using the model. This process was repeated 10 000 times for N ranging from 150 to 300. The outcome of the exercise was the estimated surveillance sensitivity of the test as a function of the sample size N and the pool size (1, 5, or 10).

Table 1. Description of the aquatic pathogens on Australia's national list of reportable diseases tested under this project.

Pathogen	Abbrev-iation	Pathogen description	Listed in the OIE Aquatic Animal Health Code (2015)	Listed regionally (OIE/NACA) (2015)	Exotic to Australia	Diagnostic assays used in the is project [6]	Target tissue collected for testing[7]
Aeromonas salmonicida		Gram - negative, nonmotile, short rods or coccobacilli occurring in pairs, chains or clumps. Cells measure 0.8 × 1.3 – 2.0 µm[1]			Yes (subsp. *salmonicida*)	primary isolation[8]	posterior kidney
Edwardsiella ictaluri		Gram - negative rod (0.5 × 1.3 µm), motile at 25°C but not 37°C[1]		Yes		primary isolation[9]	posterior kidney
dwarf gourami iridovirus (Infectious spleen and kidney necrosis virus – like virus)	DGIV	*Megalocytivirus* within the Family *Iridoviridae*; icosahedral virion with a diameter of 160-200nm and 110 kb genome with a GC content of 53–55%[2]			Yes	SYBR Green qPCR targeting MCP gene of genus *Megalocytivirus*	pool of kidney, liver and spleen (1,2)
koi herpesvirus (cyprinid herpesvirus-3)	KHV	Family *Alloherpesviridae*; virion contains a linear, double-stranded DNA genome packaged within an icosahedral nucleocapsid (capsid diameter ~100 nm) and genome is 295 kb and contains 155 genes[3]	Yes	Yes	Yes	SYBR Green qPCR targeting DNA polymerase gene specific for Cyprinid herpesvirus 3	pool of spleen, posterior kidney (1); encephalon in viral transport medium (2)
spring viraemia of carp virus	SVCV	Family *Rhabdoviridae*; enveloped helical nucleocapsid that is bullet-shaped (60–90-nm wide and 80–180-nm long) and a single-stranded RNA of negative polarity and a genome size varying between 11.0 and 11.2 kb[4]	Yes	Yes	Yes	Taqman probe RT-qPCR targeting the G gene, specific for Genogroup I	pool of spleen, posterior kidney (1); encephalon in viral transport medium (2)
viral haemorrhagic septicaemia virus	VHSV	Family *Rhabdoviridae*; enveloped helical nucleocapsid that is bullet-shaped (approximately 200 × 75 nm) and a single-stranded RNA of negative polarity and a genome size varying between 11.0 and 11.2 kb[4]	Yes	Yes	Yes	Taqman probe RT-qPCR targeting the N gene of 4 genotypes	pool of spleen, posterior kidney (1); encephalon in viral transport medium (2)
nervous necrosis virus	NNV	Genus *Betanodavirus*; two single-stranded, positive-sense RNAs, which are capped but not polyadenylated; genome size of betanodavirus is 4.5 kb[5]		Yes		Taqman probe RT-qPCR targeting the capsid protein gene of 4 genotypes	brain and eyes (retina) (1,2)

[1] Roberts, 2012; [2] Hick et al. 2016; [3] Hanson et al. 2016; [4] LaPatra et al. 2016; [5] Chi et al. 2016; [6] See Appendix 3, Table 3 for specific details for each assay; [7] target organ for presumptive testing (1) and target organ preserved for future testing (2); [8] Australian Department of Agriculture 2014c; [9] OIE, 2009

Table 2. The number of laboratory accessions received from each exporting country for specific pathogen testing summarized by fish host.

Fish species (Common name)[1]	Pathogen tested	Exporting country									Total
		Indonesia 1	Indonesia 2	Indonesia 3	Malaysia 1	Singapore 1	Singapore 2	Sri Lanka 1	Sri Lanka 2	Thailand 1	
Marine											
Amphiprion bicintus (Twoband anemonefish)	MCV, NNV			1							**1**
Amphiprion ocellaris (Clown anemonefish)	MCV, NNV		1	1							**2**
Amphiprion sebae (Sebae anemonefish)	MCV, NNV					1			1		**2**
Cheilodipterus quinquelineatus[2] (Five-lined cardinalfish)	MCV, NNV		3						1		**4**
Chromis viridis[2] (Blue green damselfish)	MCV, NNV		1								**1**
Ostorhinchus compressus[2] (Ochre-striped cardinalfish)	MCV, NNV		1								**1**
Pterapogon kauderni (Banggai cardinal fish)	MCV, NNV	1	1			1					**3**
Sphaeramia nematoptera[2] (Pajama cardinalfish)	MCV, NNV			1							**1**
Zoramia leptacantha (Threadfin cardinalfish)	MCV, NNV			1							**1**
Freshwater											
Betta splendens[2] (Siamese fighting fish)	MCV				1				1		**2**
Carassius auratus (Goldfish)	KHV; SVCV; VHSV; *A. salmonicida*				2		2			3	**7**
Danio rerio (Zebra danio)	SVCV; VHSV; *E. ictaluri*				1	1	1[3]	1			**4**
Helostoma temminckii[2] (Kissing gourami)	MCV					1			1	1	**3**
Pethia conchonius (Rosy barb)	*E. ictaluri*				1		1			2	**4**
Poecilia reticulata (Guppy)	MCV, NNV				1	1	2	1	1	2	**8**
Pterophyllum scalare[2] (Freshwater angelfish)	MCV				1						**1**

Fish species (Common name)[1]	Pathogen tested	Exporting country									Total
Marine		**Indonesia 1**	**Indonesia 2**	**Indonesia 3**	**Malaysia 1**	**Singapore 1**	**Singapore 2**	**Sri Lanka 1**	**Sri Lanka 2**	**Thailand 1**	
Trichogaster lalius[2] (Dwarf gourami)	MCV				1	1				1	**4**
Trichopodus leerii[2] (Pearl gourami)	MCV							1		1	**2**
Trichopodus trichopterus[2] (Three spot gourami)	MCV				1		1	1	1	2	**6**
Xiphophorus helleri[2] (Green swordtail)	MCV				1				1		**2**
Xiphophorus maculatus[2] (Southern platyfish)	MCV						1		1	1	**3**
Total		**1**	**7**	**4**	**10**	**6**	**8**	**5**	**8**	**13**	**62**

[1] Fish species and common names were sourced from FishBase.org on 30 August 2016.

[2] These fish species were purchased and tested to meet the minimum order weight and volumes for ornamental fish exporters. Fish species were selected based on prior knowledge of susceptibility to the pathogen of interest or evolutionary relationship with known fish host species. These fish species were tested in addition to the ones nominated in the project application.

[3] only tested for SVCV and VHSV

Table 3. Summary of imported ornamental fish under quarantine used in Objective 4.

Export country (Lab accession)	Date received	Species	Common name	Mean weight ± SD (g)	Mean total length ± SD (mm)	Proportion of positive fish[1]	Percent positive (95% CI)
Thailand (SVC 15/122)	May 2015	*Trichogaster lalius*	Dwarf gourami	1.29 ± 0.33	39.12 ± 3.32	178/178	100 (97.9-100)
Singapore (SVC 15/108)	May 2015	*Helostoma temminckii*	Kissing gourami	2.2 ± 0.64	50.2 ± 5.12	114/172	66.3 (58.7-73.3)

[1] tested by qPCR for the detection DGIV

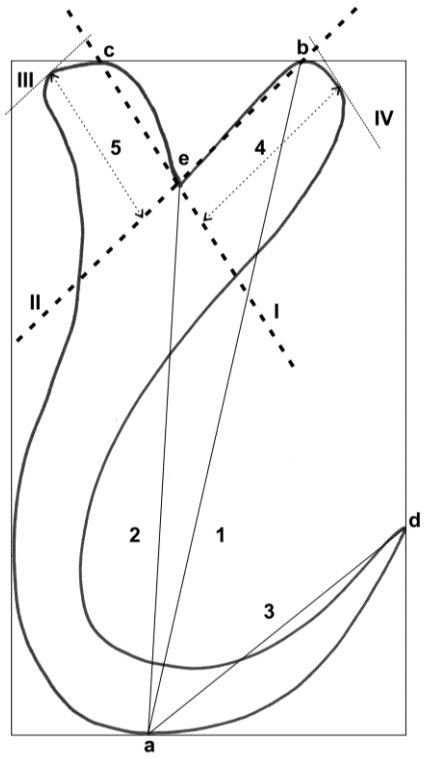

Figure 1. Representative measurements of *Dactylogyrus* spp. hamuli. Points a-d describe the location where the superimposed rectangle touches the hamulus. Point e describes the lowest point of the root saddle. Thick dotted lines (I and II) are drawn between points b-e and and c-e, respectively; lines III and IV are drawn perpendicular to lines II and I, respectively, touching the highest point of each root (see lines III and IV). 1 = total hamulus length; 2 = basal hamulus length; 3 = total blade length; 4 = inner root length; 5 =outer root length; 6 = total gap length; 7 = Gap ratio (gap lenth/ blade length). Image credit: David Vaughn.

Results and Discussion

Objective 1. Determining if pathogens of potential biosecurity concern on the national list are entering QAPs in Australia through the trade in ornamental fish

In total, 12 consignments of marine and freshwater fish from five different countries were received at the Approved Arrangements (formerly Quarantine Approved Premise) laboratory at the University of Sydney. The consignments were received from nine different exporters that contained a variety of eight marine and 12 freshwater species. A total, 62 populations of ornamental fish were collected (Table 2).

Detection of *Megalocytivirus* (MCV)

Megalocytivirus was detected in 92% (11/12) of consignments of ornamental fish (Table 4). This represented five marine and eight freshwater fish species received from eight of the nine exporters from all five countries. One consignment of marine fish from Indonesia in October 2015 was the only one in which all fish were test negative with an upper 95% confidence limit of 11.6% (Table 4).

Approximately 52% (24/46) of the populations of fish tested for MCV were positive. Megalocytivirus-positive populations were detected during each sampling event throughout 2015. Of the nine species of marine fish tested for MCV, 5 species had at least one positive population. These included *Amphiprion sebae*, *Cheilodipterus quinquelineatus*, *Chromis viridis*, *Ostorhinchus compressus*, *Pterapogon kauderni* (Table 4). With the exception of *Betta splendens*, all freshwater species tested for MCV had at least one positive population (Table 4). Virus sequence analysis of representative positive samples determined that ISKNV-like genotypes were present from both freshwater and marine fish species (Appendix 3 Table 4).

All of the freshwater species that were positive for MCV fall under the Biosecurity Advice 2014/011 requiring batch testing as of 1 March 2016. None of the marine fish species require batch testing under this policy. See Appendix 4 for the health certificate requirements for freshwater and marine ornamental fish as of October 2016.

It is important to note that the requirement for freedom from MCV and batch testing was originally planned to begin 1 March 2015. This would have allowed this project to test fish being imported

immediately prior to and following the implementation of the new requirements. However, the requirement for freedom from MCV infection was delayed by twelve months and thus all consignments were received prior to the requirement for freedom from infection with MCV.

Detection of nervous necrosis virus (NNV)

NNV was detected in three of 23 (13%) populations of marine fish that were received from three exporters located in Indonesia (Table 4). The positive fish consisted of two populations of Banggai cardinal fish (*Pterapogon kauderni*) and Threadfin cardinalfish (*Zoramia leptacantha*). All other consignments and populations of fish tested were negative (Table 4). Guppies were the only freshwater fish species tested, and no positive results were obtained from any fish in 9 populations.

Tests for koi herpesvirus (KHV)

A total of seven populations of Goldfish (*Carassius auratus*) were received from three exporters each representing separate countries (Table 5). Three and four consignments were received in May and October 2015, respectively. All Goldfish tested negative for KHV with an upper confidence limit for the proportion infected ranging from 2.2 to 3.5% (Table 5).

Tests for spring viraemia of carp virus (SVCV)

A total of seven populations of Goldfish were received from three exporters each representing separate countries (Table 5). Three and four consignments were received in May and October 2015, respectively. All goldfish tested negative for SVCV with an upper confidence limit for the proportion infected ranging from 2.2 to 3.5% (Table 5).

A total of four populations of Zebra danio (*Danio rerio*) were received from four exporters representing three countries (Table 6). Three and one consignment(s) were received in May and October 2015, respectively. All fish tested negative for SVCV with an upper confidence limit for the proportion infected ranging from 1.8 to 2.4% (Table 6).

Tests for viral haemorrhagic septicaemia virus (VHSV)

A total of seven populations of Goldfish were received from three exporters each from separate countries (Table 5). Three and four consignments were received in May and October 2015, respectively.

All Goldfish tested negative for VHSV with an upper confidence limit for the proportion infected ranging from 2.2 to 3.5% (Table 5).

A total of four populations of Zebra danio (*Danio rerio*) were received from four exporters from three countries (Table 6). Three and one consignment(s) were received in May and October 2015, respectively. All fish tested negative for VHSV with an upper confidence limit for the proportion infected ranging from 1.8 to 2.4% (Table 6).

Tests for *Aeromonas salmonicida*

A total of seven populations of Goldfish were received from three exporters each representing separate countries (Table 7). Three and four consignments were received in May and October 2015, respectively. All fish tested negative for *A. salmonicida* with an upper confidence limit ranging from 8.8 to 11.6% (Table 7).

Tests for *Edwardsiella ictaluri*

Three populations of Zebra danio were received from three exporters each representing separate countries (Table 8). Negative cultures were observed in fish received from Singapore and Malaysia in May and October 2015, respectively with an upper confidence limit ranging from 6.0 to 8.8% (Table 8). One population of Zebra danio received from Sri Lanka in May 2015 had 3 fish that grew bacilli that were Gram negative and oxidase negative. PCR testing of representative colonies fixed in 70% ethanol was completed by the Western Australia Department of Agriculture and Food Diagnostics Laboratory Services. All samples tested negative for *E. ictaluri* (Table 8).

Four populations of Rosy barb were received from three exporters representing three countries (Table 8). Negative cultures were observed in fish received from Singapore and Thailand in May 2015 with an upper confidence limit ranging of 7.3% (Table 8). Another population of Rosy barb Malaysia in October 2015 were negative with an upper confidence limit of 8.8%. One population of Rosy barb received from Thailand in October 2015 had 16 fish that grew colonies that were Gram negative and oxidase negative. PCR testing was completed as above. Representative colonies from three fish were found to be from the *Edwardsiella* genus but were not *E. ictaluri*. All fish tested negative for *E. ictaluri* (Table 8).

Objective 2. Determine if current import conditions for goldfish requiring freedom from specific pathogens are being met

The current import conditions for Goldfish require additional veterinary certification to be free of SVCV, *Aeromonas salmonicida* (other than Goldfish ulcer disease strains) and infestation with gill flukes (*Dactylogyrus vastator* and *D. extensus*). The complete specification for the health certificate is available in Appendix 4. With respect to SVCV and *Aeromonas salmonicida*, there was no evidence to indicate that these pathogens were present in Goldfish immediately upon arrival in Australia and under quarantine. Five populations of Goldfish met the criteria for freedom from infection at 2% minimum expected prevalence at 95% confidence for SVCV. For *A. salmonicida*, four populations were of sufficient size to reach this level of confidence. Other populations of Goldfish were negative for these pathogens with upper confidence limits for proportion infected ranging from 2.4 to 3.5%.

The current import conditions for Goldfish were not effective for the elimination of *Dactylogyrus vastator*. Three Goldfish populations received from the same exporter from Thailand were infected with *Dactylogyrus vastator*. The morphology of *D. vastator* is highly conserved and distinct from all other dactylogyrids infecting cyprinids (Ling et al., 2016). *Dactylogyrus extensus*, the other species of dactylogrid listed on the health certification for freshwater ornamental finfish imported into Australia, was not found on any of the Goldfish consignments. However, more than six dactylogyrid monogenean species were found in five of the seven populations from south-east Asia and an additional monogenean species, *Gyrodactylus kobayashii* was found infecting the gills and skin of imported Goldfish (Table 9; Figure 2). The recovery of numerous *Dactylogryus* species indicates that the chosen paraciticide(s) by exporting countries was not effective in eliminating monogenean infections, or that the recommended treatments were not administered correctly (Table 10). *Dactylogyrus vastator* has low host specificity. It has been reported from 16 fish species representing four fish families and therefore presents a biosecurity risk to industry and natural resources.

Parasiticides have been trialed against *Gyrodactylus* species in aquaculture for decades (Santamarina et al. 1991; Hagen et al. 2014). However, the efficacy of various parasiticides (trichlorfon, formaldehyde and sodium chloride) against dactylogyrid monogenean infections and their various life stages has not been examined. *Dactylogyrus* spp. can attach deep within the gill lamellae of their host and thus chemical bath treatment may not confer 100% efficacy. Alternatively, the water that fish are

transported in may be contaminated with eggs or larval stages of *Dactylogyrus* species. Dactylogyrid monogenean eggs have a proteinaceous shell which makes them highly resistant to most chemical treatments. Monogenean larvae (oncomiracidia) can directly reinfect fish. Moreover, dactylogyrid monogeneans are hermaphrodites and are probably capable of producing viable eggs from a single individual. These parasites may also exhibit short generation times and thus are able to contaminate environments in only a few days.

Objective 3. Document parasites of potential biosecurity concern that are associated with imported ornamental fish

During the three sampling periods in 2015, a total of 990 fish representing 18 ornamental species from 33 populations were surveyed for parasite fauna. All populations of fish examined had at least one parasite taxon observed. Protozoan and metazoan parasite fauna detected included two scuticociliates and four other ciliates, three coccidians, 10 myxozoan species, five nematodes, five trematodes, seven monogeneans, and one crustacean (Table 11).

Of this diversity, five findings indicate potential biosecurity concerns because they may present a risk to Australian aquatic animal industries and natural resources:

1. *Argulus japonicus* (Crustacea) in water transporting *C. auratus* from Singapore and Thailand.
2. *Gyrodactylus* spp. (Monogenea) infected *C. auratus* from Singapore, Thailand and Malaysia and; *X. maculatus* from Singapore.
3. *Trichodina* sp. infected: *P. conchonius*, *Danio rerio* and *T. trichopterus* from Sri Lanka; *C. auratus* from Thailand and Malaysia.
4. Morphologically distinct myxozoans from the genera *Ceratomyxa*, *Henneguya*, *Kudoa, Myxobolus* and *Myxidium* infected fish surveyed in this study.
5. *Centrocestus* sp. was found in all goldfish consignments. Trematode metacercaria were found in *Amphiprion sebae, Helostoma temmincki, Ostorhinchus compressus, Pethia conchonius, Xiphophorus maculatus, Zoramia leptocantha*.

Host specificity is a significant component of parasite ecology and transmission dynamics because it can determine whether it has the potential to become established in a new environment and on novel hosts. Exposure to new environments may impact pathogen and parasite virulence by either promoting exponential growth or possibly eliminating conditions necessary for some species to survive. Conditions

associated with captive aquatic animals mean that only a limited diversity of disease agents can successfully propagate, proliferate and harm stock. The best indication of risk is by a thorough evaluation of host-specificity, although this information is not always available. Risk assessments should also include an understanding of the life cycle requirements of the parasitic agent and the history of the agent (or similar agents) as an invasive elsewhere.

***Argulus japonicus* (Crustacea)**

Argulus japonicus was thought to have been introduced with aquarium fish from Asia and has now been reported from many localities around the world. *Argulus japonicus* is a branchiurid crustacean, commonly called a fish louse, and is a temporary ectoparasite. Live specimens were observed swimming in the water transporting Goldfish, *C. auratus*, from Singapore and Thailand (Populations 15/126, 15/199 and 15/200). There were approximately 50 to 60 Goldfish in one bag from which the *A. japonicas* was observed. Morphological identification was in agreement with the original description for this taxon by Yamaguti (1937) (Figure 3).

Argulus species have been recognized as pests of farmed trout in Europe and carp in China for centuries. The most notorious species include *A. coregoni* and *A. japonicus*. *Argulus japonicus* is thought to have been introduced to Australia and New Zealand through the ornamental trade of goldfish. The earliest identification of *A. japonicus* was by Heegaard 1962 infecting *C. auratus* in New South Wales (Heegaard 1962). Four years later, *A. japonicus* was recorded in New Zealand in 'goldfish' recently imported from South East Asia (Pilgrim 1967). Museum collections in Australia include 12 holdings of *Argulus japonicus* from wild and captive goldfish collected from Victoria, New South Wales and Queensland (Hutson, unpublished data). This species also infects captive koi carp, *Cyprinus carpio*, in Western Australia (Hutson, unpublished data).

Argulus japonicus exhibits low host-specificity, has a direct life cycle and a history of being a harmful invasive species, which makes it a biosecurity concern for Australian industries and natural resources. *Argulus japonicus* primarily infests goldfish and other cyprinids (18 species), but has also been found to infect 11 non-cyprinid fish species representing Cichlidae, Clariidae, Clupeidae, Gasterosteidae, Ictaluridae, Percichthyidae, Percidae, Salmonidae and Siluridae. Transmission is direct since they have no intermediate host. They are excellent swimmers and will actively seek fish and attach themselves. The parasites have prominent suckers used for principal attachment which are supplemented by a

variety of hooks and spines (Figure 3). Once attached to the host, they use specialised mouth parts to either pierce the host's skin and suck blood and other internal fluids, or feed on mucus and skin sloughed off by the host. Infested fish become lethargic, cease feeding, lose condition and may try to remove parasites by rubbing against the substrate. In chronic infections the skin becomes opaque, ulcerative lesions develop and the fins become frayed. Mortalities are usually associated with hundreds of argulids per fish, likely as a result of a breakdown in epithelial integrity. Argulids have been implicated in the transfer viruses and other parasitic organisms (Ahne 1985).

***Gyrodactylus* spp. (Monogenea)**

Gyrodactylus kobayashii was found infecting the skin and gills of goldfish from Singapore, Thailand and Malaysia (Table 11, Figure 4) while *Gyrodactylus* sp. was found on the skin of one *Xiphophorus maculatus* individual from Singapore (Lab accession 15/117, prevalence= 3.3%). Infection of *Gyrodactylus kobayashii* on Goldfish has been associated with high mortality previously. In one incident in China, fish transferred from a fish farm to aquariums experienced approximately 80% mortality (3,335 goldfish; Tu et al. 2015). Examination showed that no other pathogens were found in this batch of fish except for *G. kobayashii* and mean parasite burdens were 265 parasite individuals per fish (range 100–450).

Although gyrodactylids are potentially highly pathogenic, the pathogenicity of different *Gyrodactylus* species is variable and parasite-induced host death is clearly dependent on host species and size. Gyrodactylids are viviparous monogenean worms – they develop the embryo in their uterus, give birth to the daughter which is fully developed and already carrying the next generation in the uterus. This mode of reproduction, hyperviviparity, has been colloquially termed "Russian Doll" and permits exponential population increases on susceptible hosts. Parasites are spread from direct physical contact between hosts or alternatively they can detach and survive for a few days before infecting a new host. Transmission from dead hosts to live hosts may also occur.

Ciliates

Ciliated protozoa are relatively common in marine and freshwater ecosystems; however some can be significant opportunistic parasites of fish. For example, species of *Tetrahymena* and *Trichodina* are common inhabitants of freshwater environments in Australia. Introduction of these organisms in aquaculture ponds or recirculating systems can result in rapid numbers of these ciliates proliferating due

to high stocking densities and nutrient input. Significant mortalities to cultured fish can occur when high numbers of ciliates cause pathology associated with feeding and tissue invasion.

Trichodina spp. infected four fish host species (*P. conchonius*, *Danio rerio*, *T. trichopterus*) from Sri Lanka and *C. auratus* from Thailand and Malaysia (Table 11). As ectoparasites, *Trichodina* spp. can cause fouling, irritation and local lesions and sometimes penetrating wounds. In fish confined to ponds, tanks or aquaria, *Trichodina* that live freely in the water column can form dense populations on fish resulting in morbidity and mortality. Asexual reproduction occurs by transverse binary fission. Little is known about the potential impacts to freshwater environments by invasion of non-native *Trichodina* spp. These ciliates are important components of freshwater ecosystems; therefore the risk to native ciliate populations may be significant.

Myxozoans

Myxozoans are obligate parasites that use invertebrate and vertebrate hosts as part of their life cycle and can occur in marine and freshwater environments. They are markedly diverse in marine and freshwater ecosystems worldwide, with over 2,000 described species in over 60 genera. Morphologically distinct myxozoans from the genera *Ceratomyxa*, *Henneguya*, *Kudoa, Myxobolus* and *Myxidium* were identified during this survey (Figure 5 and 6). These taxa are found principally in the muscle, brain and gall bladder of fish. Myxozoa form complex valved spores with polar capsules containing extrudible filaments which are used for attachment to the host. Transmission requires a developmental phase in an invertebrate host (generally oligochaetes in freshwater and polychaetes in marine ecosystems), although rare cases of direct fish to fish transmission have been reported. Infections by myxozoans may be asymptomatic, but some may cause severe tissue hyperplasia, unsightly macroscopic cysts, erosive and necrotic lesions, enzymatic lysis of fish tissue (i.e. myoliquefaction), deformities and even death. The most pathogenic species are known from the genera *Myxobolus*, *Sphaerospora*, *Ceratomyxa*, *Kudoa*, *Unicapsula*, *Henneguya*, *Enteromyxum* and *Tetracapsuloides*. Some fish-borne myxozoan species (i.e. *Kudoa* spp.) can cause food poisoning in humans.

Trematode metacercariae (Heterophyidae)

Two marine species (*A. sebae*, *O. compressus*) and five freshwater fish (*C. auratus, H. temmincki, P. conchonius, X. maculatus, Z. leptocantha*) were infected with trematode metacercariae in gill and muscle tissues. Many of the marine species examined had wide range of larval trematode metacercariae

encysted in their tissues (e.g. species of the families Bucephalidae, Cryptogonimidae), which are common parasites encountered in the wild reef ecosystems where these fish would have originated. However, the majority of the trematode metacercariae observed encysted in various tissues (e.g. gills, muscle) the imported freshwater fish examined were from the Heterophyidae (Figure 7). This family of trematodes is relatively common in freshwater ecosystems, but importantly contains a number of species that are significant pathogens of birds and mammals (and zoonotic). Among these are *Centrocestus formosanus*, which is a zoonotic trematode from Asia and has been associated as cause of mortalities in cultured fish. *Centrocestus formosanus* is a minute trematode species inhabiting the intestine of fish-eating birds and mammals (Scholz and Salgado-Maldonado 2000). It exhibits a complex life cycle where the larval stage reproduces asexually in a gastropod, while the second intermediate host includes various species of freshwater fish. *Centrocestus* species are known to infect imported ornamental fish and become co-invasive in native environments, affecting native fish fauna (Mitchell et al. 2005). All *C. auratus* consignments were observed with a high prevalence of putative *Centrocestus* spp., which display low host specificity and can infect a wide range of fish species (Gibson et al. 2005).

Objective 4. Develop efficient pooled sample strategies for testing imported fish

Sensitivity of qPCR for pooled tissue homogenates

The pooled tissue homogenate samples were prepared from a random selection of 292 gourami that were positive for MCV when tested individually and 508 test negative fish. The prevalence of infection in the source populations was 66% - 100% and the range of virus load was $1.97 \times 10^2 – 6.79 \times 10^9$ copies of the MCV major capsid protein gene per gram of fish tissue. The Ct values for these positive samples when tested individually were in the range 13.3 – 38.9 (average 24.5 +/- 5.6). This provided authentic pooled samples that were reflective of the sample matrix and virus load of apparently healthy imported ornamental fish.

The sensitivity of the qPCR assay to detect pools that were expected to be positive based on the results for the contributing individual samples was substantially reduced. This reflected the ratio of positive to negative samples contributing to the pool (Table 12). The sensitivity for a single positive sample in a pool of 5 was 68.3% (95% CI, 55.0 - 79.7%) and for a single positive in a pool of 10 was 63.3% (95% CI, 50.7 - 74.4%).

The generalized mixed-effects model identified a significant impact of the population from which the samples were sourced on the pooled sensitivity ($p<0.01$). Therefore, predictions for pooled sensitivity derived from the experimental data were stratified for the two different host fish species (Figure 8).

Simulation exercise: test for freedom from infection using pooled samples

The different pooled sensitivity for the different source populations was extremely influential in determining the surveillance sensitivity (Table 13, Figure 9). Simulations for tests of a population for freedom from infection were undertaken according to OIE guidelines for 95% confidence of detecting a minimum expected prevalence of 2% with the assumption that the qPCR tests of individual fish provided 100% sensitivity and 100% specificity. For the Dwarf gourami (*T. lalius*) source population, it was demonstrated that the nominal surveillance sensitivity could be achieved using 40 pools of 5 fish (200 fish, 40 tests) or 26 pools of size 10 (260 fish, 26 tests) in place of 150 tests of individual fish (Table 13, Figure 9). However, the surveillance sensitivity for the Kissing gourami (*H. temminckii*) source population did not reach the desired level of confidence for any sample size with pools of 5 or 10 fish (Table 13, Figure 9).

The applicability of pooled testing was limited to one of the two source populations in this study. This result might reflect a species specific difference in the sample matrix. However, it is more likely influenced by the pathobiology of MCV infection. Higher pooled sample sensitivity was obtained when pools were prepared using dwarf gourami that were present in a population with 100% prevalence and a virus load $5.6 \times 10^2 – 3.1 \times 10^9$, median 9.6×10^6 major capsid protein gene copies per gram of fish tissue (Ct range 14.5 – 36.78; average 23.91 +/- 5.0). Comparatively, the MCV positive kissing gourami (*H. temminckii*) were derived from a population with 66% prevalence and a relatively lower virus load $1.97 \times 10^2 - 6.79 \times 10^9$, median 6.2×10^4 major capsid protein gene copies per gram of fish tissue (Ct range 13.30 - 38.29; average 27.8 +/- 6.3).

Table 4. Summary of the number of ornamental fish collected immediately upon arrival in Australia and under quarantine for the detection of megalocytiviruses (MCV) and viral nervous necrosis viruses (NNV). Diagnostic tests were applied according to at risk fish species as indicated in Table 2.

Date	Exporting Country	Lab Accession	Fish Species	Common Name	Proportion positive			
					MCV	95% CI	NNV	95% CI
January 2015	Singapore-1	15/019	*Pterapogon kauderni*	Banggai cardinal fish	39/39	91.0-100	0/39	0-9.0
		15/023	*Poecilia reticulata*	Guppy	0/150	0-2.4	not done	
		15/022	*Trichogaster lalius*	Dwarf gourami	0/150	0-2.4		
	Indonesia-1	15/024	*Pterapogon kauderni*	Banggai cardinal fish	22/60	24.6-50.1	1/60	0-8.9
	Indonesia-2	15/025	*Pterapogon kauderni*	Banggai cardinal fish	2/60	0.4-11.5	1/60	0-8.9
		15/030	*Cheilodipterus quinquelineatus*	Five-lined cardinalfish	15/60	14.7-37.9	0/60	0-6.0
		15/032	*Cheilodipterus quinquelineatus*	Five-lined cardinalfish	19/60	20.3-45.0	0/60	0-6.0
		15/031	*Chromis viridis*	Blue green damselfish	3/150	0.7-5.7	0/150	0-2.4
May 2015	Singapore-1	15/107	*Amphiprion sebae*	Sebae anemonefish	2/30	0.8-22.1	0/30	0-11.6
		15/108	*Helostoma temmincki*	Kissing gourami	114/172	58.7-73.3		
	Indonesia-2	15/109	*Ostorhinchus compressus*	Ochre-striped cardinalfish	1/60	0-8.9	0/60	0-6.0
		15/110	*Cheilodipterus quinquelineatus*	Five-lined cardinalfish	0/50	0-7.1	0/50	0-7.1
		15/111	*Amphiprion ocellaris*	Clown anemonefish	0/60	0-6.0	0/60	0-6.0
	Sri Lanka-1	15/112	*Poecilia reticulata*	Guppy	1/100	0-5.4	0/100	0-3.6
		15/113	*Trichopodus trichopterus*	Three spot gourami	0/142	0-2.6		
		15/114	*Trichopodus leerii*	Pearl gourami	2/150	0.4-4.7		
	Singapore-2	15/115	*Poecilia reticulata*	Guppy	0/196	0-1.9	0/196	0-1.9
		15/116	*Poecilia reticulata*	Guppy	0/197	0-1.9	0/197	0-1.9
		15/117	*Xiphophorus maculatus*	Southern platyfish	10/167	2.9-10.7		
		15/128	*Trichopodus trichopterus*	Three spot gourami	9/212	2-7.9		
	Thailand-1	15/118	*Trichopodus trichopterus*	Three spot gourami	115/136	77.7-90.2		
		15/119	*Poecilia reticulata*	Guppy	0/182	0-2.0	0/182	0-2.0
		15/120	*Xiphophorus maculatus*	Southern platyfish	7/150	1.9-9.4		
		15/121	*Trichopodus leerii*	Pearl gourami	0/181	0-2.0		
		15/122	*Trichogaster lalius*	Dwarf gourami	178/178	97.9-100		
October 2015	Malaysia-1	15/176	*Trichopodus trichopterus*	Three spot gourami	49/150	25.2-40.8		
		15/177	*Xiphophorus helleri*	Green swordtail	113/173	57.7-72.4		
		15/178	*Betta splendens*	Siamese fighting fish	0/91	0-4.0		

	15/179	*Poecilia reticulata*	Guppy	0/151	0-2.4	0/151	0-2.4
	15/181	*Pterophyllum scalare*	Freshwater angelfish	31/102	21.7-40.3		
	15/182	*Trichogaster lalius*	Dwarf gourami	17/150	6.7-17.5		
Indonesia-3	15/183	*Sphaeramia nematoptera*	Pajama cardinalfish	0/30	0-11.6	0/30	0-11.6
	15/184	*Ostorhinchus compressus*	Ochre-striped cardinalfish	0/30	0-11.6	5/30	5.6-34.7
	15/185	*Amphiprion ocellaris*	Clown anemonefish	0/60	0-6.0	0/60	0-6.0
	15/186	*Amphiprion bicintus*	Twoband anemonefish	0/60	0-6.0	0/60	0-6.0
Sri Lanka-2	15/187	*Amphiprion sebae*	Sebae anemonefish	0/31	0-11.2	0/31	0-11.2
	15/190	*Cheilodipterus quinquelineatus*	Five-lined cardinalfish	0/32	0-10.9	0/32	0-10.9
	15/191	*Poecilia reticulata*	Guppy	0/126	0-2.9	0/126	0-2.9
	15/192	*Xiphophorus helleri*	Green swordtail	60/154	31.2-47.1		
	15/193*	*Xiphophorus maculatus*	Southern platyfish	120/152	71.6-85.1		
	15/194*	*Betta splendens*	Siamese fighting fish	0/150	0-2.4		
	15/196*	*Helostoma temminckii*	Kissing gourami	0/41	0-8.6		
	15/197*	*Trichopodus trichopterus*	Three spot gourami	15/114	7.6-20.8		
Thailand-1	15/201	*Helostoma temminckii*	Kissing gourami	0/152	0-2.4		
	15/202	*Trichopodus trichopterus*	Three spot gourami	157/158	96.5-100		
	15/203	*Poecilia reticulata*	Guppy	0/150	0-2.4	0/150	0-2.4

*These populations were seized at AQIS and euthanized. We received the dead fish in a plastic bag with no water.

Table 5. Summary of the number of Goldfish (*Carassius auratus*) collected immediately upon arrival in Australia and under quarantine for the detection of koi herpesvirus (KHV), spring viraemia of carp virus (SVCV) and viral haemorrhagic septicaemia virus (VHSV).

Date	Exporting Country	Lab accession	Proportion positive			95% CI
			KHV	SVCV	VHSV	
May 2015	Singapore-2	15/104	0/150	0/150	0/150	0 - 2.4
	Singapore-2	15/126	0/172	0/172	0/172	0 - 2.2
	Thailand-1	15/106	0/105	0/105	0/105	0 - 3.5
October 2015	Malaysia-1	15/180	0/134	0/134	0/134	0 - 2.8
	Malaysia-1	15/205	0/150	0/150	0/150	0 - 2.4
	Thailand-1	15/199	0/150	0/150	0/150	0 - 2.4
	Thailand-1	15/200	0/150	0/150	0/150	0 - 2.4

Table 6. Summary of the number of Zebra danio (*Danio rerio*) collected immediately upon arrival in Australia and under quarantine for the detection of spring viraemia of carp virus (SVCV) and viral haemorrhagic septicaemia virus (VHSV).

Date	Exporting Country	Lab accession	Proportion positive		95% CI
			SVCV	VHSV	
May 2015	Singapore-1	15/100	0/209	0/209	0 - 1.8
	Sri Lanka-1	15/102	0/152	0/152	0 - 2.4
	Singapore-2	15/129	0/208	0/208	0 - 1.8
October 2015	Malaysia-1	15/204	0/150	0/150	0 - 2.4

Table 7. Summary of the number of Goldfish (*Carassius auratus*) collected immediately upon arrival in Australia and under quarantine for the detection of the notifiable bacterial pathogen, *Aeromonas salmonicida*.

Date	Exporting Country	Lab Accession	Proportion positive fish	95% CI
May 2015	Singapore-2	15/104	0/30	0-11.6
	Singapore-2	15/126	0/30	0-11.6
	Thailand-1	15/106	0/30	0-11.6
October 2015	Malaysia 1	15/205	0/40	0-8.8
	Malaysia 1	15/180	0/40	0-8.8
	Thailand 1	15/199	0/40	0-8.8
	Thailand 1	15/200	0/40	0-8.8

Table 8. Summary of the number of ornamental fish collected immediately upon arrival in Australia and under quarantine for the detection of the notifiable bacterial pathogen, *Edwardsiella ictaluri*.

Date	Exporting Country	Lab Accession	Species	Common Name	Proportion positive fish	95% CI
May 2015	Singapore-1	15/100	*Danio rerio*	Zebra danio	0/60	0-6.0
	Sri Lanka-1	15/102	*Danio rerio*	Zebra danio	0/150[1]	0-2.5
	Singapore-2	15/103	*Pethia conchonius*	Rosy barb	0/49	0-7.3
	Thailand-1	15/105	*Pethia conchonius*	Rosy barb	0/50	0-7.1
October 2015	Malaysia 1	15/204	*Danio rerio*	Zebra danio	0/40	0-8.8
	Malaysia 1	15/206	*Pethia conchonius*	Rosy barb	0/40	0-8.8
	Thailand 1	15/198	*Pethia conchonius*	Rosy barb	0/40[2]	0-8.8

1 representative colonies that were Gram negative and oxidase negative from 3 fish were sent for molecular testing and were all negative
2 representative colonies that were Gram negative and oxidase negative from 16 fish were sent for molecular testing. Three samples were from the *Edwardsiella* genus but were not *E. ictaluri*.

Table 9. Monogenean parasites infecting Goldfish, *Carassius auratus* received from Singapore, Thailand and Malaysia. Parasite prevalence indicates the proportion of fish infected in each population (n = 30).

Parasite species	**Exporting country**	**Laboratory Accession**	**Prevalence (%)**
Dactylogyrus vastator	Thailand-1	15/199	13.3
		15/200	16.6
		15/106	6.6
Dactylogyrus anchoratus	Thailand-1	15/199	23.3
		15/200	3.3
Dactylogyrus intermedius	Malaysia-1	15/205	3.3
	Thailand-1	15/106	20.0
		15/199	30.0
		15/200	30.0
Dactylogyrus formosus	Malaysia-1	15/205	6.6
	Thailand-1	15/199	6.6
		15/200	40.0
Dactylogyrus baueri	Singapore-2	15/126	3.3
	Thailand-1	15/199	13.3
		15/200	60.0
Dactylogyrus sp.	Malaysia-1	15/205	3.3
	Singapore-2	15/126	3.3
	Thailand-1	15/106	10.0
		15/199	10.0
		15/200	23.3
Gyrodactylus kobayashii	Malaysia-1	15/180	3.3
		15/205	13.3
	Singapore-2	15/126	6.6
	Thailand-1	15/106	13.3

Table 10. Summary of parasite treatments as stated on importation documentation for Goldfish.

Date	**Exporting Country**	**Lab accession**	**Description of parasite treatment**
May 2015	Singapore-2	15/104	Malachite green
	Singapore-2	15/126	Malachite green
	Thailand-1	15/106	Formalin at 40 ppm
October 2015	Malaysia-1	15/180	Sodium chloride at 3g/L
	Malaysia-1	15/205	Sodium chloride at 3g/L
	Thailand-1	15/199	Formalin at 40 ppm
	Thailand-1	15/200	Formalin at 40 ppm

Table 11. Summary of parasite fauna found in ornamental fish collected immediately upon arrival in Australia and under quarantine. Parasite prevalence indicates the proportion of fish infected in each population (n = 30).

Lab accession	Fish species	Environment	Exporting country	Parasite identification	Parasite location	Prevalence (%)
15/019	*Pterapogon kauderni*	Marine	Singapore-1	Ciliate	Gall bladder	13.3
				Ceratomyxa sp.	Gall bladder	46.7
				Trematode	Digestive tract	6.7
				Myxidium sp.	Gall bladder	3.3
15/022	*Trichogaster lalius*	Freshwater	Singapore-1	Myxozoa	Gall bladder	30
				Ceratomyxa sp.	Gall bladder	3.3
				Actinosporea	Gall bladder	3.3
				Ciliate	Gall bladder	3.3
15/024	*Pterapogon kauderni*	Marine	Indonesia-1	*Ceratomyxa* sp.	Gall bladder	56.7
				Procamallanus sp.	Digestive tract	6.7
				Palliatus sp.	Gall bladder	3.3
				Henneguya sp.	Gall bladder	3.3
				Myxidium sp.	Gall bladder	3.3
15/025	*Pterapogon kauderni*	Marine	Indonesia-2	*Ceratomyxa* sp.	Gall bladder	60
				Myxidium sp.	Gall bladder	3.3
				Henneguya sp.	Gall bladder	3.3
				Procamallanus sp.	Digestive tract	16.7
15/030	*Cheilodipterus quinquelineatus*	Marine	Indonesia-2	*Kudoa* sp.	Muscle tissue	23.3
				Trematode	Digestive tract	3.3
15/107	*Amphiprion sebae*	Marine	Singapore-1	Trematode metacercaria	Gill lamella	33.3
				Tetraphyllidean metacestode	Digestive tract	10
				Anerbachia sp.	Gall bladder	6.66
				Ceratomyxa sp.	Gall bladder	3.3

Lab accession	Fish species	Environment	Exporting country	Parasite identification	Parasite location	Prevalence (%)
15/108	*Helostoma temmincki*	Freshwater	Singapore-1	*Myxidium* sp.	Gall bladder	16.66
				Cryptosporidium sp.	Digestive tract	86.6
				Trematode metacercaria	Gill lamella	3.3
15/109	*Ostorhinchus compressus*	Marine	Indonesia-2	*Kudoa* sp.	Muscle tissue	20
				Ceratomyxa sp.	Gall bladder	3.3
				Trematode metacercaria	Gill lamella	3.3
15/110	*Cheilodipterus quinquelineatus*	Marine	Indonesia-2	*Kudoa* sp.	Muscle tissue	43.3
				Ceratomyxa sp.	Gall bladder	13.3
15/111	*Amphiprion ocellaris*	Marine	Indonesia-2	*Trichodina* sp.	Skin scrape	3.7
15/101	*Pethia conchonius*	Freshwater	Sri Lanka-1	*Trichodina* sp.	Skin scrape	30
				Dactylogyrus ostraviensis	Gill lamella	33.3
15/102	*Danio rerio*	Freshwater	Sri Lanka-1	*Trichodina* sp.	Skin scrape	3.3
15/113	*Trichopodus trichopterus*	Freshwater	Sri Lanka-1	*Trianchoratus aecleithrium*	Gill lamella	6.6
				Trichodina sp.	Skin scrape	16.6
15/114	*Trichopodus leerii*	Freshwater	Sri Lanka-1	*Trianchoratus aecleithrium*	Gill lamella	17.2
15/104	*Carassius auratus*	Freshwater	Singapore-2	*Centrocestus* sp.	Gill lamella	63.3
				Myxobolus sp.	Gall bladder	30
				Dactylogyrus sp.	Gill lamella	66.6
				Apicomplexa	Muscle tissue	3.3
15/126	*Carassius auratus*	Freshwater	Singapore-2	*Argulus japonicus*	Filter paper	
				Centrocestus sp.	Gill lamella	66.6
				Dactylogyrus sp.	Gill lamella	33.3
				Gyrodactylus kobayashii	Skin scrape	6.6
				Myxobolus sp.	Gall bladder	30

Lab accession	Fish species	Environment	Exporting country	Parasite identification	Parasite location	Prevalence (%)
15/103	*Pethia conchonius*	Freshwater	Singapore-2	Trematode metacercaria	Gill lamella	46.6
				Dactylogyrus ostraviensis	Gill lamella	20
15/117	*Xiphophorus maculatus*	Freshwater	Singapore-2	*Camallanus* sp.	Digestive tract	3.3
				Centrocestus sp.	Gill lamella	73.3
				Gyrodactylus sp.	Skin scrape	3.3
				Myxozoa	Gall bladder	36.6
				Trianchoratus aecleithrium	Gill lamella	16.6
15/105	*Pethia conchonius*	Freshwater	Thailand-1	Coccidia	Skin scrape	80
				Dactylogyrus ostraviensis	Gill lamella	40
				Protozoa	Skin scrape	3.3
15/106	*Carassius auratus*	Freshwater	Thailand-1	*Centrocestus* sp.	Gill lamella	80
				Myxobolus sp.	Gall bladder	46.6
				Dactylogyrus sp.	Gill lamella	13.3
				Gyrodactylus kobayashii	Skin scrape	13.3
15/118	*Trichopodus trichopterus*	Freshwater	Thailand-1	*Trianchoratus aecleithrium*	Gill lamella	30
15/120	*Xiphophorus maculatus*	Freshwater	Thailand-1	Trematode metacercaria	Gill lamella	90
				Coccidia	Skin scrape	10
15/183	*Sphaeramia nematoptera*	Marine	Indonesia	*Cryptosporidium* sp.	Digestive tract	26.6
				Nematode	Digestive tract	3.3
				Trematoda	Digestive tract	13.3
				Myxobolus sp.	Gall bladder	3.3
15/185	*Amphiprion ocellaris*	Marine	Indonesia	*Cryptosporidium* sp.	Digestive tract	3.3
				Kudoa sp.	Muscle tissue	13.3
				Myxozoa	Gall bladder	3.3
15/186	*Amphiprion bicintus*	Marine	Indonesia	*Cryptosporidium* sp.	Digestive tract	73.3
				Protozoa	Skin scrape	13.3

Lab accession	Fish species	Environment	Exporting country	Parasite identification	Parasite location	Prevalence (%)
15/184	*Zoramia leptocantha*	Marine	Indonesia	*Ceratomyxa* sp.	Gall bladder	20
				Nematoda	Digestive tract	3.3
				Kudoa sp.	Muscle tissue	6.6
				Trematode metacercaria	Gill lamella	3.3
15/200	*Carassius auratus*	Freshwater	Thailand	*Centrocestus* sp.	Gill lamella	73.3
				Dactylogyrus baueri	Gill lamella	60
				Dactylogyrus intermedius	Gill lamella	30
				Dactylogyrus formosus	Gill lamella	40
				Dactylogyrus vastator	Gill lamella	16.6
				Dactylogyrus spp.	Gill lamella	23.3
				Myxobolus sp.	Gall bladder	40
				Trichodina sp.	Skin scrape	13.3
				Argulus japonicus	Filter paper	
				Nematode eggs	Digestive tract	10
				Protozoa 1	Muscle tissue	10
				Protozoa 2	Muscle tissue	3.3
15/199	*Carassius auratus*	Freshwater	Thailand	*Centrocestus* sp.	Gill lamella	86.6
				Dactylogyrus anchoratus	Gill lamella	26.6
				Dactylogyrus baueri	Gill lamella	23.3
				Dactylogyrus intermedius	Gill lamella	70
				Dactylogyrus formosus	Gill lamella	16.5
				Dactylogyrus vastator	Gill lamella	30
				Argulus japonicus	Filter paper	
				Scuticociliate	Gall bladder	10
				Myxobolus sp.	Gall bladder	16.6
15/198	*Pethia conchonius*	Freshwater	Thailand	*Dactylogyrus ostraviensis*	Gill lamella	46.6

Lab accession	Fish species	Environment	Exporting country	Parasite identification	Parasite location	Prevalence (%)
15/191	*Poecilia reticulata*	Freshwater	Sri Lanka	*Urocleioides reticulatus*	Gill lamella	86.6
15/180	*Carassius auratus*	Freshwater	Malaysia	*Centrocestus* sp.	Gill lamella	90
				Dactylogyrus sp.	Gill lamella	26.6
				Gyrodactylus kobayashii	Skin scrape	3.3
				Scuticociliate	Gall bladder	6.6
				Myxobolus sp.	Gall bladder	50
				Myxozoa	Gall bladder	10
				Clonorchis sp.	Digestive tract	3.3
15/205	*Carassius auratus*	Freshwater	Malaysia	*Dactylogyrus* sp.	Gill lamella	40
				Gyrodactylus kobayashii	Skin scrape	13.3
				Trichodina sp.	Skin scrape	3.3
				Centrocestus sp.	Gill lamella	53.5
				Myxobolus sp.	Gall bladder	46.4

Table 12: Summary of the results for pooling tissue homogenates from ornamental fish for the detection of megalocytivirus.

Pool		Expected frequency			Result				
Size	No. of positive homogenates	Prevalence 2%	Prevalence 10%	Prevalence 20%	No. of pools prepared	Expected positive tests	No. of positive tests	Probability of correct test (%)	95% Confidence limits
5	0	0.90	0.59	0.32	60	0	0	100	94.0-100
	1	0.10	0.34	0.42	60	60	41	68.3	55.0-79.7
	2	0	0.07	0.21	75	75	60	80	69.6-87.5
	3	0	0.01	0.05	60	60	54	90	79.9-95.3
10	0	0.81	0.34	0.10	60	0	1	98.3	91.1-99.7
	1	0.18	0.40	0.27	60	60	38	63.3	50.7-74.4
	2	0.01	0.20	0.31	75	75	65	86.7	77.2-92.6
	3	0	0.05	0.21	60	60	56	93.3	84.1-97.4
	4	0	0.01	0.09	60	60	51	85	73.9-91.9
	5	0	0	0.02	60	60	59	98.3	91.1-99.7

Table 13. Surveillance sensitivity using different combinations of sample size and pool size from a population with a prevalence of 2% for the detection of megalocytivirus in Kissing gourami (*H. temminckii*) and Dwarf gourami (*T. lalius*). The shaded boxes indicate a pool and sample size combination that achieved sensitivity greater than 95%.

Sample Size	**Pool size**					
	H. temminckii			***T. lalius***		
	1	**5**	**10**	**1**	**5**	**10**
150	0.96	0.48	0.50	0.96	0.89	0.80
160	0.97	0.50	0.52	0.97	0.91	0.83
170	0.98	0.52	0.54	0.98	0.92	0.85
180	0.98	0.54	0.56	0.98	0.93	0.86
190	0.99	0.56	0.58	0.99	0.94	0.88
200	0.99	0.58	0.60	0.99	0.95	0.89
210	0.99	0.60	0.62	0.99	0.96	0.90
220	0.99	0.62	0.63	0.99	0.97	0.92
230	1.00	0.64	0.65	0.99	0.97	0.93
240	1.00	0.65	0.67	1.00	0.98	0.93
250	1.00	0.67	0.68	1.00	0.98	0.94
260	1.00	0.68	0.70	1.00	0.98	0.95
270	1.00	0.69	0.71	1.00	0.99	0.95
280	1.00	0.71	0.72	1.00	0.99	0.96
290	1.00	0.72	0.74	1.00	0.99	0.97
300	1.00	0.73	0.75	1.00	0.99	0.97

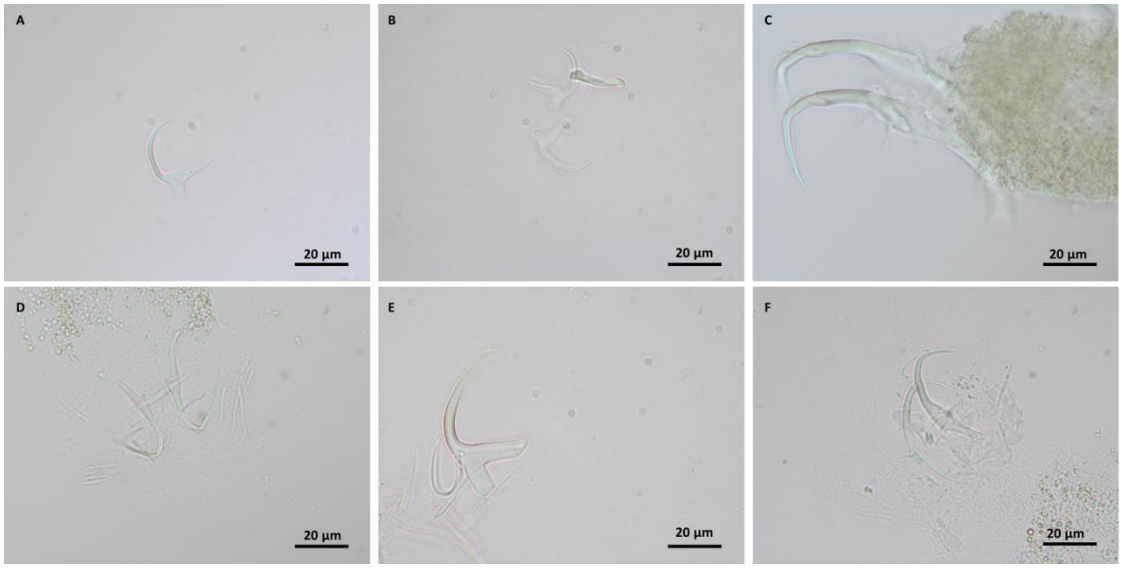

Figure 2. Hamuli morphology of *Dactylogyrus* species infecting *Carassius auratus* imported to Australia from southeast Asia. A) *Dactylogyrus intermedius*; B) *Dactylogyrus* sp.; C) *Dactylogyrus anchoratus*, D) *Dactylogyrus baueri*; E) *Dactylogyrus vastator* and; F) *Dactylogyrus formosus*. Image credit: David Vaughn.

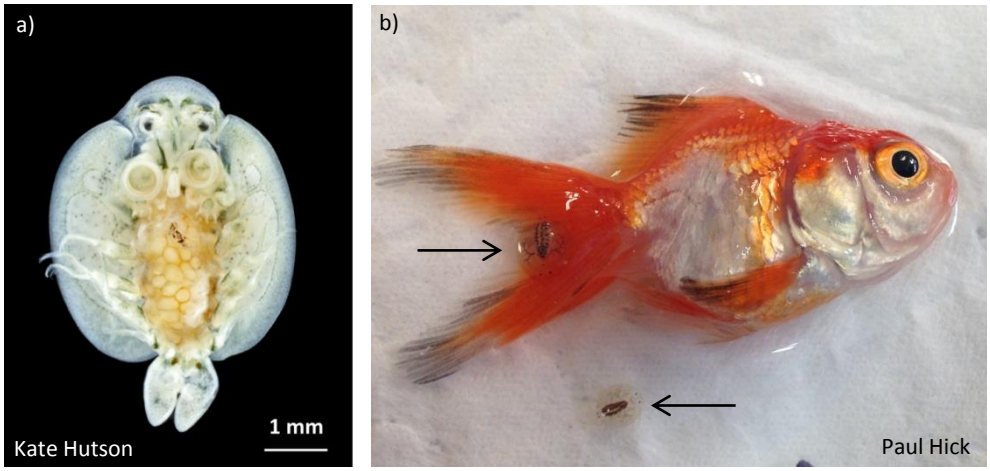

Figure 3. (a) Ventral view of a female *Argulus japonicus* (arrows) which was found in water used to transport Goldfish, *Carassius auratus* (b) from Singapore (15/126) and Thailand (15/199 and 15/200).

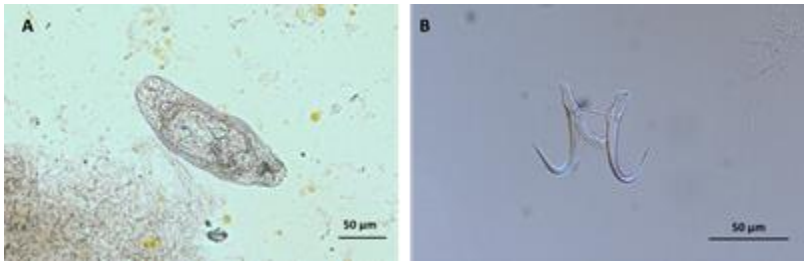

Figure 4. Representative *Gyrodactylus* sp. (A) and hamuli morphology (B) consistent with *Gyrodactylus kobayashii* collected from *Carassius auratus* consignments from Malaysia, Singapore and Thailand. Image credit: David Vaughn.

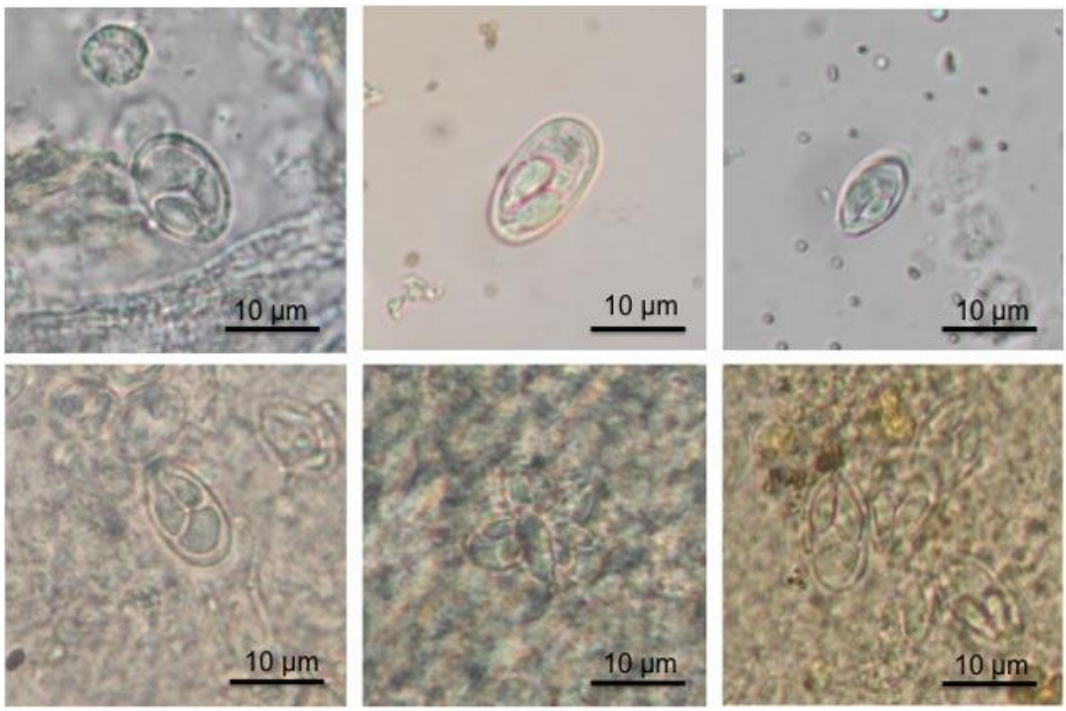

Figure 5. Representative *Myxobolus* specimens collected from *Carassius auratus*. Image credit: Alejandro Trujillo-Gonzalez.

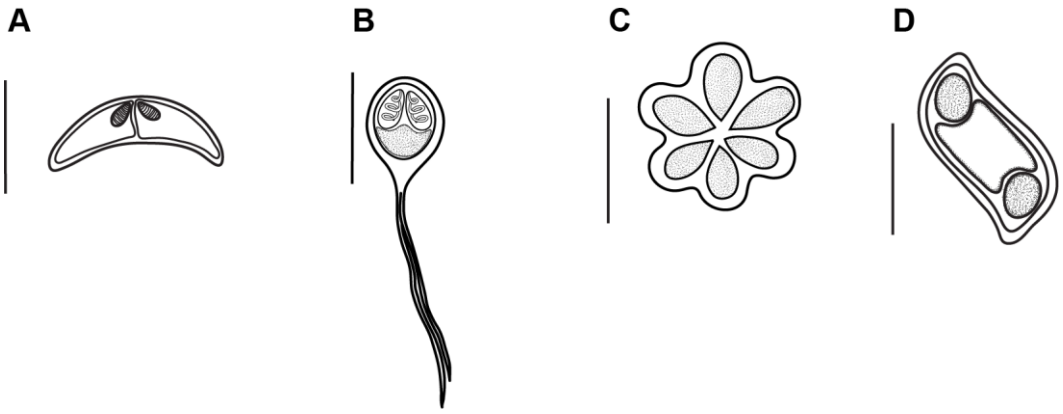

Figure 6. Representative line drawings for the myxozoan genera recovered during this survey. A) *Ceratomyxa* sp. B) *Henneguya* sp. C) *Kudoa* sp. D) *Myxidium* sp. Scale bars = 10µm. Image credit: Terrence Miller.

Figure 7. Representative specimen of larval trematodes of the family Heterophyidae found in all *Carassius auratus* populations, *Amphiprion bicintus*, *Cheilodipterus quinquelineatus*, *Helostoma temmincki*, *Ostorhinchus compressus*, *Pethia conchonius*, *Pterapogon kauderni* and *Sphaeramia leptocantha*.

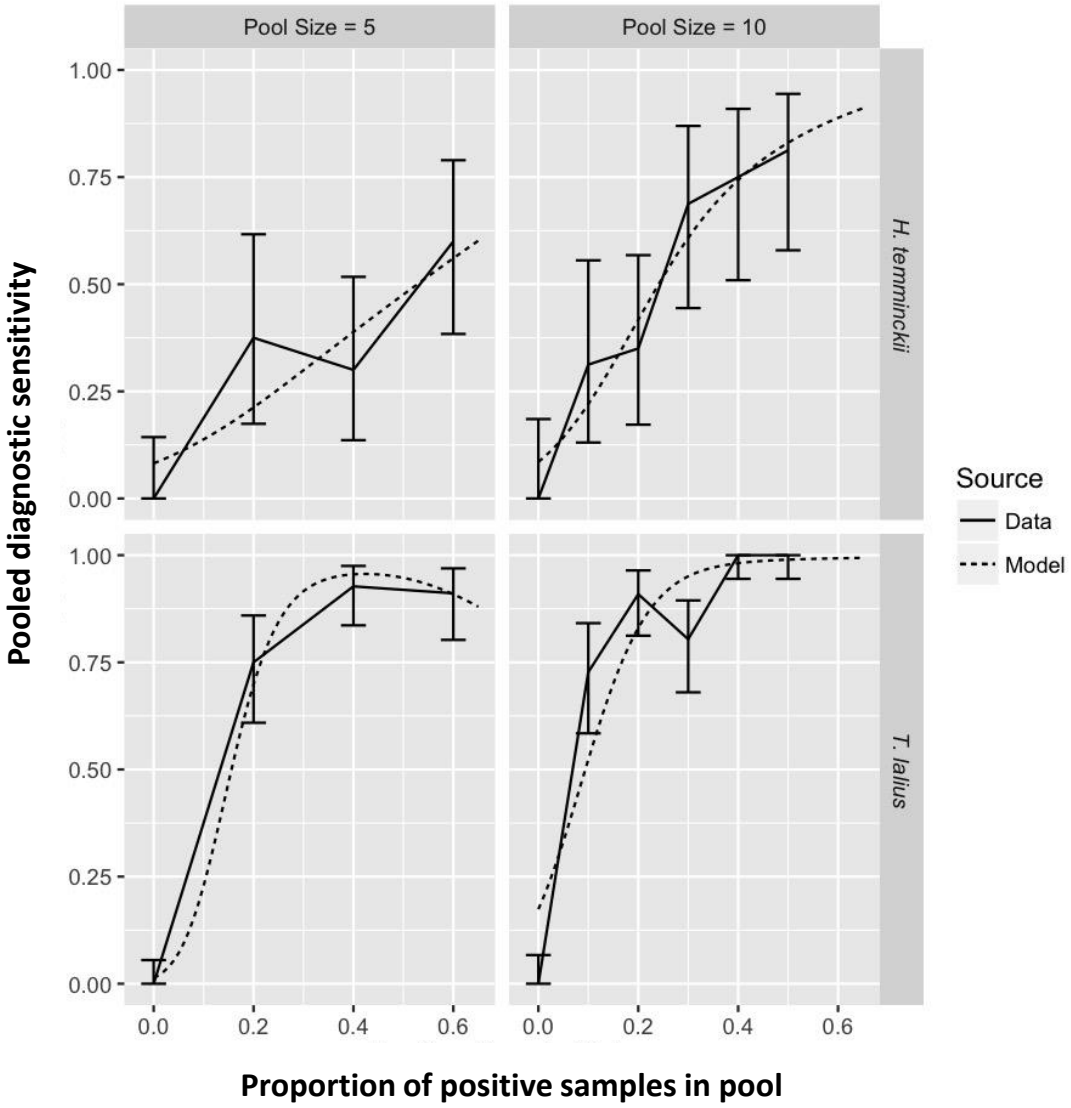

Figure 8. The generalized mixed-effects model for the diagnostic sensitivity experiment for the detection of megalocytivirus. Tissues homogenates from Kissing gourami (*H. temminckii*) and Dwarf gourami (*T. lalius*) were pooled as shown in Table 12.

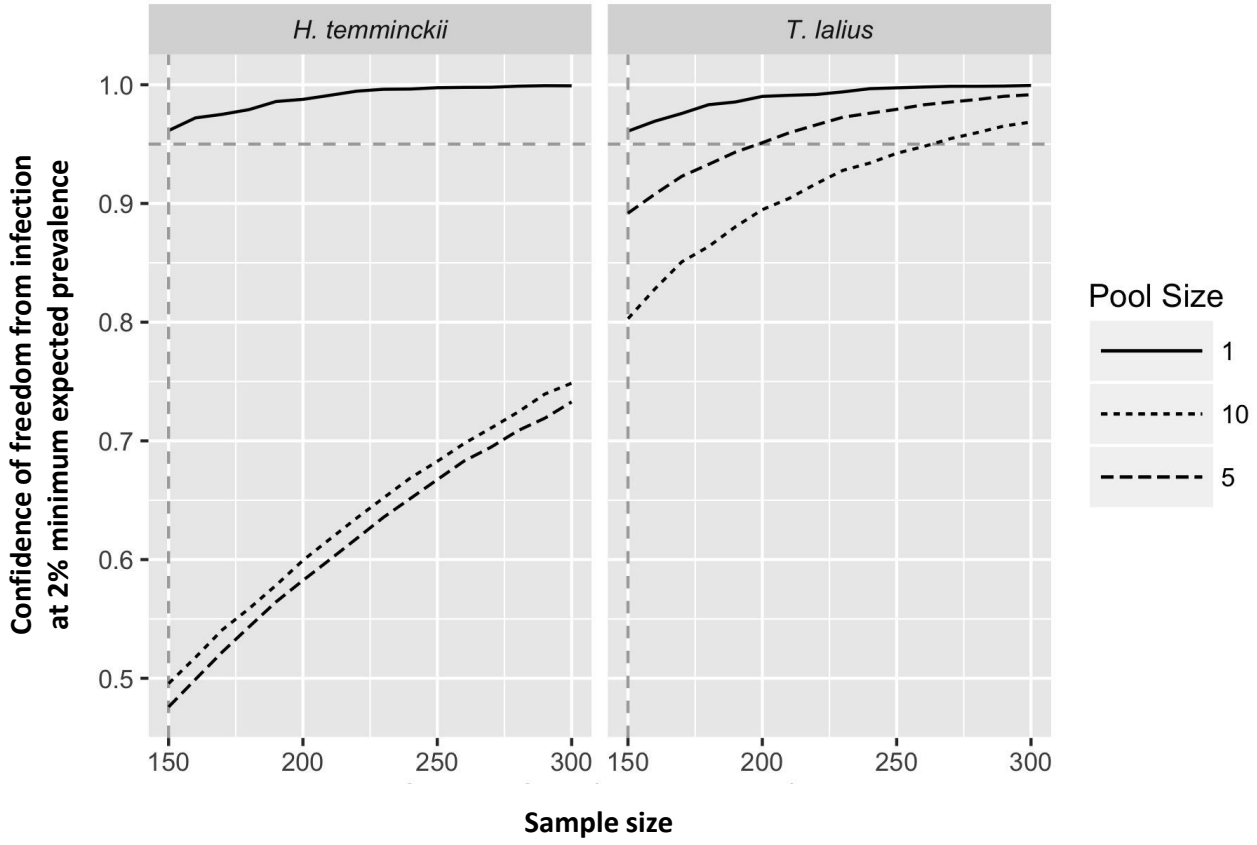

Figure 9. Simulation using the pooled sample sensitivity predicted by the generalized mixed-effects model to determine the probability of detecting an infection at 2% prevalence using different combinations of pool size and sample size.

Conclusion

Pathogens on Australia's national list of reportable disease that were not detected

Australia's biosecurity policy framework for the importation of ornamental fish was being met for the OIE-listed pathogens koi herpesvirus (KHV), spring viraemia of carp virus (SVCV) and viral haemorrhagic septicaemia virus (VHSV). All seven populations of Goldfish tested were free (within stated confidence intervals) of KHV, SVCV and VHSV. All four populations of Zebra danio were considered free (within stated confidence intervals) of SVCV and VHSV. KHV, SVCV and VHSV are considered exotic to Australia and there was no evidence of subclinical fish being imported to Australia.

Australia's biosecurity policy framework for the importation of ornamental fish was being met for the nationally-listed bacterial pathogens, *Edwardsiella ictaluri* and *Aeromonas salmonicida*. Three populations of Zebra danio and four populations of Rosy barb were considered free (within stated confidence limits) of *E. ictaluri*. All seven populations of Goldfish tested were free (within stated confidence intervals) of *A. salmonicida*. Neither bacterial species are considered exotic to Australia with the exception of *A. salmonicida* subspecies *salmonicida*.

Pathogens on Australia's national list of reportable disease that were detected

Megalocytivirus

Megalocytiviruses are known to be a cause of high mortality diseases that have seriously impacted aquaculture since they were first recognized in 1990 (Inouye et al., 1992). Megalocytiviruses infect both food fish and ornamental fish living in either marine or freshwater. Disease has occurred most frequently in east and south-east Asia (Subramaniam et al., 2012). Megalocytivirus is divided into three groups based on the sequence of the major capsid protein gene with the type species being *Infectious spleen and kidney necrosis virus* (ISKNV) (Song et al. 2008; Subramaniam et al. 2014). ISKNV is one of the viruses related to, but distinct from red sea bream iridovirus (RSIV). Megalocytiviruses are subject to regulation and notification requirements in many countries to protect regions that remain free of disease. Red sea bream iridoviral disease (RSIVD) is listed by the World Organization for Animal Health; it is defined by infection with either RSIV or ISKNV as causative agents (OIE, 2012).

In agreement with previous research (Becker et al., 2013 (FRDC 2009/044); Rimmer et al., 2015), the pre-export biosecurity measures and associated health certificate were insufficient to detect and prevent fish with subclinical infections with *Megalocytivirus* from entering Australia. *Megalocytivirus* was detected in more than half of fish populations tested for the virus. Positive fish populations were found in the consignments received from eight of nine exporters.

Of note, five of nine marine ornamental fish species were positive for Megalocytivirus. To meet the import permit conditions, all marine fish were wild caught and away from aquaculture premises. Currently, marine ornamental fish do not require additional health certification for freedom from infection with Megalocytivirus (Australian Department of Agriculture, 2014a). Eight freshwater ornamental fish species were positive for Megalocytivirus. All freshwater species are known hosts for Megalocytivirus and fall under the additional health certification for freedom from infection (Australian Department of Agriculture, 2014a)

Nervous necrosis virus

Nervous necrosis viruses have a global distribution and are the cause of high mortality disease of predominantly marine larval and juvenile fish. Less frequently, disease attributed to NNV infection has been reported in some freshwater species and in adult fish. The extent of impacts on wild fish has not been determined. NNV has been a cause of disease in marine fish hatcheries in Australia and is on the list of reportable diseases. The range of fish species susceptible to disease is broad and subclinical infection is common. This provides a reservoir for recurrence of disease in endemic locations. Presently, just one of the four genotypes of NNV is present in Australia. Regulations are used to prevent translocation of NNV from areas where it is abundant to epidemiological units including aquaculture facilities and waterways where it is relatively less abundant or absent. Fish infected with NNV identified in this study were exported from Indonesia and were wild caught Banggai cardinalfish and Threadfin cardinalfish. NNV was not detected in populations of fish received Singapore, Sri Lanka, Thailand or Malaysia.

Biosecurity risk mitigation needed for parasites associated with the importation of goldfish

Current import conditions for goldfish were not effective to eliminate undesired dactylogyrid gill monogeneans. Specifically, *Dactylogyrus vastator* was found infecting goldfish in three separate fish populations. Although *Dactylogyrus extensus* was not detected, at least five other *Dactylogyrus* species and one *Gyrodactylus* species survived chemical treatment of goldfish seven days prior to importation. There are several explanations for this observation including: 1) the chemical treatments applied may not be effective against these species; 2) the appropriate dose of the chemical was not supplied or; 3) the adult parasites or egg stage is resistant to chemical treatment. An appropriate method of eradication that accounts for multiple mechanisms of invasion, needs to be identified to ensure import conditions are being met.

Biosecurity risk mitigation needed for parasites associated with the importation of ornamental fish

Live ornamental fishes present a high risk group for introducing aquatic animal diseases into Australia because they are vectors of parasitic diseases of high quarantine significance. This study showed that significant parasitic disease agents (ciliates, myxozoans, helminths and a crustacean) infected freshwater and marine pre-import ornamental fishes. It is clear from previous studies that disease agents including ciliates, helminths and cestodes have been spread into native Australian fish populations from alien ornamental fish released into the wild, causing significant disease and ecological damage (e.g. Ashburner 1976; Langdon 1988; 1990; Rowland & Ingram 1991; Humphrey 1995a; 1995b; Dove 1998; 2000; Dove & Ernst 1998; Dove & Fletcher 2000; Dove & O'Donoghue 2005). In all cases, these adverse effects are most likely to be permanent and irreversible. This indicates the need for strategic and pragmatic treatment of fish to limit unreasonable restrictions on importers of ornamental fish.

Strategies for pooled sample testing for *Megalocytivirus* by qPCR

The pooling of fish for virus testing should be considered for surveillance for freedom from infection. The reduction of pooled diagnostic sensitivity compared to diagnostic sensitivity for individual fish can be overcome to achieve the desired surveillance sensitivity when the sample size can be feasibly increased above 150. This can reduce laboratory costs with a lower number of PCR tests. However, pooled sensitivity is strongly influenced by the pathobiology of the infection and the range in expected virus load between fish with sub-clinical infection. Pooled

testing is highly effective when the prevalence is >10% which might be informed by prior knowledge and risk based surveillance or this might be used as a screening test to rapidly identify populations with high prevalence of infection. Thus, the applicability of pooling should be considered on a case-by-case basis.

Implications

Biosecurity risk mitigation needed for Megalocytivirus

Improved understanding of the pathobiology of MCV infection and disease requires research to identify the range of prevalence and virus load that impact detection by qPCR and how these relate to fish that pose a risk of biosecurity breach through release of infectious viruses. Evaluation of different assay protocols could identify improvements in test sensitivity. Even more advantageous for improved biosecurity will be extension activities to improve testing in the responsible laboratories in exporting countries. Further knowledge is needed about the risk pathways for MCV. This will be used to inform a risk-based surveillance system which can inform an increase in the minimum expected prevalence. Under this scenario consignments can be screened for infected fish populations and reduce the need for resource intensive tests.

Pooled testing may decrease the cost of biosecurity intervention for some MCV surveillance scenarios, because fewer tests applied to pools of fish can provide nominal sensitivity with fewer tests applied to a larger number of fish. At present, pooling is not suitable for certification of freedom from infection at 2% minimum expected prevalence with 95% confidence for all potential scenarios. Further knowledge is required to utilise pooled testing.

The detection of NNV in imported ornamental fish demonstrates that this is a potential pathway for introduction of this pathogen. This should be considered in protecting at risk epidemiological units including aquaculture facilities that maintain freedom from infection and waterways in which potentially susceptible native fish spawn.

Biosecurity risk mitigation needed for parasitic agents

The results of this study show that freshwater and marine ornamental fish imports arrived to Australia with a diversity of external and internal parasite fauna. While pre-import fish were evaluated in this study, it is apparent that the subsequent conditions of Australian quarantine are unlikely to detect or eradicate the diversity of parasite agents infecting imported ornamental fish. Indeed, parasites may live on or within a host for all or part of their lives but not cause clinical disease. The risk of parasitic disease outbreaks for the ornamental trade industry is considerable, but is higher for agents where captive conditions may exacerbate amplification and spread. Consumers may also be impacted when they purchase infected fish,

because heavy parasite burdens can inhibit growth or longevity of stock. The subsequent risk to the aquaculture industry and our natural resources results from the irresponsible disposal of infected or moribund fishes.

Recommendations

1. Revision of biosecurity policy to prevent incursions of exotic megalocytiviruses from imported ornamental fish into domestic stocks. The project findings indicate that the health certification at exporting countries was insufficient to prevent fish with Megalocytivirus being exported to Australia. These results are of particular relevance to marine ornamental fish species as they are not included under existing policy. Currently, there is no requirement for marine fish to be specifically declared free of *Megalocytivirus*. We recommend that policy revision and new policy development include the requirement for marine species belonging to the Family *Apogonidae*, Genus *Amphiprion* and Genus *Chromis* to require additional health certification to be free of *Megalocytivirus*. Additional taxonomic groupings of fish species should be added to the biosecurity policy framework as new species-specific knowledge becomes available. There is a need for research to determine the risk of transmission that is posed by fish with subclinical infection and a survey of other marine species that are imported to Australia.

2. Revision of biosecurity policy to prevent incursions of parasitic agents. The project findings indicate that the health certification at exporting countries was insufficient to prevent fish external and internal parasites from being exported to Australia. We recommend a requirement of an effective parasite treatment within three days prior to export for all freshwater and marine ornamental fish. The health certificate requirements should include a description of the parasite treatment including, chemical name and manufacturer, chemical concentration, dosage rate and exposure time to chemical.

3. The health certification requirements for freshwater ornamental finfish imported to Australia should be reconsidered with respect to the relative biosecurity risk presented by parasitic disease agents. Parasitic agents with broad host specificity such as the crustaceans *Lernaea cyprinacea* (reported from 59 species of fish representing 23 families; but not detected in this study) and *Argulus japonicus* (29 species from 10 fish families; detected in this study) are likely become co-invasive and present a high risk to natural resources and aquaculture industries. We recommend that freedom from *A. japonicus* and

L. cyprinacea be specifically listed on the health certification for the import conditions for freshwater ornamental fish.

4. The risk ornamental fish imports present to Australian aquatic animal industries and natural resources is high. This is illustrated by the detection of live crustaceans (*Argulus japonicus*) in goldfish populations in this study. Further examination of museum records provided evidence for multiple introductions of *A. japonicus* through the ornamental trade since the 1960s. It is well known that the release of Goldfish, which are native to eastern Asia, has been widespread in Australia and feral populations are widespread in the Murray-Darling Basin. In view of this finding, and numerous other freshwater and marine parasite agents, it is recommended that transport water and fish should be treated for external and internal parasite infections by Australia ornamental importers.

5. From the project findings, visual inspection of consignments was not effective at detecting misidentified fish species and did not prevent importation of live parasite fauna. A total of 170 individually bagged marine fish were misidentified as members belonging to the genus *Apogon*. The fish were identified by project staff to be *Cheilodipterus quinquelineatus*. The number of ornamental species allowed for import and phenotypic diversity within each fish species is vast. Further, the consignments for which live *Argulus japonicus* were collected contained 50 to 60 Goldfish (3 cm) in a single bag for air transport from Thailand. It is recommended that the health certification require a standardization of the naming of fish species used (such as fishbase.org) and taxonomy training for quarantine staff to identify species. It is recommended that a maximum number of fish per bag be specified in the import conditions. Factors such as fish species and fish size/length should be considered in determining the maximum number of fish per bag. The new proposed risk based surveillance program for ornamental fish relies heavily on visual inspection as the first step in the cascade at quarantine for identifying suspect consignments.

6. The pooling of fish for MCV testing should be considered only with more detailed knowledge of the range of infection scenarios. Pooling of tissue homogenates was used for *T. lalius* to achieve nominal surveillance sensitivity with fewer tests (but more fish sacrificed). However, for *H. temminckii* pooling was not always acceptable due to a different virus load, stage of infection or host fish species. The impact of pooling using

different molecular assays for MCV remains to be determined. It should be determined using experiment designs with diagnostic samples in addition to evaluations of analytical sensitivity.

Further development

1. Ornamental fish were shown to be subclinical carriers of MCV. There is limited information available regarding the pathogenesis of ISKNV in subclinically-infected fish and under what host and environmental conditions they can shed live virus and infect other individuals. An experimental challenge study has shown that Murray cod held at 27°C will succumb to infectious spleen and kidney necrosis when cohabitated with Dwarf gourami subclinically-infected with MCV (Go and Whittington, 2006). Further research is needed to identify under what conditions MCV-infected fish shed this pathogen (or undergo disease recrudescence). Practical, industry-relevant environmental factors (e.g. sudden changes in temperature, high nitrogen conditions, organic loading, crowding) need to be evaluated under controlled conditions. Trials should simulate realistic conditions that ornamental fish are likely to encounter throughout the supply chain (e.g. long and short haul transportation, wholesale and retail holding facilities).

2. A follow up survey of pre-import freshwater and marine ornamental fish under quarantine is needed now that the health certification requires the freedom from infection with ISKNV-like viruses. The importation of marine ornamental fish is a pathway for introduction of MCV and NNV into Australia. From the project findings, there is a need for a repeated cross sectional survey to test imported marine ornamental fish for subclinical infections with MCV and NNV. Of particular note is the need to test for red sea bream iridovirus, which is listed by the OIE. Research is needed to identify marine ornamental fish species that can carry subclinical MCV infections. This will support the revision of biosecurity policy to prevent incursions of exotic megalocytiviruses.

3. The factors that impact the sensitivity of surveillance using qPCR tests of pooled samples should be determined. Detailed evaluation of two different populations in this study identified that empirical recommendations and simple extrapolation of analytical data did not predict pooled diagnostic sensitivity. The ability to detect populations infected at low prevalence is likely to depend on viral load, stage of infection and host fish species as well as the assay used. Knowledge of the importance of these factors will help develop the most efficient surveillance strategies

4. Quantify the success of anthelminthic treatments on specific parasite groups to determine whether mandatory approved treatments are having the desired effect. High host-specificity exhibited by the majority of parasite species means that only a limited number of host-species may be vulnerable to parasite epidemics. However, many of the parasite taxa identified in this survey have low host specificity, which suggests they may threaten native fauna if an incursion occurs. This is difficult to predict and has devastating implications for naïve fish hosts that have no adaptive immunity. For example, the goldfish anchor 'worm' *Lernaea cyprinacea* has been reported from 59 species of fish from 23 families).

5. There is a need to quantify the release pathways for ornamental fish infected with pathogens of biosecurity significance in Australia. In particular, there is a need to assess the human-assisted release pathways for ornamental fish into natural waterways. This knowledge will support the proposed changes to the way that the Department of Agriculture and Water Resources manages the disease risks associated with imported ornamental fish. The proposed changes include the removal of quarantine and the introduction of on-arrival health surveillance based on visual inspection.

Extension and Adoption

The project findings are of significance to national biosecurity policy and will have direct benefit to many stakeholders. The findings indicate the need for a revision of quarantine policy to prevent incursions of high risk pathogens (e.g. *Megalocytivirus*, *Dactylogyrus vastator*, *Argulus japonicus*) from imported ornamental fish becoming established in Australia. Extension of the key outcomes of the project will be ongoing through consultations with the Department of Agriculture and Water Resources and the domestic aquaculture industry.

During the project, extension of the key findings to government and industry stakeholders was achieved by conference presentations. During the project, there were numerous informal and formal contacts with staff from the Department Agriculture and Water Resources in Sydney and Canberra. After the project, extension of the key findings will be mainly through scientific articles and conference presentations.

Project coverage

Print media

Becker, J.A. 2016. Why You Should Never Put A Goldfish In A Park Pond Or Down The Toilet, The Conversation; https://theconversation.com/why-you-should-never-put-a-goldfish-in-a-park-pond-or-down-the-toilet-56808

Becker, J.A. 2016. Why You Should Never Put A Goldfish In A Park Pond Or Down The Toilet, iflscience.com; http://www.iflscience.com/plants-and-animals/why-you-should-never-put-goldfish-park-pond-or-down-toilet/

Radio

Dr Joy Becker from the Faculty of Veterinary Science spoke to 891 ABC Adelaide about caring for pet fish

Dr Joy Becker from the Faculty of Veterinary Science was interviewed on 720 ABC Perth about the origins and life expectancy of gold fish and how best to care for them.

Project materials developed

Conference Abstracts

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Competition

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Appendices

Appendix 1. List of researchers and project staff

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Kate Hutson
Terrence Miller
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Appendix 2: Intellectual Property
This project has not developed any intellectual property that requires legal protection.

Appendix 3: Supplemental information

Table 1. From the project application for FRDC 2014/001, the proposed list of aquatic pathogens and their associated ornamental fish hosts.

Fish species	Common name	Aquatic pathogen on Australia's list of reportable diseases							Total number of assays per fish species
		Aeromonas salmonicida	**dwarf gourami iridovirus DGIV**	***Edwardsiella ictaluri* ESC**	**koi herpesvirus KHV (CyHV3)**	**spring viraemia of carp virus SVCV**	**viral haemorrhagic septicaemia virus VHSV**	**viral nervous necrosis virus NNV**	
Carassius auratus	Goldfish	✔			✔	✔	✔		4
Danio rerio	Zebrafish			✔		✔	✔		3
Pethia conchonius	Rosy barb			✔					1
Poecilia reticulata	Guppy		✔					✔	2
Pterapogon kauderni	Banggai cardinal fish		✔					✔	2
Amphiprion ocellaris	Common clownfish		✔					✔	2
Total number of assays per pathogen		1	3	2	1	2	2	3	

Table 2. Summary of sampling events for FRDC 2014/001.

Sampling Event	Dates	JCU Staff and Students	USyd Staff and Students	Number of consignments received
1	14 - 23 January 2015	Dr Kate Hutson, Alejandro Truijllo Gonzalez, Josh Allas	Drs Joy Becker, Paul Hick and Richard Whittington; Alison Tweedie, Anna Waldron, Rebecca Mauer, Vickie Patten, Sophia Johnson	3
2	25 May – 6 June 2015	Drs Kate Hutson and Terry Miller; Alejandro Truijllo Gonzalez, Josh Allas	Drs Joy Becker, Paul Hick, Richard Whittington, Alison Tweedie, Anna Waldron, Rebecca Mauer, Vickie Patten, Ann-Michele Whittington, Sophia Johnson	5
3	20 – 31 October 2015	Drs Kate Hutson and Terry Miller; Alejandro Truijllo Gonzalez, Josh Allas	Drs Joy Becker, Paul Hick and Richard Whittington; Alison Tweedie, Anna Waldron, Rebecca Mauer, Vickie Patten, Ann-Michele Whittington, Sophia Johnson	4

Table 3: Details of primers and probes used in qPCR assays for viral pathogens.

Assay		**Primer**			**Reference**
Virus	**Detection chemistry**	**Name**	**5'-3' sequence**	**Concentration (nM)**	
Megalocytivirus (MCV)	SYBR Green	C1073	AATGCCGTGACCTACTTTGC	500	Rimmer et al., 2012
		C1074	GATCTTAACACGCAGCCACA	500	
Nervous necrosis virus (NNV)	Hydrolysis probe	QR2TF	CTTCCTGCCTGATCCAACTG	250	Hick and Whittington, 2010
		QR2TR	GTTCTGCTTTCCCACCATTTG	250	
		QR2probe	6-FAM-CAACGACTGCACCACGAGTTG-BHQ1	200	
Koi herpesvirus (KHV)	SYBR Green	KHV_C1149	GTACGTGAGGAACACGGTCA	500	Whittington et al., 2009
		KHV_C1150	GTGTAGGTCTCGAAGCGGAC	500	
Spring viraemia of carp virus (SVCV)	Hydrolysis probe	SVCV_UP	CATTCAAAGGATTGCATCAG	250	Zhang, et al., 2009
		SVCV_LOW	TTCTGTTCATTTAGAGCCATATG	250	
		SVCV_Probe	6-FAM-TCCCCCTCAAAGTTGCGGATGG-BHQ1	200	
Viral hemorrhagic septicaemia virus (VHSV)	Hydrolysis probe	VHSV-FW	AAACTCGCAGGATGTGTGCGTCC	900	Jonstrup, et al., 2013
		VHSV-BW	TCTGCGATCTCAGTCAGGATGAA	900	
		VHSV-probe	6-FAM-TAGAGGGCCTTGGTGATCTTCTG-BHQ-1	250	

Table 4. Sequence analysis for selected MCV positive samples all identified as ISKNV-like genotypes. Data are the number of bases different to the ISKNV reference sequence (GenBank accession AF371960) / the number of bases with quality sequence data within each amplification target. Differentiation of ISKNV from RSIV-like genotypes was based on the presence of a 9 base deletion in the ATPase gene sequence of ISKNV and less than 5 single nucleotide polymorphism in common with the RSIV reference strain (BD143114) out of 31 SNPs in the concatenated regions sequenced.

Sample				Gene fragment sequenced[1]			Genotypic
Date	Lab ID	Common name	Country	MCP	IRB6	ATPase	Identity
Jan.	15/019 -11	Banggai cardinal fish	Singapore-1	0 / 157	3 / 545	6 / 455	ISKNV-like
2015	15/019 -34	Banggai cardinal fish	Singapore-1	0 / 161	3 / 545	6 / 462	ISKNV-like
	15/024 -5	Banggai cardinal fish	Indonesia-1	0 / 163	n/d	n/d	ISKNV-like
	15/024 -47	Banggai cardinal fish	Indonesia-1	0 /163	n/d	n/d	ISKNV-like
	15/030 -8	Five-lined cardinalfish	Indonesia-2	0 / 166	n/d	n/d	ISKNV-like
	15/030 -21	Five-lined cardinalfish	Indonesia-2	0 / 166	n/d	n/d	ISKNV-like
	15/030 -57	Five-lined cardinalfish	Indonesia-2	0 / 118	n/d	n/d	ISKNV-like
	15/030 -59	Five-lined cardinalfish	Indonesia-2	0 / 166	n/d	n/d	ISKNV-like
	15/030 -60	Five-lined cardinalfish	Indonesia-2	0 / 166	n/d	n/d	ISKNV-like
	15/032 -13	Five-lined cardinalfish	Indonesia-2	0 / 166	n/d	n/d	ISKNV-like
	15/032 -14	Five-lined cardinalfish	Indonesia-2	0 / 166	n/d	n/d	ISKNV-like
	15/032 -16	Five-lined cardinalfish	Indonesia-2	3 / 166	1 / 545	1 / 462	ISKNV-like
	15/032 -28	Five-lined cardinalfish	Indonesia-2	0 / 166	n/d	n/d	ISKNV-like
	15/031 -76	Blue green damselfish	Indonesia-2	3 / 166	n/d	n/d	ISKNV-like
	15/031 -85	Blue green damselfish	Indonesia-2	3 / 166	n/d	n/d	ISKNV-like
	15/031 -86	Blue green damselfish	Indonesia-2	3 / 166	n/d	n/d	ISKNV-like
May	15/107 -26	Sebae anemonefish	Singapore-1	n/p	n/p	n/p	Not determined
2015	15/107 -28	Sebae anemonefish	Singapore-1	n/p	n/p	n/p	Not determined
	15/108 -92	Kissing gourami	Singapore-1	low quality	0 / 518	0 / 462	ISKNV-like
	15/108 -173	Kissing gourami	Singapore-1	low quality	0 / 387	0 / 462	ISKNV-like
	15/109 -48	Ochre-striped cardinalfish	Indonesia-2	n/p	n/p	n/p	Not determined
	15/112 -171	Guppy	Sri Lanka-1	n/p	n/p	n/p	Not determined
	15/114 -121	Pearl gourami	Sri Lanka-1	n/p	n/p	n/p	Not determined
	15/117 -144	Southern platy	Singapore-2	2 / 96	1 / 219	0 / 462	ISKNV-like
	15/128 -209	Three spot gourami	Singapore-2	n/p	n/p	n/p	Not determined
	15/128 -174	Three spot gourami	Singapore-2	n/p	n/p	n/p	Not determined
	15/128 -192	Three spot gourami	Singapore-2	n/p	n/p	n/p	Not determined

Sample Date	Lab ID	Common name	Country	Gene fragment sequenced[1]			Genotypic Identity
				MCP	IRB6	ATPase	
	15/128 -200	Three spot gourami	Singapore-2	n/p	n/p	n/p	Not determined
	15/118 -44	Three spot gourami	Thailand-1	low quality	0 / 509	0 / 462	ISKNV-like
	15/120 -74	Southern platyfish	Thailand-1	low quality	low quality	0 / 462	ISKNV-like
	15/122 -19	Dwarf gourami	Thailand-1	low quality	1 / 220	0 / 462	ISKNV-like
	15/122 -81	Dwarf gourami	Thailand-1	4 / 125	0 / 480	0 / 462	ISKNV-like
Oct.	15/176 -71	Three spot gourami	Malaysia-1	low quality	low quality	0 / 462	ISKNV-like
2015	15/177 -99	Green swordtail	Malaysia-1	4 / 94	0 / 362	0 / 462	ISKNV-like
	15/181 -39	Freshwater angelfish	Malaysia-1	6 / 102	0 / 353	2 / 462	ISKNV-like
	15/182 -34	Dwarf gourami	Malaysia-1	low quality	0 / 361	0 / 462	ISKNV-like
	15/192 -92	Green swordtail	Sri Lanka-2	low quality	low quality	0 / 462	ISKNV-like
	15/193 -42	Southern platyfish	Sri Lanka-2	4 / 111	0 / 360	0 / 462	ISKNV-like
	15/197 -32	Three spot gourami	Sri Lanka-2	n/p	1 / 361	0 / 462	ISKNV-like
	15/202 -44	Three spot gourami	Thailand-1	low quality	low quality	0 / 462	ISKNV-like

[1]n/p: No amplification product in conventional PCR; n/d: sequencing not attempted; low quality: chromatograms were of insufficient quality to determine the sequence

Appendix 4: Health certification requirements for the importation of freshwater ornamental (aquarium) fish

Excerpt from the BICON database accessed on 25 October 2016:

Annex: Health certification requirements for the importation of freshwater ornamental (aquarium) fish

1. Only fish listed in the list of permitted live freshwater fish suitable for import are included in this consignment, and are documented on the attached invoice.

2. The fish in the consignment have been inspected within seven (7) days prior to export and show no clinical signs of infectious disease or pests.

3. The export premises are currently approved for export to Australia as meeting standards under the Department of Agriculture and Water Resources.

4. All fish being held at the export premises exhibit no clinical signs of significant infectious disease or pests and are sourced from populations not associated with any significant disease or pests within the 6 months prior to certification.

5. All fish in the consignment have been in approved export premises for 14 days prior to export.

6. The fish have not been kept in water in common with farmed foodfish (fish farmed for human consumption including recreational fishing) or koi carp.

7. Adequate quarantine safeguards are in place to maintain the health status of the certified fish until export. The fish are effectively isolated in holding systems that prevent infection by direct contact with other fish or indirect contact via water, equipment or any other means.

Additional health certification requirements if the consignment includes *gouramis*[1], *bettas*, paradise fish, *cichlids*, and *poeciliids* exported to Australia
(Strike through and initial the option 1. or 2. that does not apply):

1. Source Population Freedom
The fish originate from a country, zone or export premises determined by the Competent Authority to be free from megalocytiviruses consistent with the procedures described in Additional health certification criteria and procedures for *gouramis, bettas,* paradise fish, *cichlids and poeciliids*, exported to Australia or

2. Batch Test Negative
The batch of consigned fish have been tested by the Competent Authority and found negative for megalocytiviruses consistent with definitions and testing methodology described in Additional health certification criteria and procedures for *gouramis, bettas,* paradise fish, *cichlids and poeciliids* exported to Australia.

[1]*Gouramis*, *bettas* and paradise fish include *all* species belonging to subfamilies *Luciocephalinae* and *Macropodusinae* of the family *Osphronemidae* listed in the list of permitted live freshwater fish suitable for import.

Additional health certification requirements if the consignment includes goldfish (*Carassius auratus*)

1. The goldfish originate from a country, zone or export premises (the population) determined to be free from spring viraemia of carp virus (SVCV) and *Aeromonas salmonicida* (other than goldfish ulcer disease strains) based on:

   1.1. the absence of clinical, laboratory or epidemiological evidence of these agents in the source fish population in the previous 2 years, and;

   1.2. a system of monitoring and surveillance for the previous 2 years acceptable to the Competent Authority and consistent with theAdditional health certification criteria and for goldfish exported to Australia.

2. All goldfish in the consignment have been treated with an effective parasiticide (e.g. trichlorfon, formaldehyde, sodium chloride) during the seven (7) days prior to export to Australia to eliminate infestation by the gill flukes *Dactylogyrus vastator* and *D. extensus.*