FRDC
FISHERIES RESEARCH & DEVELOPMENT CORPORATION

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

Nicholas JG Moody, Peter G Mohr and Mark StJ Crane

May 2021

FRDC Project No 2015-005

© 2021 Fisheries Research and Development Corporation
All rights reserved.

ISBN **978-1-925994-27-8**

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

2015-005

2021

Ownership of Intellectual property rights
Unless otherwise noted, copyright (and any other intellectual property rights, if any) in this publication is owned by the Fisheries Research and Development Corporation and CSIRO Australian Centre for Disease Preparedness

This publication (and any information sourced from it) should be attributed to Moody NJG, Mohr PM and Crane MStJ, CSIRO, 2021, Aquatic Animal Health and Biosecurity Subprogram: Determining the susceptibility of Australian *Penaeus monodon* and *Penaeus merguiensis* to newly identified enzootic (YHV7) and exotic (YHV9 and YHV10) Yellow head virus (YHV) CSIRO Centre for Disease Preparedness**,** April. CC BY 3.0

Creative Commons licence
All material in this publication is licensed under a Creative Commons Attribution 3.0 Australia Licence, save for content supplied by third parties, logos and the Commonwealth Coat of Arms.

Creative Commons Attribution 3.0 Australia Licence is a standard form licence agreement that allows you to copy, distribute, transmit and adapt this publication provided you attribute the work. A summary of the licence terms is available from https://creativecommons.org/licenses/by/3.0/au/. The full licence terms are available from https://creativecommons.org/licenses/by-sa/3.0/au/legalcode.

Inquiries regarding the licence and any use of this document should be sent to: frdc@frdc.com.au

Disclaimer
The authors do not warrant that the information in this document is free from errors or omissions. The authors do not accept any form of liability, be it contractual, tortious, or otherwise, for the contents of this document or for any consequences arising from its use or any reliance placed upon it. The information, opinions and advice contained in this document may not relate, or be relevant, to a reader's particular circumstances. Opinions expressed by the authors are the individual opinions expressed by those persons and are not necessarily those of the publisher, research provider or the FRDC.

The Fisheries Research and Development Corporation plans, invests in and manages fisheries research and development throughout Australia. It is a statutory authority within the portfolio of the federal Minister for Agriculture, Fisheries and Forestry, jointly funded by the Australian Government and the fishing industry.

Researcher Contact Details

Name:	Dr Nicholas Moody
Address:	CSIRO Australian Centre for Disease Preparedness
	Private Bag 24, Geelong VIC 3220
Phone:	+61 3 5227 5749
Fax:	+61 3 5227 5555
Email:	nick.moody@csiro.au

FRDC Contact Details

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

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

Contents

Tables

Figures

Acknowledgments

The authors are grateful to Tony Charles, Australian Prawn Farmers Association (APFA) Research and Development Committee, for organizing provision of experimental prawns. Farmed *Penaeus monodon* and prawn feed were obtained from Pacific Reef Fisheries, Ayr, Queensland, and farmed *Penaeus merguiensis* were obtained from Seafarms, Cardwell, Queensland.

We appreciate the assistance from the ADCP Histology Team and Drs Ian Anderson and Roger Chong, Biological Sciences Laboratory, for expert histological examination.

The authors acknowledge the capabilities of the Australian Centre for Disease Preparedness (grid.413322.5) in undertaking this research.

Funding for this project was provided by the Fisheries Research and Development Corporation on behalf of the Australian Government.

Abbreviations

ABARES	Australian Bureau of Agricultural and Resource Economics and Sciences
AHPND	Acute hepatopancreatic necrosis disease
C_T	Threshold cycle
CVO	Chief Veterinary Officer
EtOH	Ethanol
GAV	Gill-associated virus, Yellow head virus genotype 2
HPV	Hepatopancreatic parvovirus
IHHNV	Infectious hypodermal and haematopoietic necrosis virus
IMNV	Infectious myonecrosis virus
IRA	Import Risk Analysis
MBV	*Penaeus monodon*-type baculovirus
MoV	Mourilyan virus
nPCR	nested polymerase chain reaction
OIE	Office International des Epizooties (World Organisation for Animal Health)
PBSA	Phosphate-buffered saline without Ca^{++} and Mg^{++} ions (pH 7.4)
PCR	polymerase chain reaction
qPCR	real-time (quantitative) polymerase chain reaction
RT-PCR	reverse-transcriptase polymerase chain reaction
RT-nPCR	nested RT-PCR
RT-qPCR	real-time reverse-transcriptase (quantitative) polymerase chain reaction
TSV	Taura syndrome virus
v/v	volume per volume
WSSV	White spot syndrome virus
YHV	Yellow head virus
YHV1	Yellow head virus genotype 1
YHV2	Yellow head virus genotype 2
YHV3	Yellow head virus genotype 3
YHV4	Yellow head virus genotype 4
YHV5	Yellow head virus genotype 5
YHV6	Yellow head virus genotype 6
YHV7	Yellow head virus genotype 7
YHV8	Yellow head virus genotype 8
YHV9	Yellow head virus genotype 9
YHV10	Yellow head virus genotype 10

Executive Summary

What the report is about

In recent years, new genotypes within the yellow head complex of viruses have been discovered in farmed prawns both within Australia and overseas. This report describes research undertaken at the CSIRO Australian Centre for Disease Preparedness Fish Diseases Laboratory (ACDP AFDL), Geelong, Victoria to determine the pathogenicity of three of these new genotypes to prawn species significant to the Australian prawn farming industry. As part of the project, new diagnostic tests (RT-qPCR assays) were evaluated and determined to be highly sensitive and specific and can be used for monitoring of domestic prawn populations as well as commodity prawns imported into Australia, enhancing Australia's biosecurity capability.

Background

The yellow head complex of viruses is made up of at least 10 genotypes of viruses in the genus *Okavirus*. *Yellow head virus* genotype 1 (YHV1) is the only known agent to cause yellow head disease. Until January 2013, a total of six genotypes were recognised in the yellow head disease complex, viz., YHV1, *gill associated virus*, YHV2, YHV3, YHV4, YHV5 and YHV6. YHV genotypes 3-6 were detected in healthy *Penaeus monodon*. More recently, 4 other genotypes have been discovered with very little knowledge concerning their pathogenicity and significance to the prawn fisheries and aquaculture sector.

During an investigation of diseased *P. monodon* from Queensland in January 2013, anomalous test results, relating to test specificity with the frontline real-time and conventional PCR assays, were observed. AFDL determined that the CSIRO YHV1 RT-qPCR and the OIE Protocol 2 RT-nPCR cross-reacted with GAV, resulting in false-positive results for the exotic YHV1. Coincidentally, a new genotype, YHV7, was identified in the *P. monodon* after positive results with the OIE Protocol 2 RT-PCR. This false-positive result was further evidence of the lack of specificity of this assay. All results were confirmed using OIE Protocol 3 and sequencing of amplicons. Through an awareness of specificity issues for tests detecting YHV1 and evaluation of new candidate assays specific for YHV1, two new YHV-complex genotypes (YHV-9 and YHV-10) were also discovered in imported commodity prawns from China in 2014.

The pathogenicity and impact of YHV7, YHV9 and YHV10 to Australian prawn species was not known.

Aims/objectives

1. Determine the susceptibility of P. monodon and P. merguiensis to YHV-complex Genotype 7 (YHV7)

2. Determine the susceptibility of P. monodon and P. merguiensis to YHV-complex Genotype 8 (YHV8)

3. Determine the susceptibility of P. monodon and P. merguiensis to YHV-complex Genotype 10 (YHV10)

4. Transfer protocols and controls for diagnostic tests to state diagnostic laboratories

Methodology

Susceptibility to infection was determined by exposing experimental prawns to infectious doses of each genotype and monitoring the health of the exposed prawns. The experiments were undertaken in the high-level bio-secure facility at the CSIRO ACDP where animal experiments with exotic viruses can be

undertaken with no risk of virus escape to the Australian environment. Three Experimental Trials were undertaken. Experimental Trial 1 aimed to amplify limited material and assess the pathogenicity of the different YHV genotypes by a direct, unnatural transmission route (i.e. injection) at 24°C. Based on Experimental Trial 1 results, Experimental Trial 2 investigated pathogenicity by natural routes of infection (i.e. feeding with experimentally generated infected material and co-habitation with experimentally infected prawns) for YHV1 and YHV2 at 29°C. Prawns were again exposed to YHV9, and a new inoculum preparation of YHV10 with a lower C_T value, by injection due to an absence of disease by either genotype after infection at 24°C in Experimental Trial 1. Experimental Trial 3 repeated the exposure of *P. monodon* to YHV7 by injection, co-habitation and feeding at 24°C, to confirm the observed increase in pathogenicity of YHV7 at this temperature, based on the results from Experimental Trials 1 and 2.

Prawns were monitored daily for signs of disease, including mortality, and with samples collected for analyses including PCR testing to determine the presence of the viruses in dead, dying and surviving prawns, and histopathological examination to identify any pathology caused by exposure to the different YHV genotypes. Prawns were also exposed to YHV1 in Experimental Trials 1 and 2 as a positive infection control and to confirm the pathogenicity of this exotic YHV genotype to *P. monodon* and *P. merguiensis*.

The protocols developed during this project can be used as a model to assess the pathogenicity of new and emerging pathogens.

Results/key findings

1. *P. monodon* and *P. merguiensis* are highly susceptible to infection with YHV1. Infection demonstrated that YHV1 is highly pathogenic to both species as acute mortalities of 100% in both species were observed after 4 to 6 days after exposure to YHV1 by injection and feeding and 9 to 10 days after exposure by co-habitation.

2. *P. monodon* were susceptible to infection with YHV7, after exposure by injection, co-habitation and feeding, although the pathogenicity of YHV7 was lower compared to infection with YHV1, as the mortalities observed were less acute than mortalities observed after infection with YHV1. Infection of *P. monodon* with YHV7 at 24°C resulted in higher mortalities than infection at 29°C so YHV7 is more pathogenic to *P. monodon* at 24°C than at 29°C.

3. While mortalities were observed after infection of *P. merguiensis* at both temperatures, the levels of mortality were similar to those observed in negative control animals. As high levels of YHV7 RNA were detected in prawns, *P. merguiensis* appear to be susceptible to infection with YHV7, but refractory to disease. Therefore, while *P. merguiensis* are susceptible to infection with YHV7, this YHV genotype does not appear to be pathogenic to *P. merguiensis* as infection did not result in appearance of clinical signs of disease.

4. *In situ* hybridization using YHV2- and YHV7-specific probes is required to confirm whether the lesions observed were caused by either YHV2 or YHV7 or both YHV genotypes.

5. Both *P. monodon* and *P. merguiensis* are susceptible to infection with YHV9, but refractory to disease.

6. *P. monodon* and *P. merguiensis* are not susceptible to infection with YHV10 based on the trials undertaken at ACDP.

7. The detections of YHV7, YHV9 and YHV10 and issues with specificity of the YHV2 RT-qPCR, highlight the need to fully investigate anomalous test results and confirm detections of pathogens

by real-time PCR screening methods, particularly those associated with disease, by conventional PCR and sequence analysis.

Implications for relevant stakeholders

The results presented in this report are based on experimental infections in an artificial environment. However, the occurrence of disease (demonstrated by mortalities, molecular test results and histological analysis) confirm the highly pathogenic nature of the exotic YHV1 to both *P. monodon* and *P. merguiensis*.

While based on experimental infections, the mortalities generated after exposure to *P. monodon,* by all routes of infection, indicate that YHV7 is pathogenic to *P. monodon*. Disease, based on mortalities observed after exposure, was more severe at 24°C than at 29°C. Screening for YHV7 should be included in the suite of pathogens tested for as part of disease investigations into increased mortalities in Australian prawn farms.

Recommendations

1. Australian stocks of *P. monodon* and *P. merguiensis* are highly susceptible to infection with YHV1 and biosecurity protocols need to retain YHV1 as a significant pathogen to be excluded.

2. As YHV7 has been added to several prawn translocation and health screening protocols, with an increasing number of laboratories undertaking testing for YHV7, consideration should be given to including YHV7 as a pathogen within the crustacean panel of the DAWE-funded National Aquatic Proficiency Testing Program.

3. A number of recommendations for additional research have been provided in this report, including generating a better understanding of the pathogenicity of YHV7 at different temperatures, improving our knowledge of the pathology associated with observed mortalities and improving the specificity of diagnostic tests.

Keywords
Yellow head virus, YHV1, YHV2, YHV7, YHV9, YHV10, *Penaeus monodon*, *Penaeus merguiensis*, pathogenicity, bioassay, real-time PCR, qPCR.

Introduction

Australian prawn production, 25 kilotonnes in 2016/17, is valued at AU$400 million. The prawn fishery is an important natural resource that supports a substantial export industry. Prawn aquaculture is a significant industry in northern Australia and accounts for approximately 20% of the total volume of Australian prawn production (ABARES, 2018). Two species of prawns, *Penaeus monodon* and *Penaeus merguiensis*, form the basis for the prawn farming industry in Australia. Previous research on viral diseases of prawns has demonstrated that these species are susceptible to several known viral pathogens (Walker and Winton 2010, Gudkovs et al. 2015), including the OIE-listed yellow head virus genotype 1 (OIE 2015).

Yellow head virus genotype 1 (YHV1) emerged in the early 1990s as the cause of mass mortalities of giant tiger prawns (*P. monodon*) in Thailand (Boonyaratpalin et al. 1993). In Thailand, YHV1 continues to cause production losses and has become established as a pathogen in the Pacific white prawn (*Penaeus vannamei*) (Senapin et al. 2010). YHV1 has subsequently been reported from most *P. monodon* farming regions and has emerged in Pacific blue prawn (*Penaeus stylirostris*) in the Gulf of California (Castro-Longoria et al. 2008). While YHV1 is exotic to Australia, a closely related genotype, *gill-associated virus* (GAV; YHV2), was discovered not long after the discovery of YHV1 (Spann et al. 1995). YHV2 is enzootic to Australia, can occur at high prevalence in wild and farmed *P. monodon* in Eastern Australia and can cause disease on farms (Spann et al. 1997). Until January 2013, a total of six genotypes were recognised in the Yellow Head Disease complex, viz., YHV1, YHV2, YHV3 YHV4, YHV5 and YHV6 (Wijegoonawardane et al. 2008). YHV genotypes 3-6 were detected in healthy *P. monodon*. Subsequently, YHV7, YHV8 YHV9 and YHV10 were identified (described in detail below). A summary of the detections of YHV1 to YHV10 and their phylogenetic relationship to each other is presented in Appendix 1.

YHV1 is OIE-listed and PCR assays are described in the *OIE Manual of Diagnostic Tests for Aquatic Animals*: OIE YHV Protocol 1 (RT-PCR specific for YHV1), OIE YHV Protocol 2 (RT-nPCR specific for YHV1 and YHV2 with differentiation of YHV1 and YHV2 in the nested PCR), and OIE YHV Protocol 3 (RT-nPCR specific for YHV1 to YHV7) (OIE, 2019). An unpublished real-time PCR assay, CSIRO YHV RT-qPCR, has also been used as a screening assay (Cowley et al. pers. comm.). During an investigation of diseased *P. monodon* from Queensland in January 2013, anomalous test results, relating to test specificity, with the CSIRO YHV RT-qPCR and the OIE YHV Protocol 2 RT-nPCR were obtained at the CSIRO-ACDP Fish Diseases Laboratory (AFDL). AFDL determined that the CSIRO YHV RT-qPCR and the OIE YHV Protocol 2 RT-nPCR cross-reacted with YHV2, resulting in false-positive results for the exotic YHV1. This cross-reactivity was more likely to occur if the nested PCR for OIE Protocol 2 was not multiplexed, which can be done to increase test sensitivity to generate amplicons for sequence confirmation of where real-time PCR screening generates high C_T values. When the nested PCR is run as a singleplex assay, YHV2 will generate an amplicon of almost the expected size using the primer specific for YHV1. Coincidentally, a new genotype, YHV7, was identified in the *P. monodon* samples following positive results with the OIE YHV Protocol 2 RT-nPCR (Mohr et al. 2015). In this case, an amplicon was generated in the primary RT-PCR of the OIE YHV Protocol 2 RT-nPCR, with no subsequent amplicons generated for the nested PCRs. Generating a primary PCR amplicon but no nested PCR amplicons flagged the result as atypical and requiring further investigation, which led to the identification of YHV7. This false-positive result was further evidence of the lack of specificity of this assay. All results were confirmed using OIE YHV Protocol 3 RT-nPCR and sequencing of the PCR amplicons.

A FRDC Tactical Research Fund project (2013/036; Cowley et al. 2015) was funded in 2013 with objectives including: To determine what YHV genotypes exist and their prevalence in wild *P. monodon* in NT/WA/QLD, and to revise PCR assay designs to ensure their specificity, particularly in differentiating the highly virulent YHV1 genotype. As work was being undertaken to evaluate new candidate assays specific for YHV1, opportunistic testing of submissions resulted in the detection and identification of two new YHV-complex genotypes (YHV9 and YHV10) in commodity prawns imported from China in 2014. As

appropriate, the Australian CVO advised the Chinese CVO of the detection of YHV9 and YHV10 in imported prawns of Chinese origin.

An additional yellow head virus genotype (YHV8) was published by Chinese scientists (Liu et al. 2014) and its sequence submitted to GenBank. From discussions with Chinese contacts, YHV8 is apparently highly pathogenic to at least some species of farmed prawns. There are currently three recognized species within the *Okavirus* genus of the Family *Roniviridae*; *Gill-associated virus* (GAV; YHV-2); *Yellow head virus* (YHV-1) and *Okavirus* 1 (YHV-8) with a further five related, unclassified viruses; YHV-3, YHV-4, YHV-5, YHV-6 and YHV-7 (Walker et al., 2021). Mohr et al. (2015) reported that within the ORF1b region, YHV-7 shared <86% nucleotide identity with YHV genotypes 1 to 6. Additional analysis of sequences generated from YHV9 and YHV10 for this region demonstrated that these two genotypes, detected in imported commodity prawns, shared <91% nucleotide identity with YHV genotypes 1 to 7 (Authors, unpublished). For simplicity, the different YHV genotypes in this report will be referred to as YHV1, YHV2, YHV7, YHV9 and YHV10.

As mentioned above, YHV1 is exotic to Australia, is an OIE-listed pathogen, and can cause 100% mortality in 3-5 days following infection. YHV2 is enzootic, occurs commonly in *P. monodon* in Australia and has been associated with Mid-Crop Mortality Syndrome (Wijegoonawardane et al. 2008). However, the pathogenicity of the newly detected YHV-complex genotypes to Australian prawns is unknown. While YHV7 was detected in diseased *P. monodon*, its role in the disease is unknown. YHV9 and YHV10 were detected in imported commodity prawns from China.

As the virulence and pathogenicity of YHV7, YHV9 and YHV10 to Australian prawns is unknown, the aim of this project is to determine the pathogenicity of these newly discovered YHV genotypes to two prawn species, commercially farmed in Australia.

This project will generate knowledge regarding the susceptibility of Australian farmed *P. monodon* and *P. merguiensis* to the newly discovered enzootic YHV7 and exotic YHV9 and YHV10 genotypes. This information is critical for policymakers, regulators, managers and producers to implement appropriate biosecurity measures. The project aligns with Key Research Area 6.2.1 of the FRDC Aquatic Animal Health and Biosecurity Subprogram's R&D Plan "Knowledge about new and emerging infectious diseases" and value-adds to the FRDC TRF 2013/036 project.

Objectives

1. Determine the susceptibility of *P. monodon* and *P. merguiensis* to YHV-complex genotype 7 (YHV7)
2. Determine the susceptibility of *P. monodon* and *P. merguiensis* to YHV-complex genotype 9 (YHV9)
3. Determine the susceptibility of *P. monodon* and *P. merguiensis* to YHV-complex genotype 10 (YHV10)
4. Transfer protocols and controls for diagnostic tests to state diagnostic laboratories

Note: The original application referred to YHV8 in Objective 2. Between the submission of the project application and writing of this report the YHV genotype reported from China was published as YHV8. This has resulted in the isolate AFDL detected in imported commodity prawns, originally referred to as YHV8 in Objective 2 being renamed YHV9 as this is a genetically different YHV genotype based on comparison with published sequence from the Chinese YHV8

Experimental Trial 1: Pilot study and amplification of virus

The aims of Experimental Trial 1 were:

1. To attempt to amplify viral material which was in limited supply to provide sufficient material for all subsequent experimental infections.

2. To provide an initial indication of the pathogenicity of YHV1, YHV7, YHV9 and YHV10 to *P. monodon* and *P. merguiensis* through direct exposure by injection.

1.1 Methods

1.1.1 Experimental animals

Sourcing and delivery of prawns was organized through the Australian Prawn Farmers Association (APFA) Research and Development Committee. Farmed *P. monodon* (~8-10 cm in length) and farmed *P. merguiensis* (~7-9 cm in length) were obtained from two prawn farms in north Queensland. On arrival, prawns were transferred to the ACDP Large Animal Facility (LAF) and placed in round or square polypropylene tanks containing 80 L of 100-µm-filtered seawater, a canister filter and air stones. Prawns were distributed across 5 rooms (one for each of negative controls, YHV1, YHV7, YHV9 and YHV10 inocula). Each tank held 22 to 25 prawns with rooms maintained at a temperature of 24±1°C. At least one tank in each room was fitted with a data-logger to measure water temperature for the duration of the experiment. Animals were monitored and fed commercial prawn pellets daily and 50% water exchanges were undertaken every 2 days. Prior to infection, 25 animals of each species were sacrificed, 10 of which were fixed in 80% (v/v) EtOH for PCR testing and the other 15 fixed in Davidson's Fixative for histological analysis (as described below).

1.1.2 Preparation of inocula

Fresh prawn tissues containing YHV7, YHV9 and YHV10, held at -80°C at ACDP, were the primary sources of infected material for preparation of the inocula used in the infectivity trials (Table 1). Tissues were tested by PCR to determine the health status of prawns with regard to other known enzootic pathogens of prawns (Table 2). Prawn haemolymph from YHV1-infected prawns, archived in liquid nitrogen in ACDP, was used as a positive infection control as it is known that *P. monodon* are susceptible to infection with development of severe clinical disease after exposure to YHV1.

Table 1. Source of inocula

YHV Genotype	ACDP ID	Source location	Source tissue
YHV1	98-07770	Thailand (exotic)	Haemolymph
YHV7	13-00839-04	North Queensland	½ × prawn head
YHV9	14-01978-02	China	2½ × prawn tails
YHV10 Trial 1	14-01329-02	China	2 × prawn tails
YHV10 Trial 2	16-01798-08	Not provided	5 x prawn tails

On the day of injection, inocula were prepared from frozen, archived material, as follows.

- YHV1: Haemolymph, stored in liquid nitrogen, was thawed and diluted 1/1000 in PBSA (pH 7.4).
- YHV7: Pool of samples generated from soft tissues (~15g) from confirmed YHV7-infected prawns were separated from the carapace and minced into smaller pieces in a petri dish. Minced tissues were transferred to a mortar and pestle (chilled to -20°C) and ground to a paste. PBSA (50 ml) was added and when the tissue had thawed the suspension was transferred to a Schott bottle, diluted 1/10 with PBSA, and mixed well. Approximately 50 ml was poured into a stainless-steel sieve and pushed though the sieve using the plunger from a 50 ml syringe. The suspension was clarified by centrifugation at 1000 × *g* for 10 min at 18°C. The supernatant was carefully decanted, the material centrifuged again, and the second supernatant, containing any virus particles present, was filtered (0.45 µm) to remove any contaminating bacteria.
- YHV9: Pool of samples generated from prawn tail tissue which were processed as for YHV7 above, with only one centrifugation step required.
- YHV10: Pool of samples generated from prawn tail tissue which were processed as for YHV7 above, with only one centrifugation step required.

A 140 µl aliquot from each inoculum was stored at -80°C for nucleic acid extraction and PCR testing with the remainder split into 2 × 20 ml aliquots. The inocula were stored at 4°C and transported to the LAF rooms on wet ice. To prevent any cross-contamination, negative control animals and those infected with each YHV genotype were held in separate rooms in the LAF. Separate overalls and footwear were provided for personnel in each room containing prawns inoculated with each of the YHV genotypes and a 3-minute shower was required on exiting each of these rooms for the duration of the trial.

1.1.3 Injection of prawns, sampling and sample processing

Prawns were transferred to a 10 L bucket containing aerated seawater prior to injection. For injection a prawn was caught with a net and held firmly to avoid tail-flick response. Each was inoculated with 100 µl of the inoculum into the second abdominal segment using a 26-gauge needle and syringe then returned back to the original tank. For each YHV genotype, prawns in two replicate tanks for each species were infected by injection. One tank of negative control prawns for each species was sham inoculated with 100 µl PBSA (Tank A) with the second tank (Tank B) receiving no treatment.

Experimental animals were maintained in 80 L seawater (as above) and monitored for signs of acute disease for a total of 28 days. Dead or moribund prawns were removed. Tissues were sampled for PCR testing (placed in 80% EtOH or frozen) and histopathological analysis (placed in Davidson's Fixative), as follows. The gills and pleopods from one side of dead prawns were removed and placed in a labelled 5 ml container containing 80% (v/v) EtOH. The rest of the prawn was placed in a labelled zip-lock bag and stored at -80°C. The gills and pleopods from one side of moribund prawns were removed and placed in a labelled 5 ml container containing 80% (v/v) EtOH. The tail, posterior to the carapace, was removed and placed in a labelled zip-lock bag and stored at -80°C. The head was then cut in half longitudinally with both halves put in Davidson's Fixative in a labelled 50 ml specimen container or, alternatively, the head was injected with Davidson's Fixative and placed in a labelled 50 ml specimen container containing fixative. After 24 hours, tissues were transferred from Davidson's Fixative to 70% (v/v) EtOH.

1.1.4 Nucleic acid extraction

Tissues were homogenised in 2 ml Lysing Matrix D tubes, containing 600 µl PBSA buffer, using FastPrep-24 or FastPrep-5G bead-beaters (MP Biomedicals). The homogenates were clarified by centrifugation at 10 000 × *g* for 5 min and total nucleic acid extracted from 50 µl of the supernatant using the MagMAX-

96™ Viral RNA Isolation Kit on a MagMAX™ Express-96 Deep Well Magnetic Particle Processor (Life Technologies). Nucleic acid was eluted in 50 µl elution buffer and stored at -80°C prior to testing.

1.1.5 Molecular tests

Molecular tests were used for confirmation of the presence of the YHV genotypes, to determine the presence of other adventitious viruses in the inocula, to identify YHV genotypes which may have been present in the prawns used for the infectivity trials, and to test the inoculated animals are detailed in Table 2. Candidate real-time (RT-qPCR) and conventional (RT-nPCR) assays, specific for YHV1 and YHV7, have been designed at ACDP. This work was undertaken as a component of FRDC Project 2013/036 (Cowley et al. 2015). Assays for YHV9 and YHV10 were designed using the same principles, as part of this project. As samples were tested, results were used for assay evaluation and updating of validation dossiers using OIE guidelines for test validation (OIE 2019a). Complete details of all assays are provided in Appendix 2.

Table 2. Molecular assays used during the project

Assay	Reference
Real-time PCR assays	
CSIRO YHV RT-qPCR	Cowley, personal communication
AFDL YHV1 RT-qPCR	AFDL in-house
CSIRO YHV7 RT-qPCR	Cowley et al. (2019)
De la Vega YHV2 RT-qPCR	de la Vega et al. (2004)
AFDL YHV9 RT-qPCR	AFDL in-house
AFDL YHV10 RT-qPCR	AFDL in-house
OIE AHPND PirA qPCR	Han et al. (2015)
OIE TSV RT-qPCR	Tang et al. (2004)
CSIRO WSSV qPCR	Sritunyalucksana et al. (2006)
Conventional PCR assays	
AFDL YHV9 RT-nPCR	AFDL in-house
OIE 309 IHHNV PCR	Tang et al. (2007)
OIE IMNV RT-nPCR	Poulos and Lightner (2006)
MBV PCR	Surachetpong et al. (2005)
MoV RT-nPCR	Cowley et al. (2005)
HPV nPCR	Waqairatu (unpubl.), Cowley et al. (2015)
OIE Decapod PCR	Lo et al. (1996)

1.1.6 Histopathology

Tissues for histology were processed for paraffin-embedding, sectioning and H&E staining using standard procedures. Sections were examined using a compound light microscope.

1.2. Results

1.2.1 Molecular testing of the inocula

Tissues and haemolymph were initially tested by removal of a small subsample, with the primary purpose of confirming the presence of the target YHV genotype. Results of testing the inoculum are presented in Table 3. The pre-trial *P. monodon* were positive for YHV2 with relatively high C_T values (indicating a low level of infection). The YHV1 inoculum contained a Mourilyan virus (MoV) variant most closely related to Wenzhou shrimp virus-1, the YHV7 inoculum contained an hepatopancreatic parvovirus (HPV) variant most closely related to HPV2 and HPV3 and both the YHV9 and YHV10 inocula also contained an HPV strain most similar to an Australian strain of HPV. The YHV10 inoculum also tested positive using the YHV9 RT-qPCR and RT-nPCR assays. All samples were positive using the OIE Decapod PCR demonstrating the nucleic acid extractions were successful and PCR inhibition was not significant.

Table 3. Molecular test results for each YHV genotype inoculum

Assay	Inoculum Result				Pre-trial *P. monodon*	Pre-trial *P. merguiensis*
	YHV1	YHV7	YHV9	YHV10		
Real-time PCR assays						
CSIRO YHV RT-qPCR	**POS (25.2)**	Neg	**POS (28.1)**	**POS (34.6)**	Not tested	Not tested
AFDL YHV1 RT-qPCR	**POS (25.9)**	Neg	Neg	Neg	Not tested	Not tested
CSIRO YHV7 RT-qPCR	Neg	**POS (13.4)**	Neg	Neg	Neg	Neg
de la Vega YHV2 RT-qPCR	Neg	**POS (31.4)**	Neg	Neg	**POS (27-32)**	Neg
AFDL YHV9 RT-qPCR	Neg	Neg	**POS (25.4)**	**POS (32.4)**	Not tested	Not tested
AFDL YHV10 RT-qPCR	Neg	Neg	Neg	SUSPECT (Neg/36.7)	Not tested	Not tested
Han AHPND PirA qPCR	Neg	Neg	Neg	Neg	Not tested	Not tested
OIE TSV RT-qPCR	Neg	Neg	Neg	Neg	Not tested	Not tested
CSIRO WSSV qPCR	Neg	Neg	Neg	Neg	Not tested	Not tested
Conventional PCR assays						
AFDL YHV9 RT-nPCR	Neg	Neg	**POS**	**POS**	Not tested	Not tested
OIE 309 IHHNV PCR	Neg	Neg	Neg	Neg	Not tested	Not tested
OIE IMNV RT-nPCR	Neg	Neg	Neg	Neg	Not tested	Not tested
MBV PCR	Neg	Neg	Neg	Neg	Not tested	Not tested
MoV RT-nPCR	**POS**[1]	Neg	Neg	Neg	Not tested	Not tested
HPV nPCR	Neg	**POS**[2]	**POS**[3]	**POS**[4]	Not tested	Not tested
OIE Decapod PCR	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**

Numbers in brackets for the real-time PCR assays indicate mean C_T values

[1]Nucleotide sequence shared 99.6% identity with Wenzhou shrimp virus-1 (KM817719)

[2]Nucleotide sequence shared 81.7% and 81.4% identity with *Fenneropenaeus chinensis* hepatopancreatic densovirus (GU371276) and Hepatopancreatic parvovirus 2 (EU247528), respectively.

[3]Nucleotide sequence shared 97.2% identity with HPV Australia (DQ458781)

[4]Nucleotide sequence shared 94.5% identity with HPV Australia (DQ458781)

1.2.2 Mortalities in inoculated prawns

Cumulative mortalities for each species inoculated with each genotype are presented in Figures 1 to 4. No negative control (uninfected, both sham injected and untreated) *P. monodon* died, while negative control mortalities were 21.5% (sham injected) and 6.1% (untreated) in *P. merguiensis* at the end of the 28-day trial.

After injection with YHV1, in both *P. monodon* and *P. merguiensis*, 100% mortality was observed at 6- and 5-days post-injection, respectively (Figure 1a and 1b). The only clinical sign of disease observed were prawns found lying on their side.

Injection of YHV7 resulted in a mean mortality rate of 62% (60.9% and 63.6%) in *P. monodon* at the termination of the trial at 28 days (Figure 2a). Mortalities started at 9 days post-injection. This is considered an important result as the mortality observed in the negative control *P. monodon* was 0%. Mortalities between the two treatments were significantly different ($p<0.0001$, Kaplan-Meier survival analysis, MedCalc). Over the same time period, injection of *P. merguiensis* with YHV7 resulted in a mean mortality rate of only 27% (21.7 and 31.8%), which was not significantly different ($p=0.0721$, Kaplan-Meier survival analysis, MedCalc) to the mortality observed in the sham-inoculated negative control *P. merguiensis* (Figure 2b). Similar to infection with YHV1, the only clinical sign observed were prawns found lying on their sides.

No *P. monodon* died after injection with YHV9 compared to 16% in one tank of *P merguiensis* (Figures 3a and 3b). The mortality observed in the *P. merguiensis* was less than, and not significantly different to ($p=0.7142$, Kaplan-Meier survival analysis, MedCalc), the mortality observed in the tank of sham-inoculated negative control *P. merguiensis*. The replicate tank of *P. merguiensis* (Tank 2) was not included in the experimental analysis due to airline failure and death of all prawns on day 10 of the trial. As this trial was primarily designed to amplify limited material for subsequent experimental trials, few conclusions will be drawn from these results.

Low level mortalities occurred after injection of *P. monodon* with YHV10 (0% and 9.1%) with mortalities slightly higher in *P. merguiensis* (33% and 10%) (Figures 4a and 4b). The mortalities observed in YHV10-exposed and sham-inoculated P. *merguiensis* were not significantly different ($p=0.0820$, Kaplan-Meier survival analysis, MedCalc). As this trial was primarily designed to amplify limited material for subsequent experimental trials, few conclusions will be drawn from these results.

The only clinical sign of disease observed in any of the prawns were moribund individuals found lying on their sides. These prawns were euthanised and processed for testing or stored for subsequent experiments.

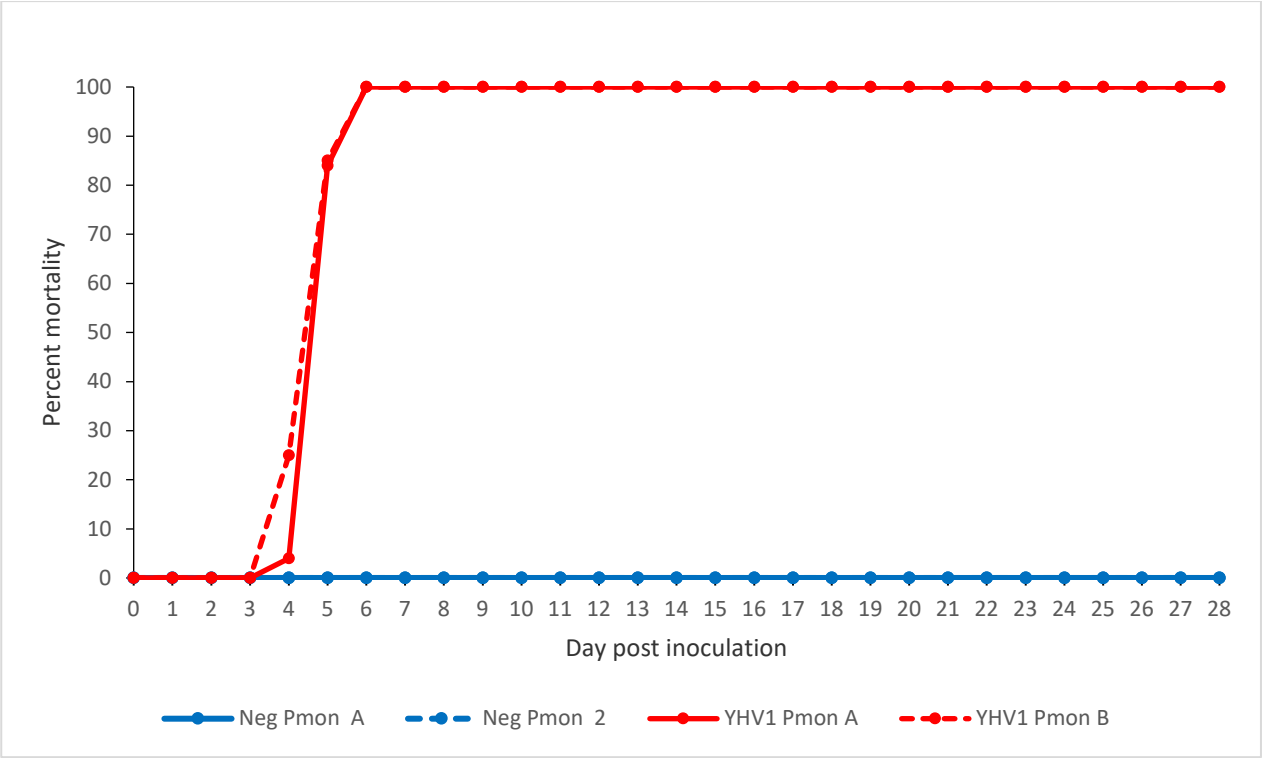


Figure 1a. Cumulative mortality in *P. monodon* after injection with YHV1 at 24°C

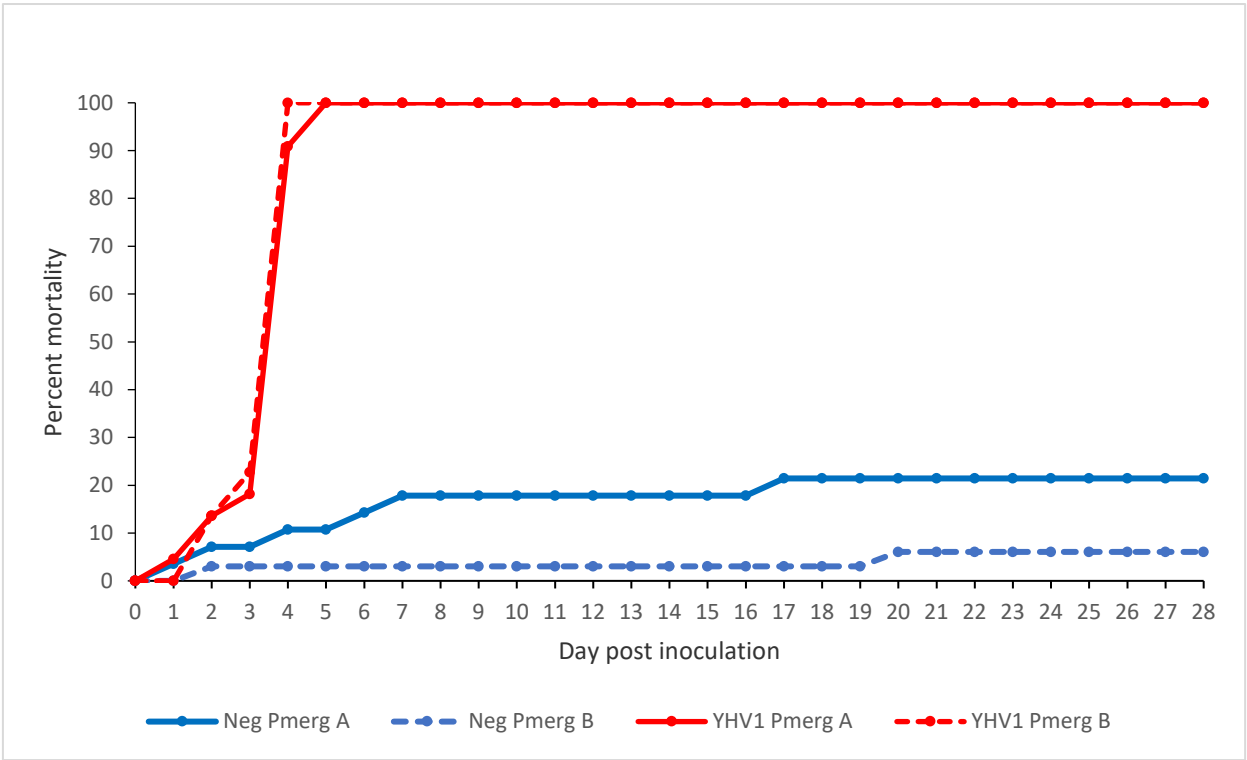


Figure 1b. Cumulative mortality in *P. merguiensis* after injection with YHV1 at 24°C

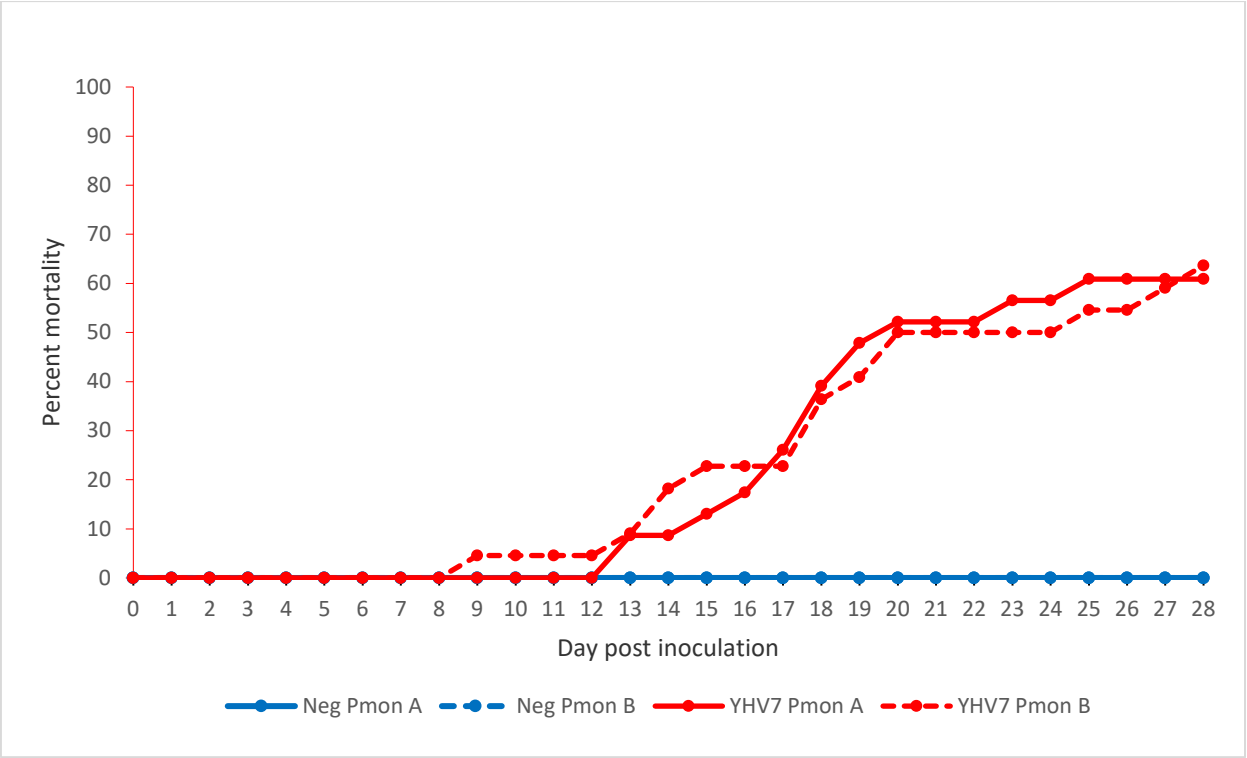


Figure 2a. Cumulative mortality in *P. monodon* after injection with YHV7 at 24°C

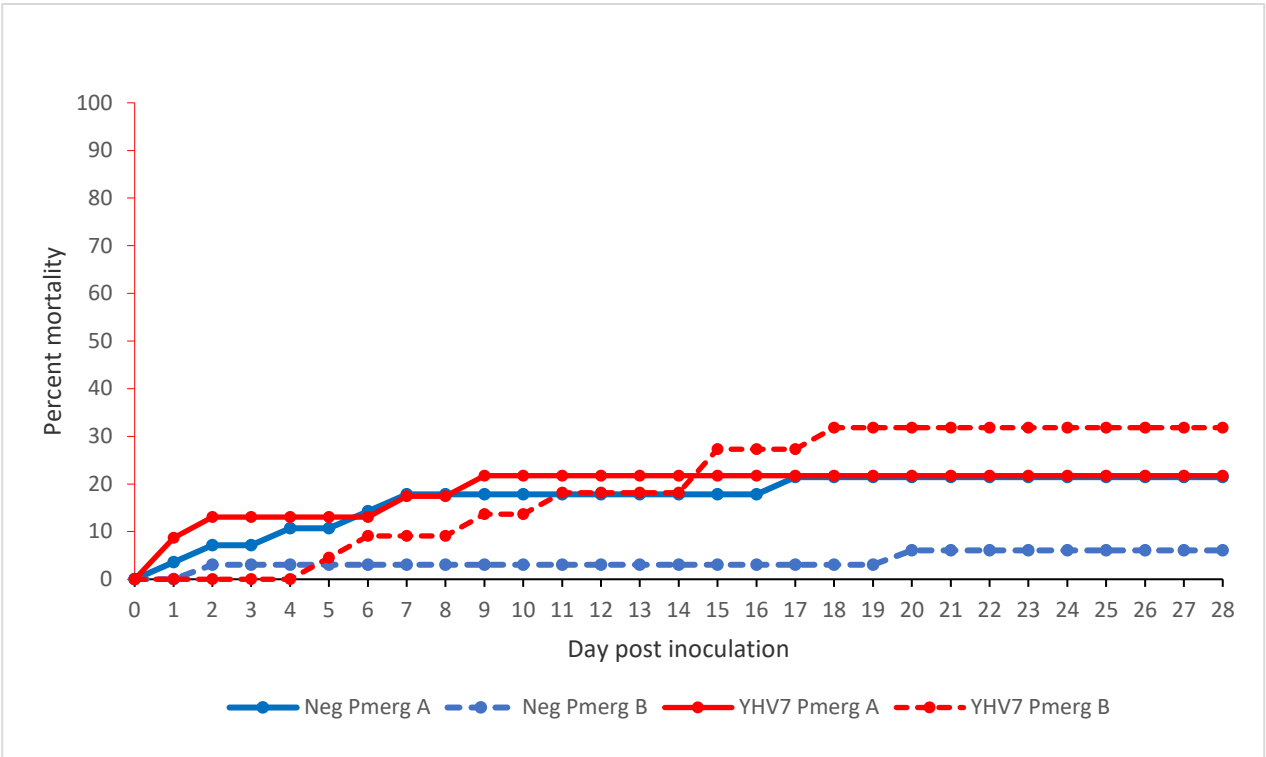


Figure 2b. Cumulative mortality in *P. merguiensis* after injection with YHV7 at 24°C

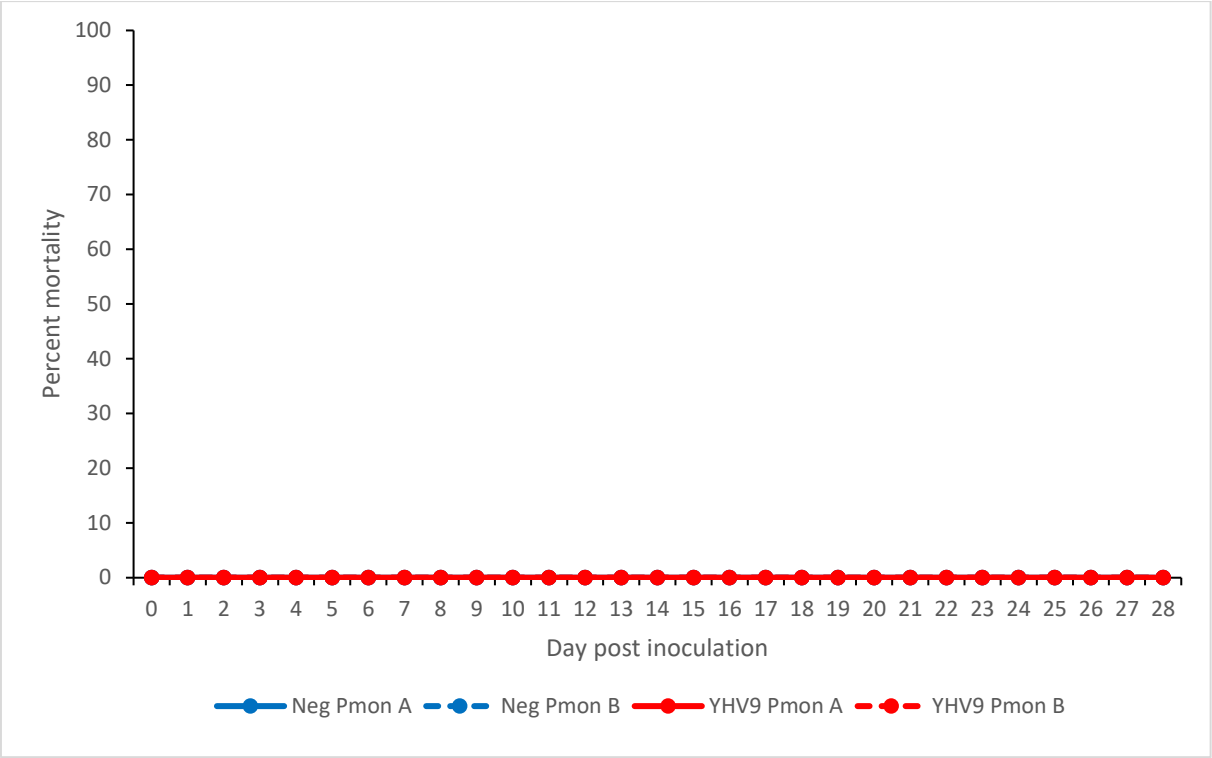


Figure 3a. Cumulative mortality in *P. monodon* after injection with YHV9 at 24°C

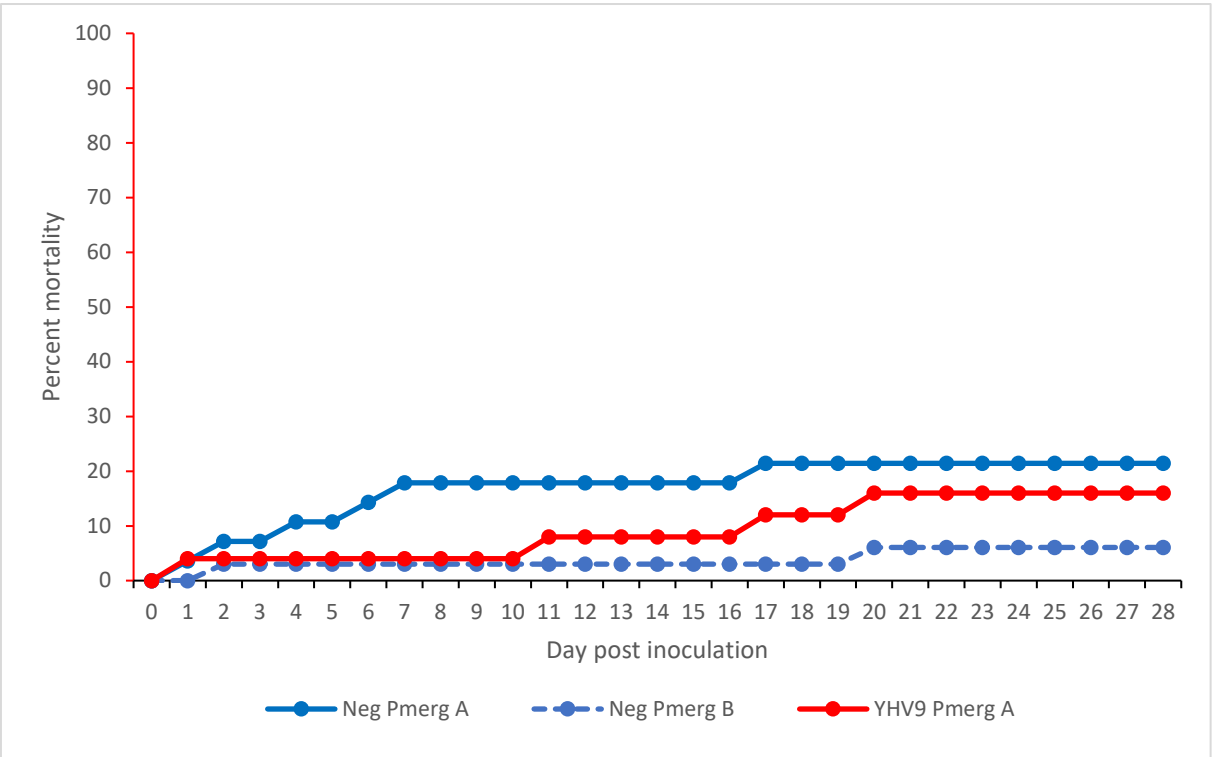


Figure 3b. Cumulative mortality in *P. merguiensis* after injection with YHV9 at 24°C

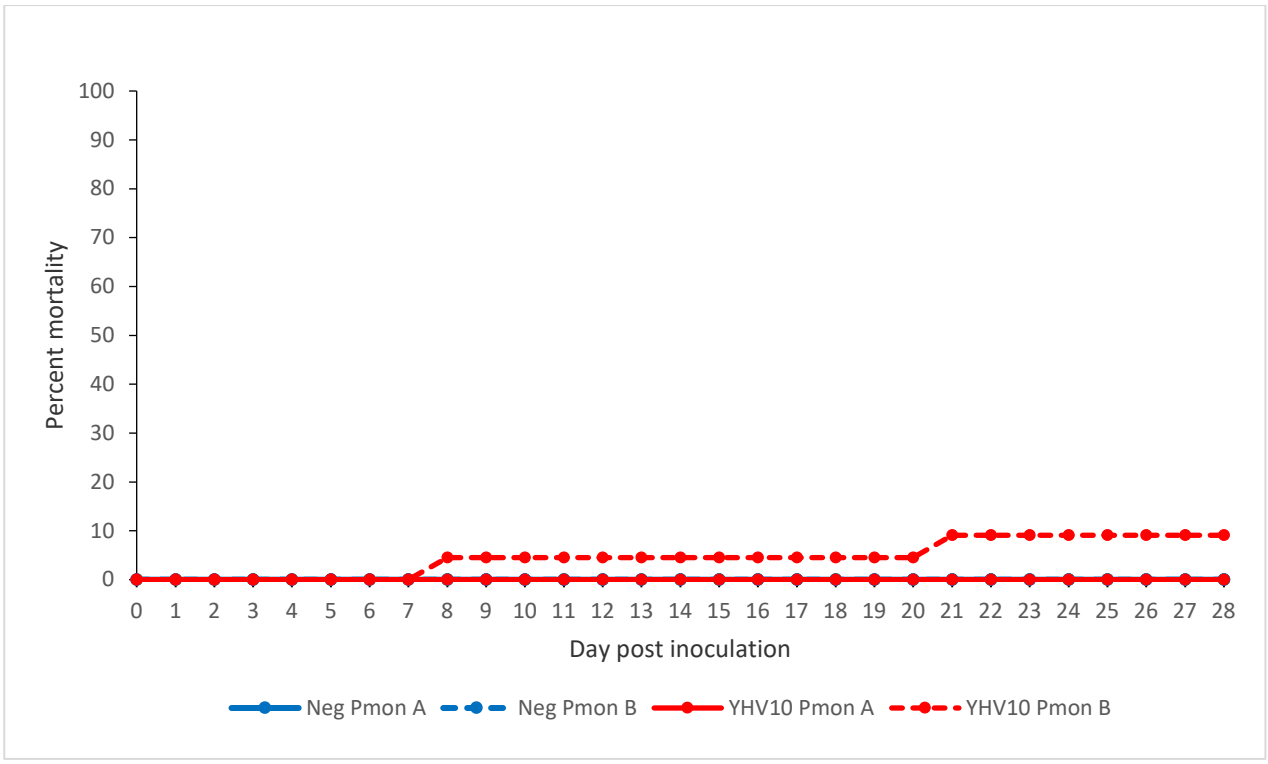


Figure 4a. Cumulative mortality in *P. monodon* after injection with YHV10 at 24°C

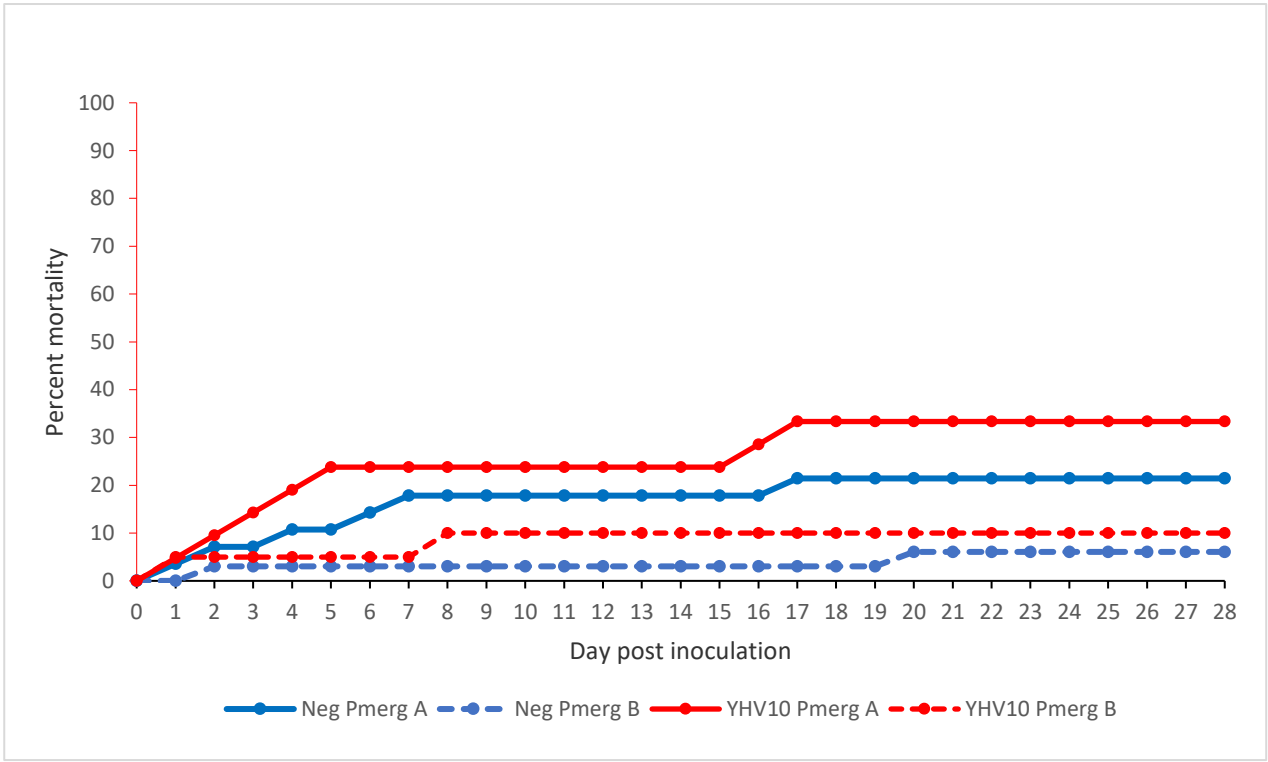


Figure 4a. Cumulative mortality in *P. merguiensis* after injection with YHV10 at 24°C

1.2.3 Molecular testing of inoculated prawns

Moribund, dead and sacrificed prawns were tested with assays for each of the specific YHV genotype present in the respective inoculum. All *P. monodon* were also tested for YHV2 as this species was shown to be positive for this genotype during pre-screening. *P. merguiensis* inoculated with YHV7 were also tested for YHV2 as the YHV7 inoculum was also test positive using the YHV2 RT-qPCR. In addition, *P. merguiensis* inoculated with YHV10 were also tested for YHV9 as this genotype was also present in the YHV10 inoculum. Results are presented as box and whisper plots in Figures 5 to 8. In each figure the C_T values, minimum, first quartile, mean, median, third quartile, and maximum values are shown. The different colours represent the different YHV genotypes and the different symbols represent the different treatments. For example, in Figure 5, specific molecular test results are in blue for YHV2, red for YHV1 and yellow for YHV7. Open blue circles represent pre-screen and negative control (unexposed) prawns, squares represent samples obtained after exposure to YHV1 and triangles represent samples obtained after exposure to YHV7.

All *P. monodon* infected with either YHV1 or YHV7 were test positive with the respective genotype-specific RT-qPCR (Figure 5). The mean C_T values for *P. monodon* inoculated with for YHV1 were 14.1 (range of 10.5 to 20.2) and for YHV7 were 17.5 (range of 12.9 to 22.6) respectively. For YHV1, the mean C_T value was lower than the C_T of the inoculum which was 25.9. While the mean C_T of prawns inoculated with YHV7 was higher than the C_T of the YHV7 inoculum, of 13.4, approximately 35% of these prawns remained alive and tested positive for YHV7 when sampled at the end of the 28-day trial. This suggests that YHV7 replicated in the prawns and further work to assess the pathogenicity by natural routes of infection (i.e. co-habitation and feeding) was justified. This also indicated that the additional stocks of YHV7 had been amplified from the limited initial material. For *P. monodon* inoculated with either YHV1 or YHV7, 91% and 100% were test positive for YHV2, respectively. The YHV2 C_T values observed were similar to those of the pre-screen and negative control prawns (Figure 5) indicating that injection with either YHV1 or YHV7 did not influence the levels of YHV2 in the prawns at 24°C.

After injection of *P. merguiensis* with either YHV1 or YHV7, 88% and 100% of all prawns were test positive with the respective genotype-specific RT-qPCR. The 12% of *P. merguiensis* that tested negative for YHV1 were mortalities 1-day post infection. The mean C_T value for *P. merguiensis* inoculated with YHV1 was 20.9 (range of 12.4 to 31.7) and for YHV7 was 20.1 (range of 15.5 to 31.9) (Figure 6). This indicates that further work to assess the pathogenicity by natural routes of infection (i.e. co-habitation and feeding) was justified. Interestingly, 79% of the *P. merguiensis* were test positive using the YHV2 RT-qPCR, which was present in the YHV7 inoculum. Negative control *P. merguiensis* were YHV2 RT-qPCR test negative. The YHV2 C_T values were similar to those observed in *P. monodon* (Figure 5 and 6).

After injection of *P. monodon*, 83% of prawns were test positive for YHV9 with a mean C_T value of 29.6 (range of 23.5 to 36.5) (Figure 7), and 70.6% of *P. merguiensis* were test positive for YHV9, with a mean C_T value of 30.1 (range of 22.0 to 38.5). As most of these samples were obtained from healthy prawns sampled at the end of the trial, the results suggest that that YHV9 had replicated in the prawns. The YHV9 material generated was utilised in additional bioassays.

No *P. monodon* or *P. merguiensis* were test positive for YHV10 (Figure 8). This was not unexpected given the high C_T values obtained for this material. The YHV10 inoculum also contained YHV9 with a C_T value of 32.4. Several *P. monodon* and *P. merguiensis* inoculated with the YHV10 inoculum were test positive with the YHV9 RT-qPCR. These were confirmed as YHV9 by conventional PCR and sequence analysis despite being injected with YHV10 inoculum . However, given the high C_T value and co-infection with YHV9, the YHV10 material was not used in subsequent experiments.

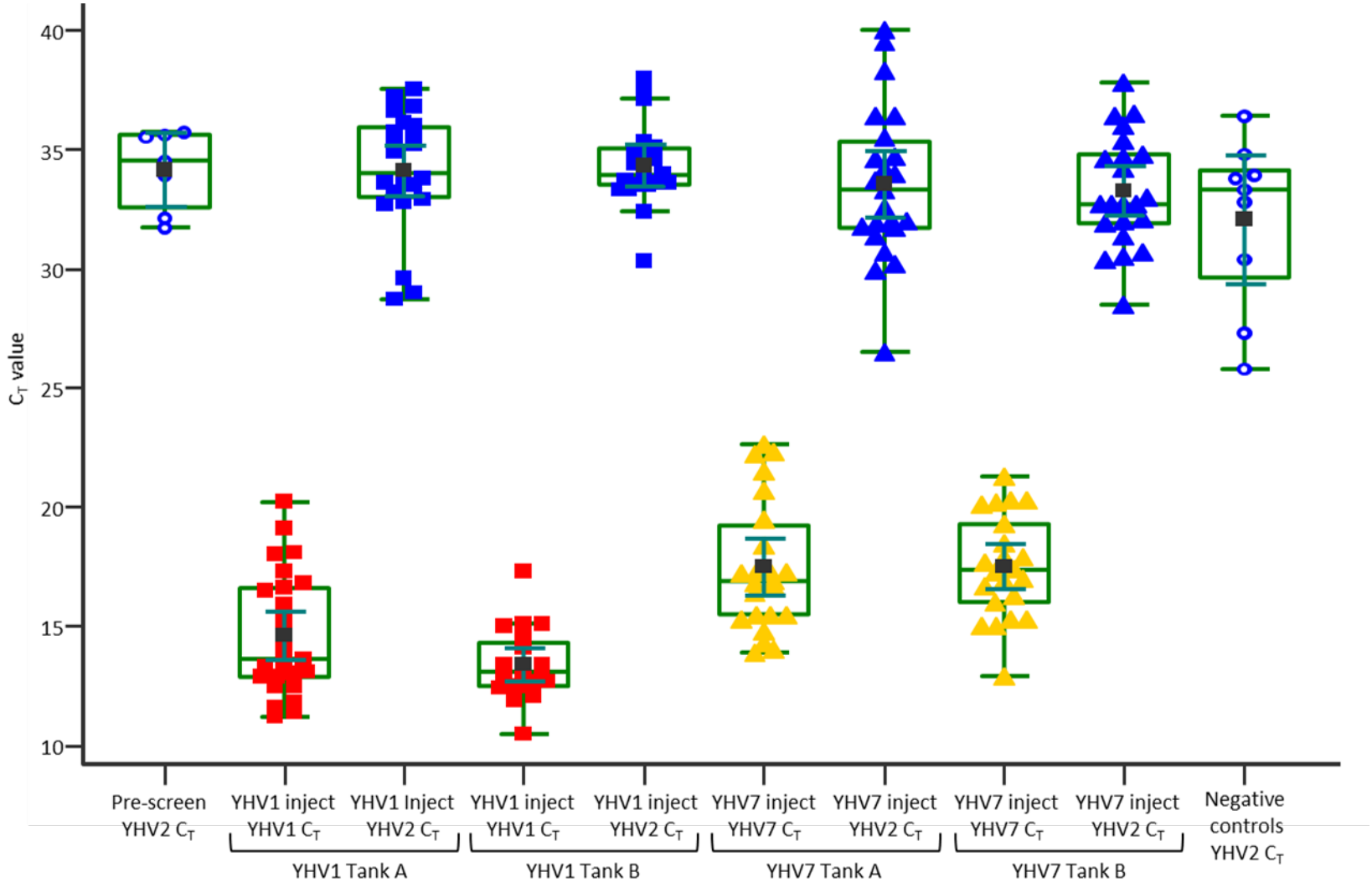


Figure 5. Molecular test results for *P. monodon* inoculated with YHV1 and YHV7 at 24°C

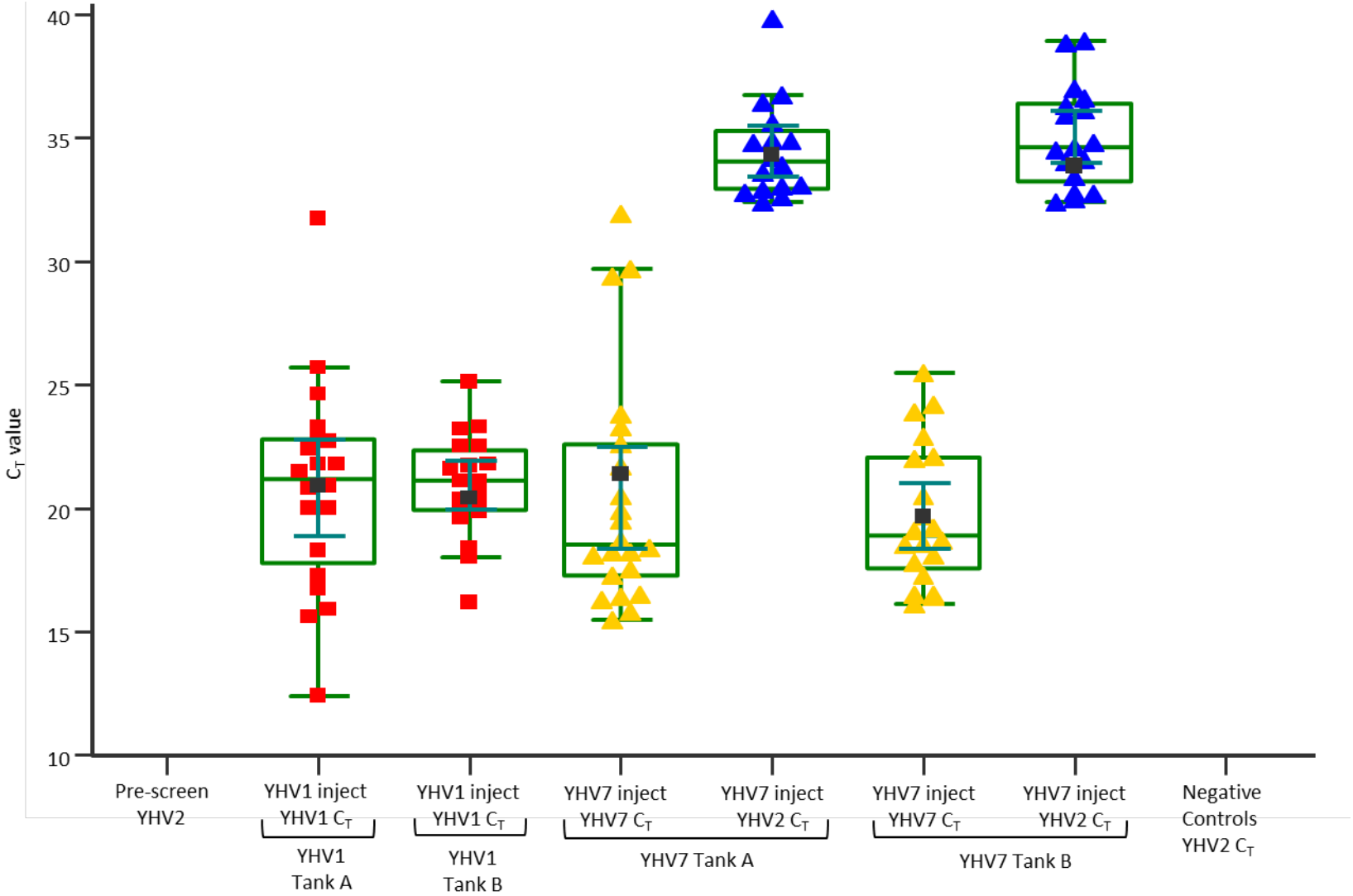


Figure 6. Molecular test results for *P. merguiensis* inoculated with YHV1 and YHV7 at 24°C

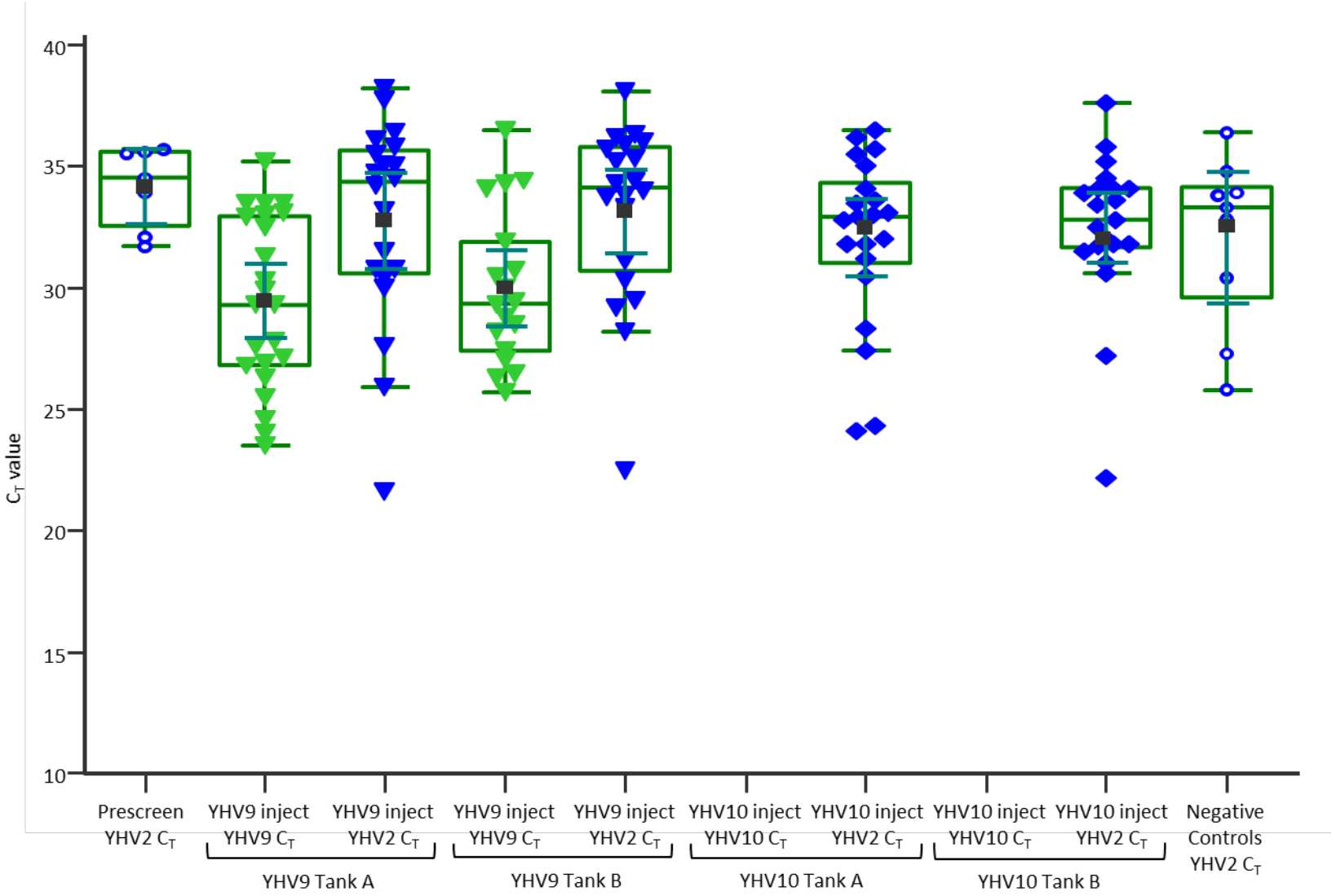


Figure 7. Molecular test results for *P. monodon* inoculated with YHV9 and YHV10 at 24°C

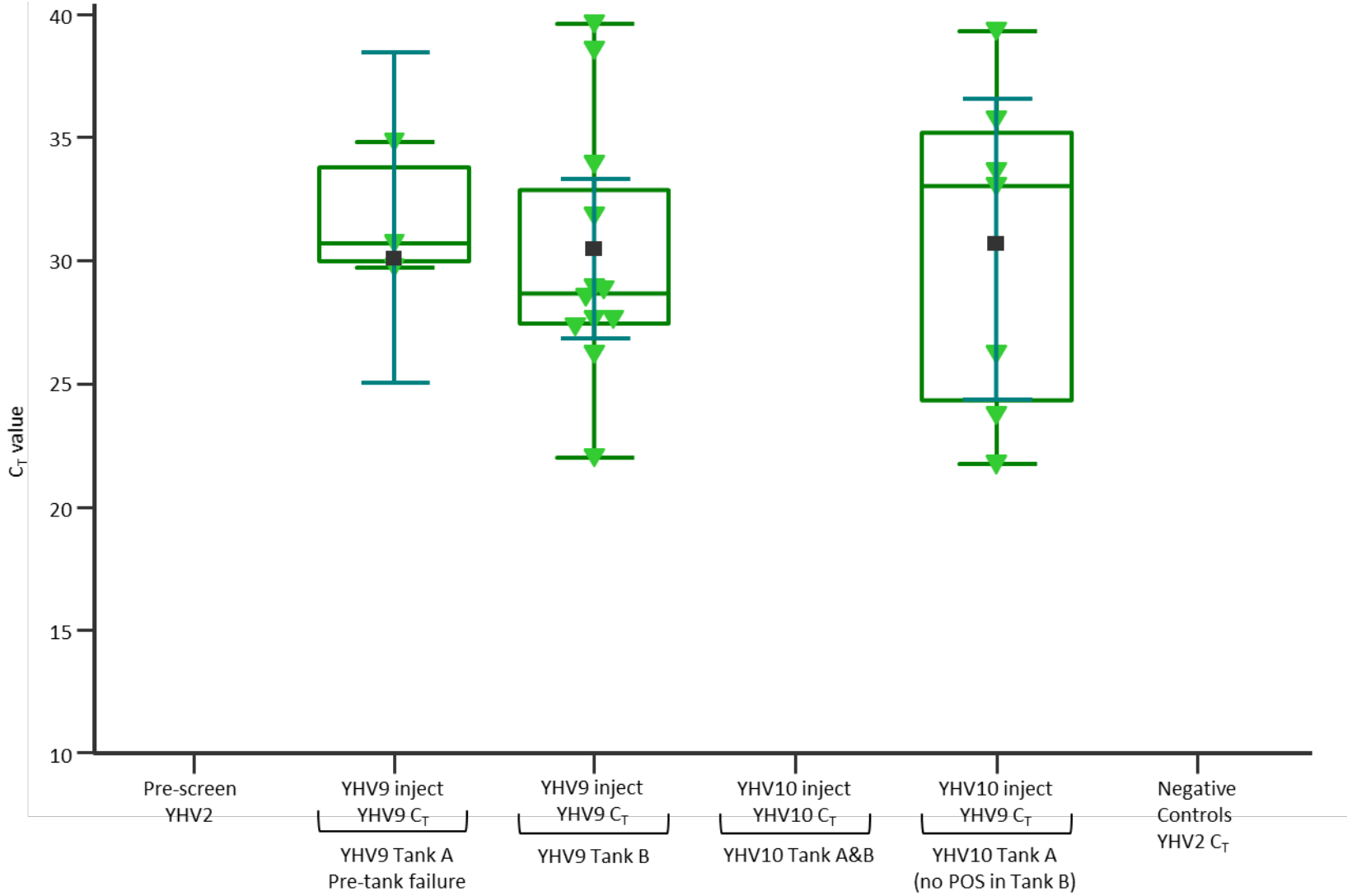


Figure 8. Molecular test results for *P. merguiensis* inoculated with YHV9 and YHV10 at 24°C

1.2.4 Histological examination of lymphoid organs of inoculated prawns

Preliminary comparative histological examination has been undertaken, targeting lymphoid organs with lymphoid organ spheroids characterised according to Hasson et al. (1999).

The lymphoid organ tubules of negative control *P. monodon* and *P. merguiensis* appeared normal (Figures 9a and 9b). Two (of five) negative control *P. monodon* contained low-level Type A lymphoid organ spheroids. No lymphoid organ spheroids were observed in negative control *P. merguiensis*. The lymphoid organs of *P. monodon* and *P. merguiensis* inoculated with YHV1 exhibited moderate-to-severe necrosis of lymphoid organ tubules (Figures 9c and 9d). Lymphoid organs of *P. monodon* inoculated with YHV7 contained a medium level of Type A and B lymphoid organ spheroids and mild necrosis in the lymphoid organ tubules (Figure 9e). *P. merguiensis* inoculated with YHV 7 contained many Type B and C lymphoid organ spheroids and mild necrosis in lymphoid organ tubules (Figure 9f). Lymphoid organs of *P. monodon* inoculated with YHV9 contained a medium level of Type B lymphoid organ spheroids (Figure 9g) and *P. merguiensis* contained a medium level of Type B and Type C lymphoid organ spheroids (Figure 9h). Similar results were observed in *P. monodon* and *P. merguiensis* inoculated with YHV10 (Figure 9i and Figure 9j) which should be considered in the context of the YHV10 inoculum also containing YHV9. No lymphoid organ tubule necrosis was observed in either *P. monodon* or *P. merguiensis* inoculated with YHV10.

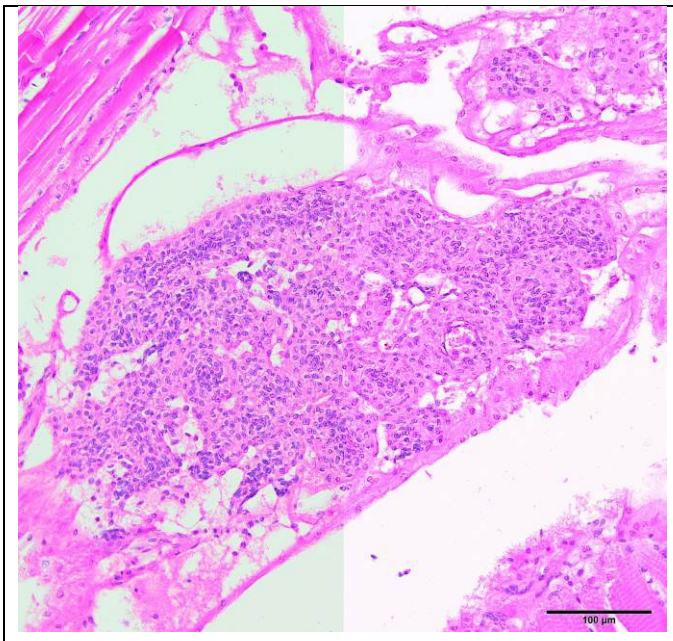


Fig 9a. Negative control *P. monodon* (H&E).

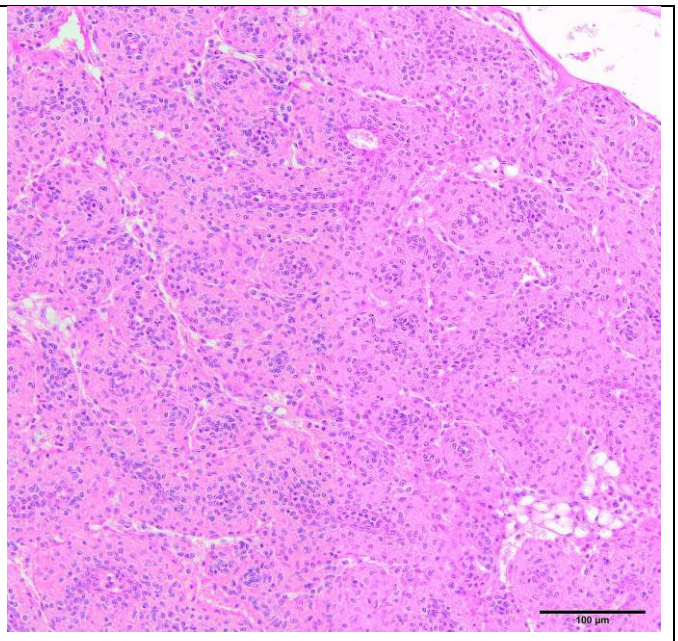


Fig 9b. Negative control *P. merguiensis* (H&E)

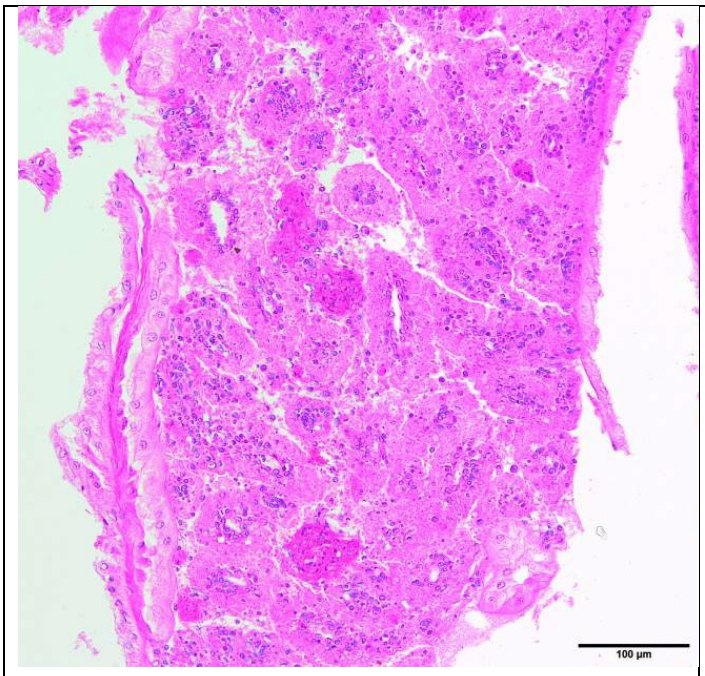


Fig 9c. *P. monodon* inoculated with YHV1 (H&E)
YHV1 C_T = 13.2, YHV2 C_T = 34.8

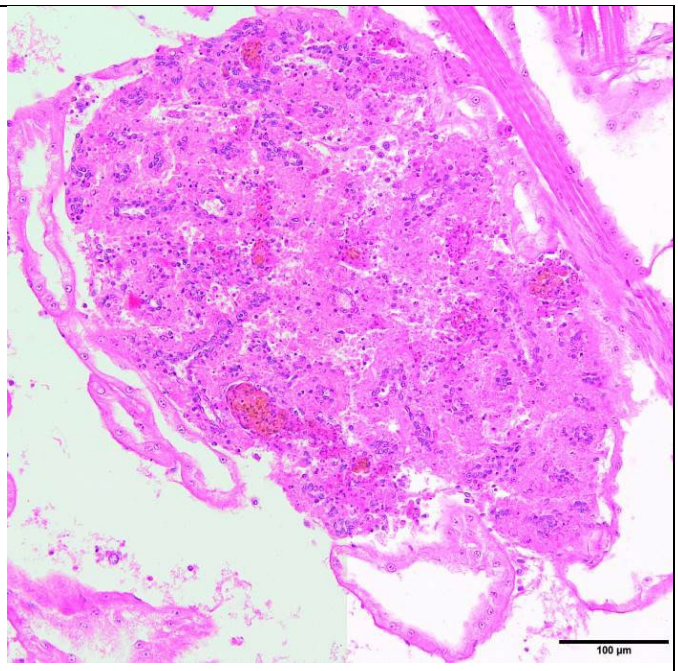


Fig 9d. *P. merguiensis* inoculated with YHV1 (H&E)
YHV1 C_T = 18.3

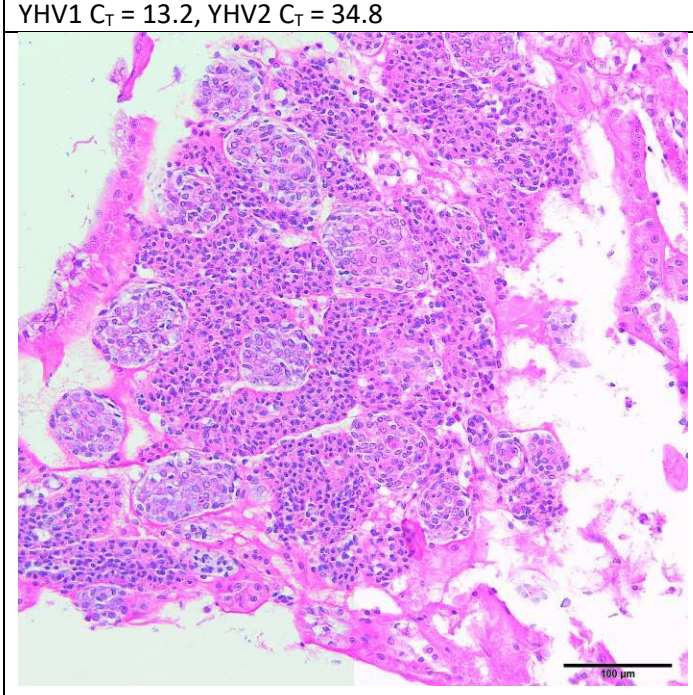


Fig 9e. *P. monodon* inoculated with YHV7 (H&E)
YHV7 C_T = 16.9, YHV2 C_T = 34.7

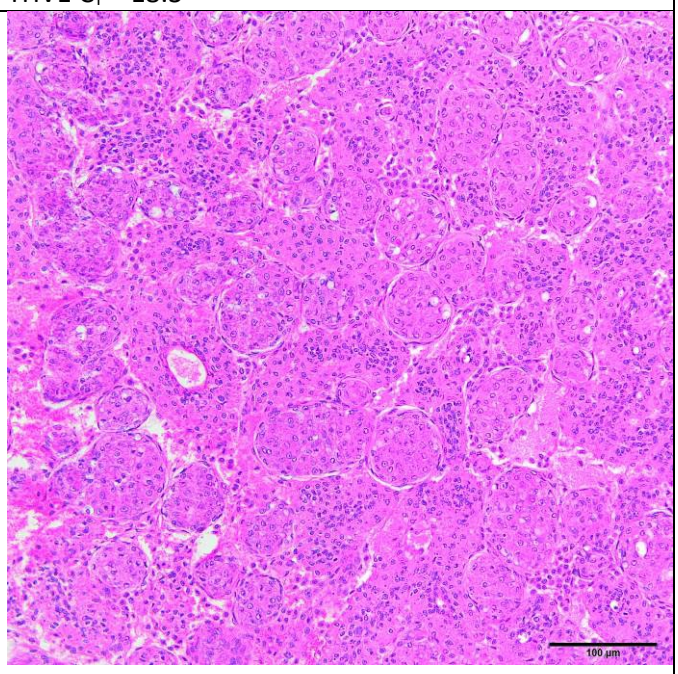


Fig 9f. *P. merguiensis* inoculated with YHV7 (H&E)
YHV7 C_T = 17.3, YHV2 C_T = 33.4

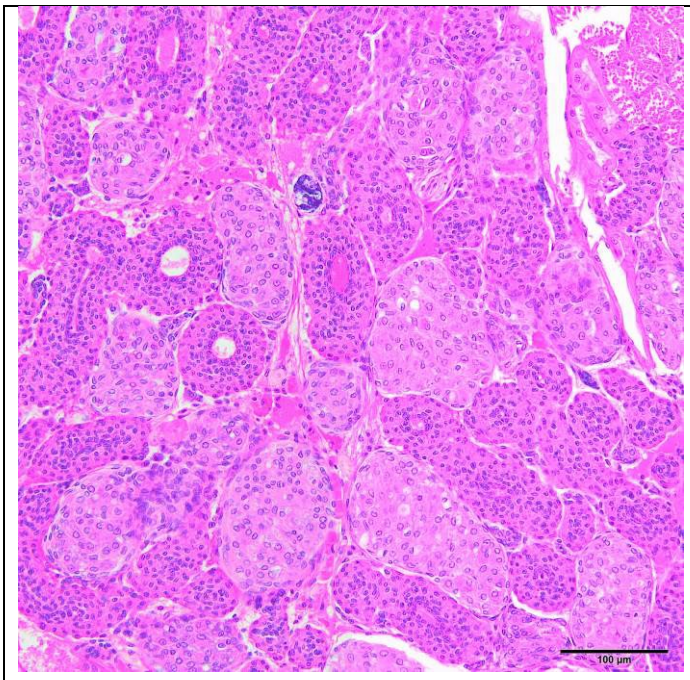


Fig 9g. *P. monodon* inoculated with YHV9 (H&E)
YHV9 C_T = 24.6, YHV2 C_T = 35.5

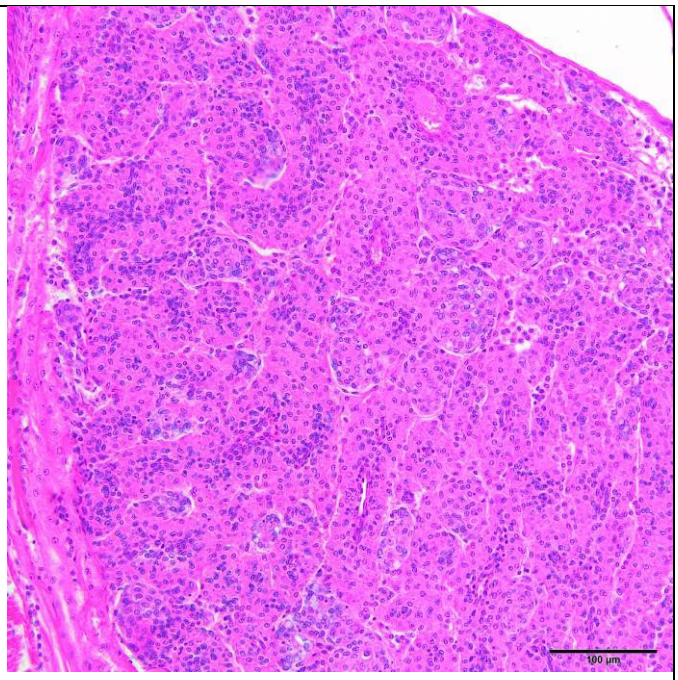


Fig 9h. *P. merguiensis* inoculated with YHV9 (H&E)
YHV9 C_T = 39.6

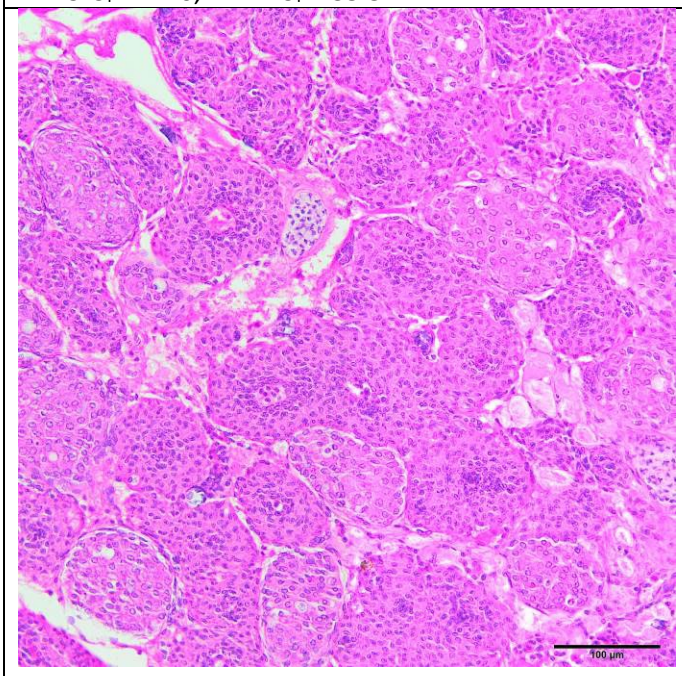


Fig 9i. *P. monodon* inoculated with YHV10 (H&E)
YHV9 C_T = 38.5, YHV2 C_T = 27.4

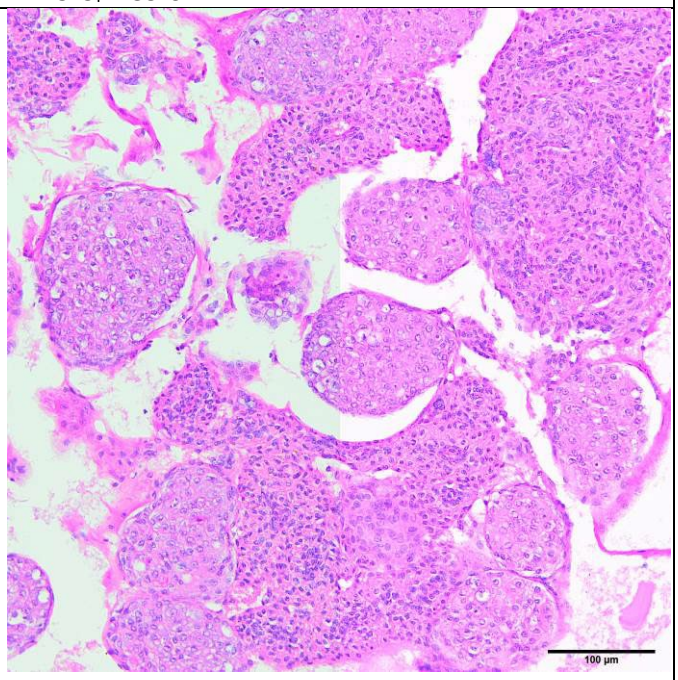


Fig 9j. *P. merguiensis* inoculated with YHV10 (H&E)
YHV9 C_T = 21.7

Figure 9. Representative examples of pathological changes observed in the lymphoid organs of *P. monodon* and *P. merguiensis* after injection with material prepared from haemolymph or tissues infected with YHV1, YHV7, YHV9 and YHV10

To clarify which YHV genotype was associated with the spheroids observed in the prawns, particularly in the *P. monodon* which were already infected with YHV2, *in situ* hybridisation needs to be undertaken, however, this is beyond the scope of this project.

1.3 Conclusion

Results of the Experimental Trial 1, which was a preliminary investigation undertaken to determine what the pathogenicity of YHV1, YHV7, YHV9 and YHV10 after infection by intra-muscular injection at 24°C can be summarised as follows:

1. Injection with YHV1 resulted in acute mortality (100% in 5-6 days) in both *P. monodon* and *P. merguiensis* with high levels of YHV1 RNA detected by RT-qPCR and pathological changes detected by histology. Both species were highly susceptible to YHV1 via intra-muscular injection.
2. Injection with YHV7 chronic mortality in *P. monodon* (63% after the 28 days of the trial). While no appreciable increase in mortality, compared to mortality observed in negative controls, were observed in *P. merguiensis*, high levels of YHV7 RNA were detected in both *P. monodon* and *P. merguiensis*. Histological changes were also observed in both species but these changes were inconclusive and further research is required to confirm whether they were a result of infection with YHV7. Based on mortalities and YHV7 specific molecular test results, *P. monodon* are susceptible to infection YHV7, with infection resulting in mortalities, although the appearance of these mortalities was less acute compared to intra-muscular injection with YHV1.
3. Injection with YHV9 caused no detectable increase in mortality compared to negative control mortality for both *P. monodon* and *P. merguiensis*. However, YHV9 RNA was detected in prawns surviving at the end of the trial, suggesting that the prawns had been infected with YHV9. Histological changes were also observed in both species. Both species may be susceptible to infection with YHV9 and additional experimental trials with YHV9 will be undertaken.
4. Intra-muscular injection with YHV10 appeared to have no effect on the prawns. However, this inoculum also contained YHV9, and molecular and histology results were similar to those observed in prawns inoculated with YHV9. It is unclear whether either species may be susceptible to infection with YHV10. The use of this YHV10 inoculum was discontinued. YHV10 had been identified in another submission and this material was later evaluated for use in subsequent trials.
5. Injection of *P. monodon* that were already naturally infected with YHV2, did not lead to a change in the level of YHV2 RNA in animals at the end of the trial period (for any of the inocula) or lead to the appearance of increased disease. No changes in YHV2 RNA levels or disease were seen in negative control prawns. Injection of the YHV2-infected *P. monodon* with the YHV7 inoculum, which also contained YHV2, did not lead to any change in the levels of YHV2 RNA at the end of the trial.

The project progressed to the investigation of susceptibility via natural routes of infection for YHV1 and YHV7. This is due to that fact that prawns inoculated with these YHV genotypes either exhibited higher mortality compared to negative control prawns, and/or tested positive at the end of the 28-day trial for the respective YHV genotype and exhibited histological changes in comparison to negative control prawns. Additional investigations were undertaken with YHV9 and new YHV10 material to determine whether or not injection of the prawns causes infection or disease at 29°C. This temperature was chosen as it is more representative of culture temperatures on prawn farms in central and north Queensland. Interestingly, infection of the prawns already infected with YHV2 did not seem to change the viral load for this YHV genotype. This is contrary to published research results. For example, de la Vega et al (2004) reported an increase in YHV2 levels and the appearance of disease in chronically infected *P. monodon* used as negative controls animals during experimental infection trials. Prawns used in future experimental trials will also be tested for YHV2 to see if infection levels (if prawns are infected with YHV2 prior to the experiment) change after infection with YHV1 and YHV7.

Experimental Trial 2: Assessment of susceptibility to infection by natural routes at 29°C

The aims of Experimental Trial 2 were:

1. To provide an indication of pathogenicity of YHV1 and YHV7 to *P. monodon* and *P. merguiensis*, after infection via a natural route (i.e. co-habitation and feeding) at approximately 30°C, a temperature more representative of prawn culture conditions.

2. To provide an indication of the pathogenicity of YHV9 after amplification in prawns during Experimental Trial 1, to *P. monodon* and *P. merguiensis*, by infection via direct and unnatural route of infection (i.e. injection) at approximately 30°C, a temperature more representative of prawn culture conditions.

3. To assess the pathogenicity of new material, containing YHV10, after exposure by inoculation of *monodon* and *P. merguiensis*, by infection via direct and unnatural route of infection and forced method (i.e. injection) at approximately 30°C, a temperature more representative of prawn culture conditions.

2.1 Methods

2.1.1 Experimental animals

Sourcing and delivery of prawns was organized through the Australian Prawn Farmers Association (APFA) Research and Development Committee. Farmed *P. monodon* (~7-9 cm in length) and farmed *P. merguiensis* (~6-8 cm in length) were obtained from two prawn farms in north Queensland. On arrival, prawns were transferred to the ACDP Large Animal Facility (LAF) and placed in round (infection by feeding) or square (infection by co-habitation) polypropylene tanks containing 80 L of 100-µm-filtered seawater, a canister filter and air stones. Prawns were distributed across 5 rooms (negative controls and treatment groups exposed to either YHV1, YHV7, YHV9 and YHV10), with a separate room used for each different treatment. Each tank held 22 to 25 prawns with water temperature maintained at 29±1°C using immersion heaters. At least one tank in each room was fitted with a data-logger to record water temperature. Animals were monitored and fed commercial prawn pellets daily (unless fed YHV1 or YHV7 positive tissue) and 30% water exchanges were undertaken every 2 days. Prior to infection, 25 animals of each species were sacrificed, 10 of which were fixed in 80% (v/v) EtOH for PCR testing and the other 15 fixed in Davidson's Fixative for histological analysis.

2.1.2 Preparation of material infected with the different YHV genotypes

Un-fixed prawn tissues, generated during Experimental Trial 1, containing YHV1, YHV7, YHV9, and a new submission containing YHV10, and held at -80°C at ACDP, were the sources of infected material for Experimental Trial 2.

On the day of inoculation, inocula were prepared from frozen, archived material, as follows.

- YHV1: The original inoculum used during Experimental Trial 1 was used to inject prawns that would act as the source of infection in co-habitation studies. *P. monodon* or *P. merguiensis* with the lowest YHV1 RT-qPCR C_T values were placed in zip-lock bags and stored at -80°C for feeding trials with the homologous species (i.e. only *P. monodon* were fed to *P. monodon*).

- YHV7: The original inoculum used during Experimental Trial 1 was used to inject prawns that would act as the source of infection in co-habitation studies. *P. monodon* or *P. merguiensis* with the lowest YHV7 RT-qPCR C_T values were placed in zip-lock bags and stored at -80°C for feeding trials with the homologous species (i.e. only *P. monodon* were fed to *P. monodon*).
- YHV9: Tails from prawns infected by injection during Experimental Trial 1 were halved and processed as described above (Section 1.1.2).
- YHV10: Imported commodity prawns (16-01798-08) were test positive by YHV10 RT-qPCR with a C_T value of 31.3 with YHV10 confirmed by conventional PCR and sequence analysis. This material was test negative by all other RT-qPCR assays, specific for YHV1, YHV7 and YHV9. Material was prepared and processed as described above (Section 1.1.2).

The inocula were stored at 4°C and transported to the LAF rooms on wet ice. To prevent any cross-contamination, negative control animals and those infected with each YHV genotype were held in separate rooms in the LAF. Separate overalls and footwear were provided for personnel in each room containing animals inoculated with each of the YHV genotypes and a 3-minute shower was required on exiting each of these rooms.

2.1.3 Inoculation of prawns, sampling and sample processing

Experimental Trial 2 was planned to run for 28 days, commencing on 11 November 2016 with a planned termination date of 9 December 2016. With the confirmed detection of WSSV in diseased prawns from a farm along the Logan River, Queensland on 30 November 2016, resources were subsequently diverted to participate in the national emergency response. Husbandry and monitoring continued to be undertaken daily, with any moribund or dead prawns sampled for testing, but the experiment was terminated on different days when resources were available to process samples appropriately. As a result the experiment was terminated for prawns exposed to YHV10 on Day 33, for prawns exposed to YHV9 on Day 41 and for prawns exposed to YHV7 on Day 44 post infection. It is for this reason Figures 12 to 14 end at either 33, 41 or 44 days.

2.1.3.1 Inoculation by injection

Prawns were injected as described above (Section 1.1.3). One tank of negative control prawns for each species was sham inoculated with 100 µl PBSA (Tank A)

2.1.3.2 Exposure by feeding

Pools of 3 or 4 frozen infected prawns were transported to the LAF and, inside each room, the prawns were sliced in half longitudinally then into pieces approximately 5 mm in length. The prawn segments were sprinkled on the surface of the water for each tank and prawns were observed to ensure each prawn was consuming prawn tissue. This generally occurred within about 30 seconds. The prawns were fed once daily with the infected prawns, for five days, and were then fed commercial prawn pellets. One tank of negative control prawns for each species was fed frozen negative control prawns (Tank B) as for exposed prawns

2.1.3.3 Exposure by co-habitation

Two square polypropylene tanks were joined by two pieces of piping and an immersible pump used to transfer water out of one tank and into the second tank to create a flow-through system (Figure 10a and b). Mesh was tied over the ends of the piping to stop movement of prawns from one tank to the other. One tank contained 25 prawns injected with either YHV1 or YHV7 (infected prawns) and the other tank contained prawns that had no treatment applied to them (co-habitant prawns).

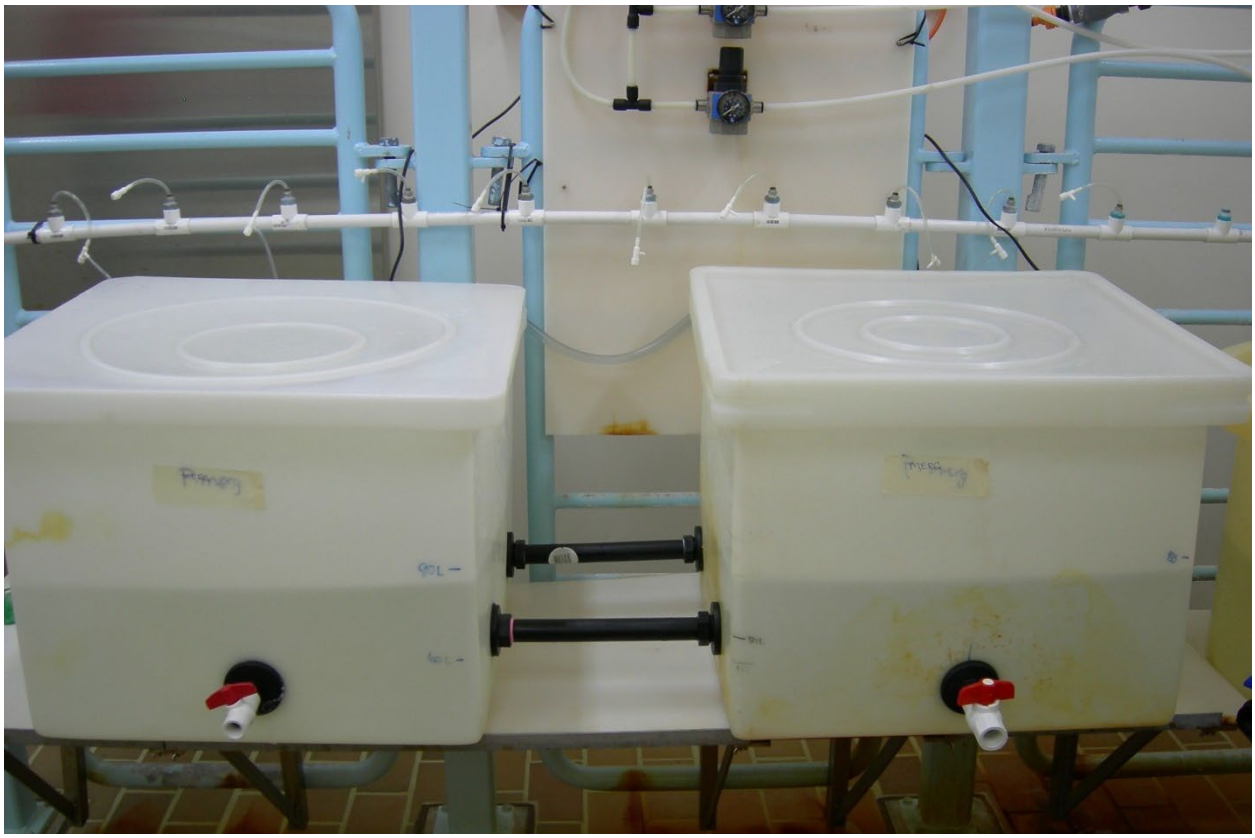


Figure 10a: Square tanks joined by tubing, used to assess infection by co-habitation

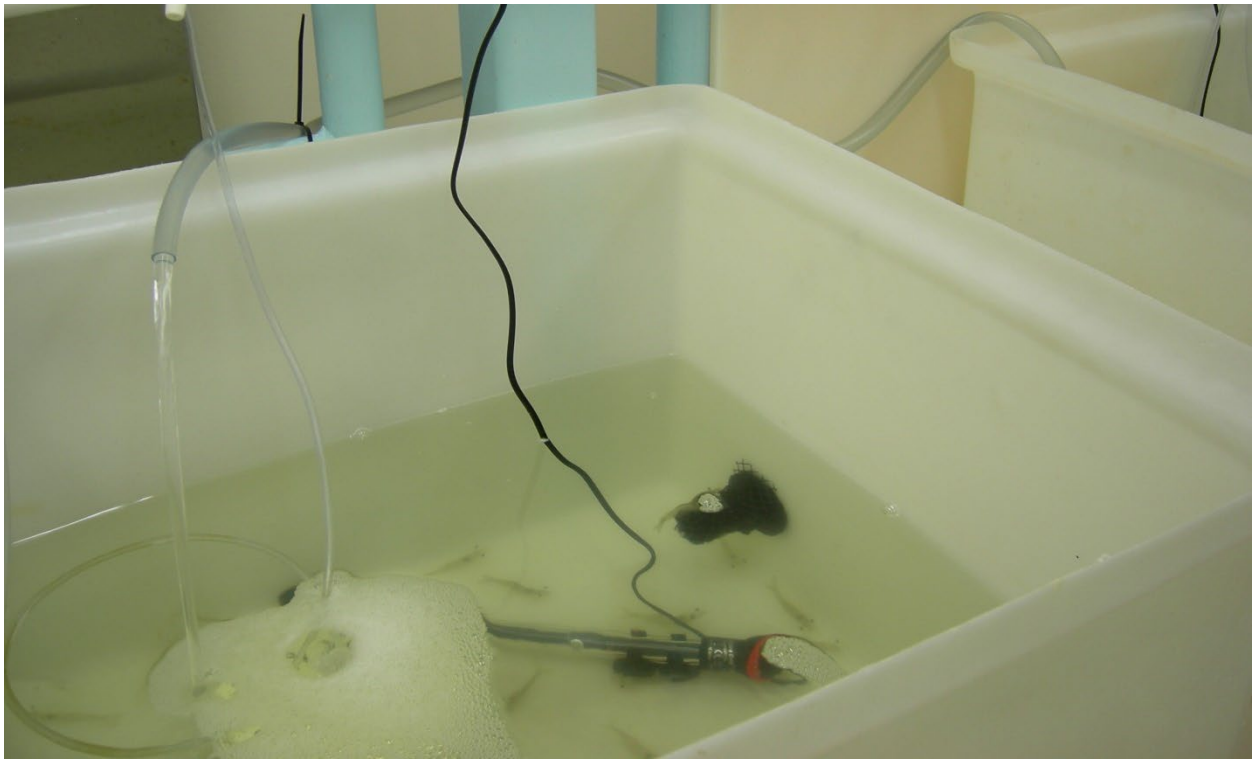


Figure 10b: Water flow from one tank to the other

2.1.4 Nucleic acid extraction

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

2.1.5 Molecular tests

Pathogen-specific PCR tests used are described above (Section 1.1.5). For Experimental Trial 2, Q-Beta was used as the positive extraction control and nucleic acids also extracted with the Q-Beta RT-qPCR to confirm nucleic acids were extracted and were free of PCR inhibitors (Moody *et al*., 2021). Complete details of all assays are provided in Appendix 2.

2.1.6 Histopathology

Fixed cephalothoraxes of *P. monodon* and *P. merguiensis*, exposed to YHV1 and YHV7, were submitted for histopathology at the Biosecurity Sciences Laboratory (BSL), Queensland Government by Drs Ian Anderson and Roger Chong. Trimming of the shrimp tissues into histology cassettes for paraffin wax embedding, processing, sectioning and hematoxylin & eosin staining was conducted as follows, depending on the presentation of the sample:

- Shrimp with whole cephalothoraxes - midline sagittal section of cephalothorax with portion of abdominal segment trimmed, 3 transverse half sections of cephalothorax, and several pereiopods, the antennal base portions and one eye and eye-stalk sagittal section included. An attempt was made to include the lymphoid organ (LO) in section, but as these are juvenile shrimp, the LO is very small and could be missed during tissue processing. Shrimp with missing LO sections were noted in the report.

- Shrimp with ½ cephalothoraxes - midline sagittal section of cephalothorax trimmed, eye stalk and any remnant appendages (pereopods, antennae) were included.

Histopathological examination covered all parts of the sectioned tissues with 1 slide used per shrimp. Histopathology reporting focused on describing lesions or abnormal changes observed in the sectioned tissues with a general statement on normal tissues as 'No other abnormalities observed'. The tissues generally included in the sections were – gills, cuticle and cuticular epithelium, hepatopancreas, foregut (stomach), anterior midgut caecum, heart, haematopoietic tissue, antennal gland, lymphoid organ, muscle, gonad, antennae, eyeball and eye stalk, ventral nerve cord, mouth parts, pereopods, pleopods. Not all tissues were present in every shrimp section due to the trimming process, the small size and variable shape or thickness of particular organs, dispersed distribution and also the presentation of the sample. The adequacy of fixation or presence of autolysis due to delayed or improper fixation, where this was an issue was reported for tissues concerned – especially the hepatopancreas.

The slides were examined by one aquatic pathologist with access to basic sample identification information about the samples e.g. species of shrimp, shrimp identification number, challenge status and method, and time post challenge. The results of the challenge were also made available to the pathologist prior to completion of the histopathology assessment. As such, this is not a blinded study, but follows the normal or standard diagnostic method for investigation of aquatic animal diseases – i.e. a full history is submitted with diagnostic samples. The processing, sectioning, staining, histological interpretation and images described in Section 2.2.4 are taken from the detailed reports provided by Drs. Anderson and Chong.

2.2 Results

2.2.1 Molecular testing of inocula

Inoculum test results after inoculation of the prawns with YHV9 and YHV10 are presented in Table 4. The YHV1 and YHV7 inoculum was the same as for Experimental Trial 1 (Table 3) which had been stored at -80°C and was not retested. All pre-trial *P. monodon* were positive for YHV2 with relatively high C_T values (indicating a low level of infection). None of the *P. merguiensis* tested positive using the using the de la Vega YHV2 RT-qPCR. The C_T values for the YHV9 and YHV10 inocula were lower than the material prepared for Trial 1 and the new YHV10 inoculum was test negative using the YHV9 RT-qPCR. However, it cannot be determined whether this was due to replication after injection during Trial 1, or simply due to the preparation of new material for this trial. All samples were positive using the Q-Beta RT-qPCR with C_T values in the expected range.

Table 4. Molecular test results for each freshly prepared YHV9 and new YHV10 inocula

Assay	Inoculum Result		Pre-trial *P. monodon*	Pre-trial *P. merguiensis*
	YHV9	YHV10		
CSIRO YHV RT-qPCR	**POS (23.3)**	**POS (33.3)**	Not tested	Not tested
AFDL YHV1 RT-qPCR	Neg	Neg	Neg	Neg
CSIRO YHV7 RT-qPCR	Neg	Neg	Neg	Neg
de la Vega YHV2 RT-qPCR	Neg	Neg	**POS (24-37)**	Neg
AFDL YHV9 V2 RT-qPCR	**POS (20.6)**	Neg	Not tested	Not tested
AFDL YHV10 RT-qPCR	Neg	**POS (32.0)**	Not tested	Not tested
Han AHPND PirA qPCR	Neg	Neg	Not tested	Not tested
OIE TSV RT-qPCR	Neg	Neg	Not tested	Not tested
CSIRO WSSV qPCR	Neg	Neg	Not tested	Not tested
OIE 309 IHHNV PCR	Neg	Neg	Not tested	Not tested
OIE IMNV RT-nPCR	Neg	Neg	Not tested	Not tested
MBV PCR	Neg	Neg	Not tested	Not tested
HPV nPCR	**POS**[1]	**POS**[2]	Not tested	Not tested
Q-Beta RT-qPCR	**POS**	**POS**	**POS**	**POS**

Numbers in brackets for the real-time PCR assays indicate mean C_T values
[1]Nucleotide sequence shared 97.2% identity with HPV Australia (DQ458781)
[2]Nucleotide sequence shared 82.8% identity with HPV3 (EU588991)

2.2.2 Mortalities in infected prawns

Cumulative mortalities for each species inoculated with each genotype are presented in Figures 11 to 14. No negative (uninfected) control *P. monodon* or *P. merguiensis* died during the 28-day trial, so negative control mortality was 0% for both prawn species.

After injection with YHV1, in both *P. monodon* and *P. merguiensis*, 100% mortality was observed at 5- and 6-days post-inoculation, respectively. The only clinical sign of disease observed were prawns found lying on their sides. After exposure by feeding, in both *P. monodon* and *P. merguiensis*, 100% mortality was observed at 5 to 6-days and 4-5 days post-inoculation in the duplicate tanks, respectively. Mortality rates in both *P. monodon* and *P. merguiensis* co-habitants reached 100% at 9- and 10- days, respectively. Mortalities were observed to start at 5- and 4-days post-exposure, and once mortalities had commenced the time until 100% mortality occurred was 4 to 6 days (Figures 11a and 11b). The only clinical sign of disease in inoculated and co-habited prawns observed were prawns found lying on their side. This was also observed in prawns fed with YHV1-infected prawn material, however a number of these prawns also had bright yellow heads.

Injection of *P. monodon* with YHV7 resulted in a mortality rate of 56% at the termination of the trial at 44 days. Several mortalities occurred at 1-day post-inoculation and accounted for 20% of the final mortality of 56% and were most likely due to the injection process and not YHV7, as they occurred 1-day post infection. Feeding of YHV7-infected material resulted in a mean mortality rate of 15.1% (12% and 20%) over the 44-day trial with co-habitation resulting in a mortality rate of 4% (Figure 12a). No mortalities were observed from day 30 to day 44 of the trial.

Over the same time period inoculation and co-habitation of *P. merguiensis* with YHV7 resulted in no mortalities while feeding resulted in mean mortalities of 6% (4% and 8%) (Figure 12b). These mortalities were not significant (p=0.0.0832, Kaplan-Meier survival analysis, MedCalc). The only clinical sign of disease observed were prawns found lying on their sides.

No *P. monodon* or *P. merguiensis* died after injection with YHV9 after 41 days (Figures 13a and 13b) when the trial was terminated.

No *P. monodon* and only two *P. merguiensis* died (both at 3-days post infection), after injection with YHV10 after 31 days (Figures 14a and 14b) when the trial was terminated.

No mortalities were observed in *P. monodon* and *P. merguiensis* negative control prawns, either sham-inoculated (Tank A) or fed frozen negative control prawns (Tank B).

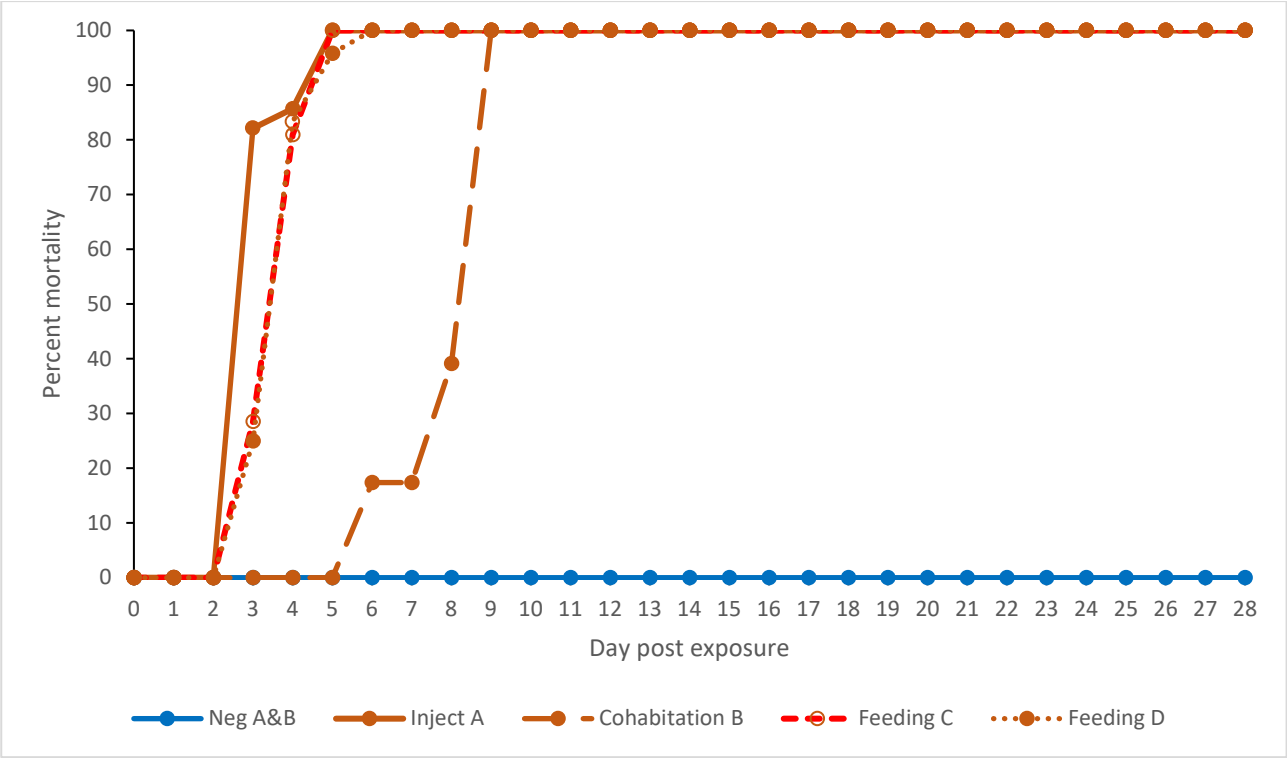


Figure 11a. Cumulative mortality in *P. monodon* after exposure to YHV1 by injection, feeding and co-habitation at 29°C

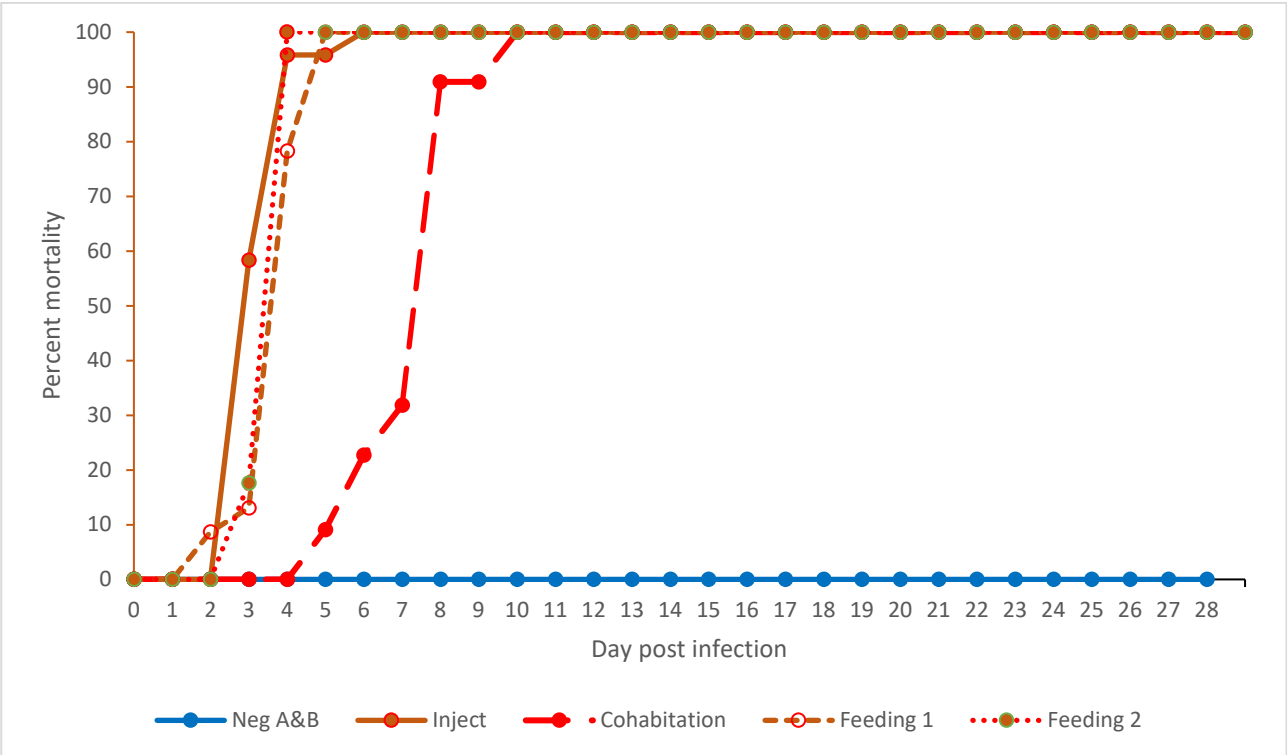


Figure 11b. Cumulative mortality in *P. merguiensis* after exposure to YHV1 by injection, feeding and co-habitation at 29°C

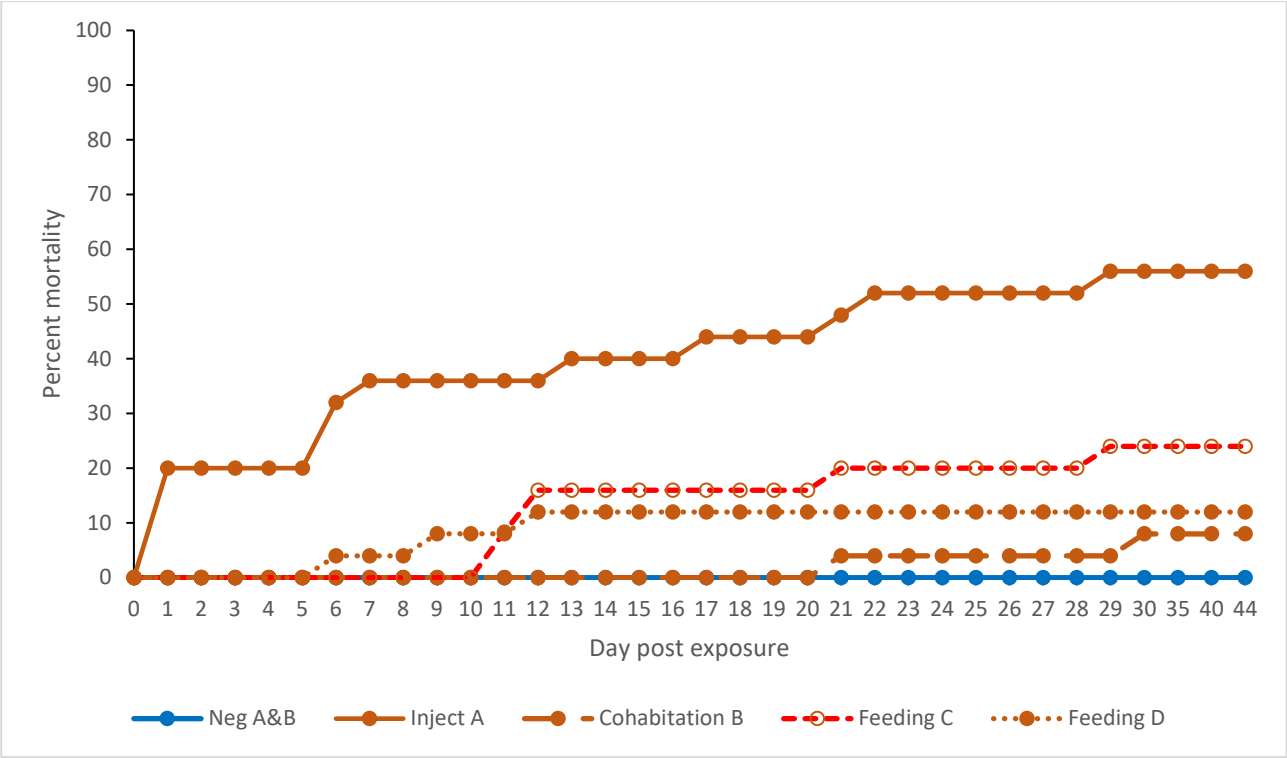


Figure 12a. Cumulative mortality in *P. monodon* after exposure to YHV7 by injection, feeding and co-habitation at 29°C

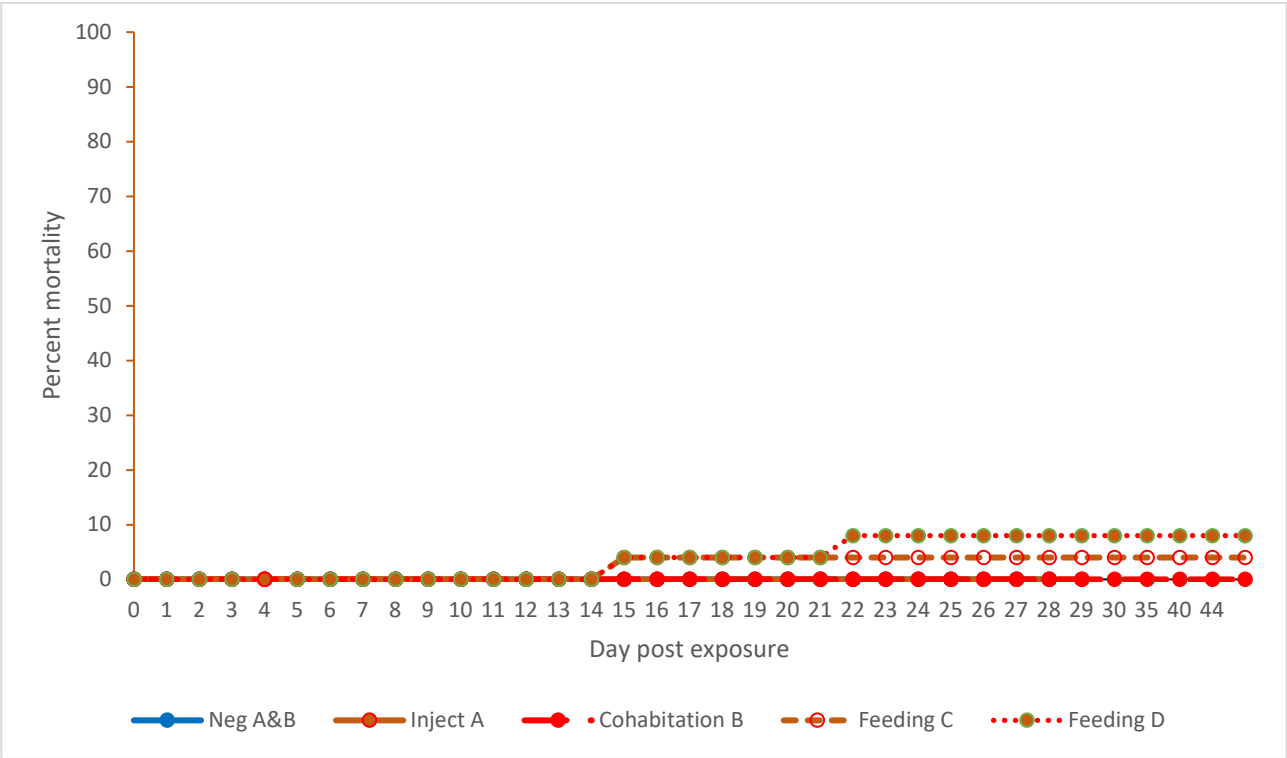


Figure 12b. Cumulative mortality in *P. merguiensis* after exposure to YHV7 by injection, feeding and co-habitation at 29°C

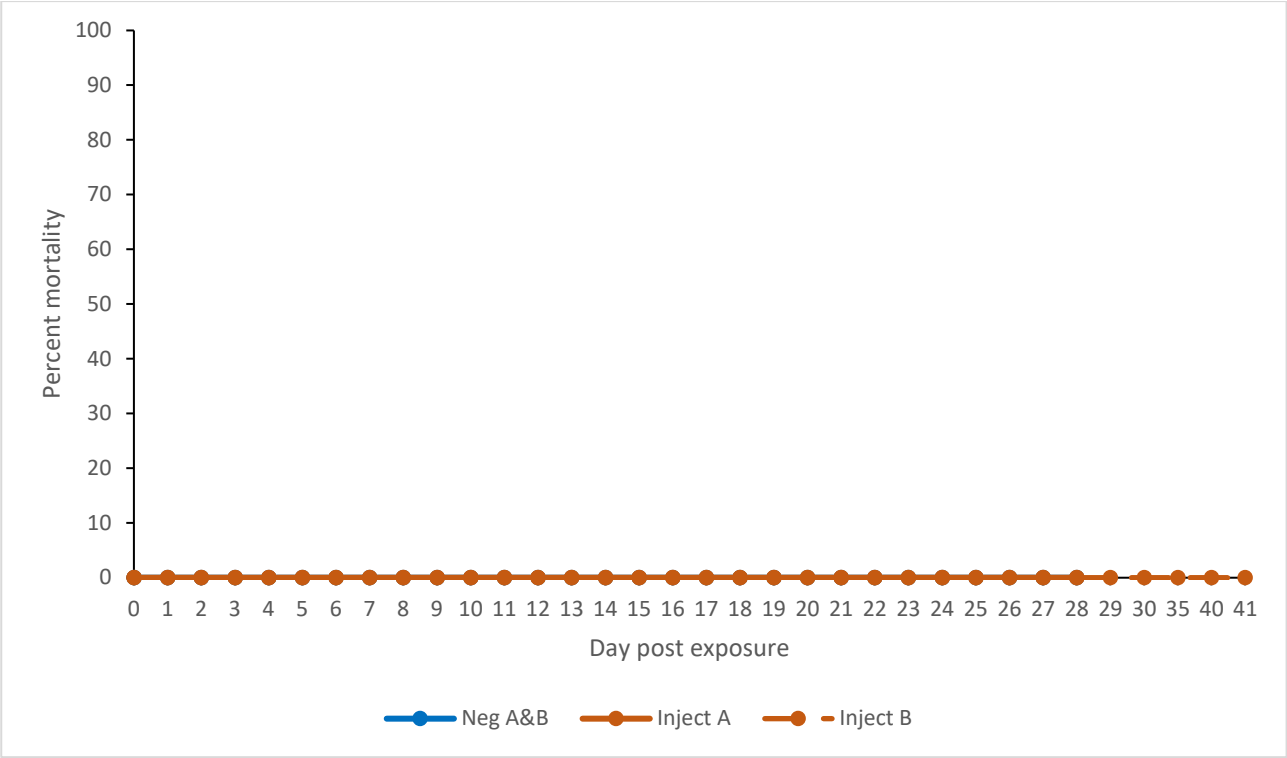


Figure 13a. Cumulative mortality in *P. monodon* after exposure to YHV9 by injection at 29°C

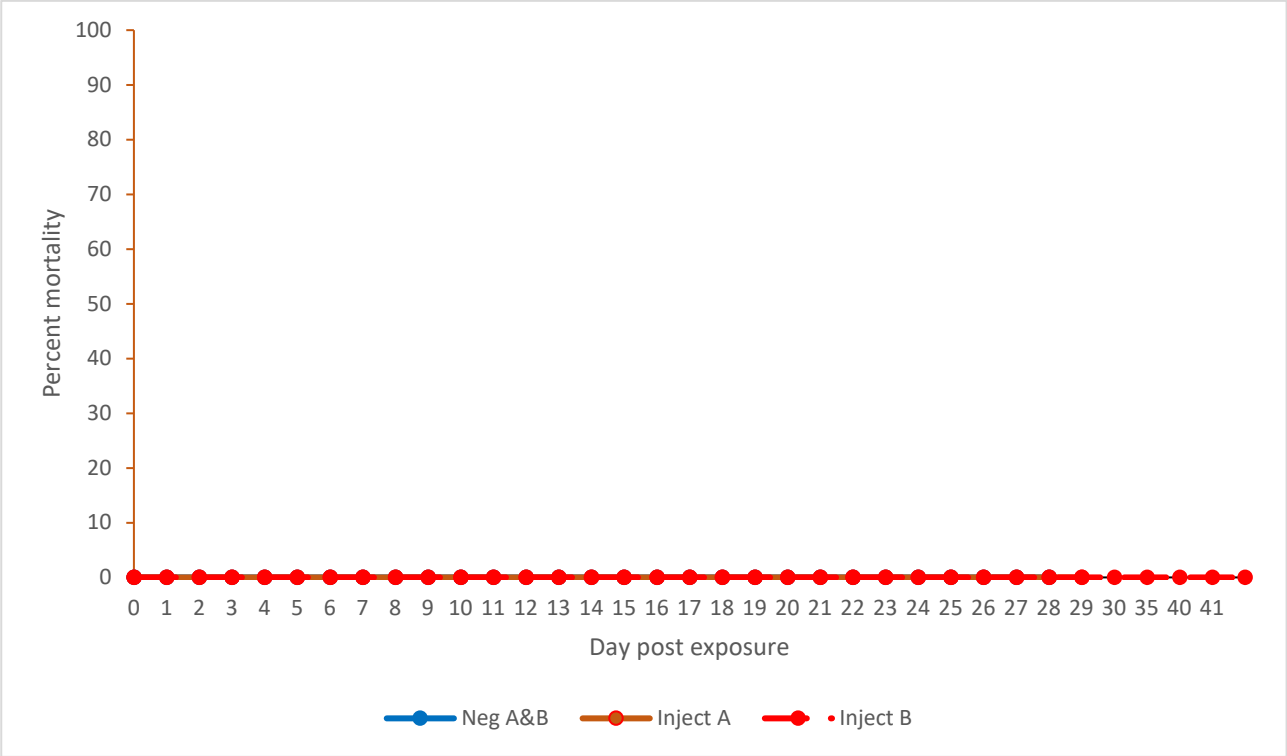


Figure 13b. Cumulative mortality in *P. merguiensis* after exposure to YHV9 by injection at 29°C

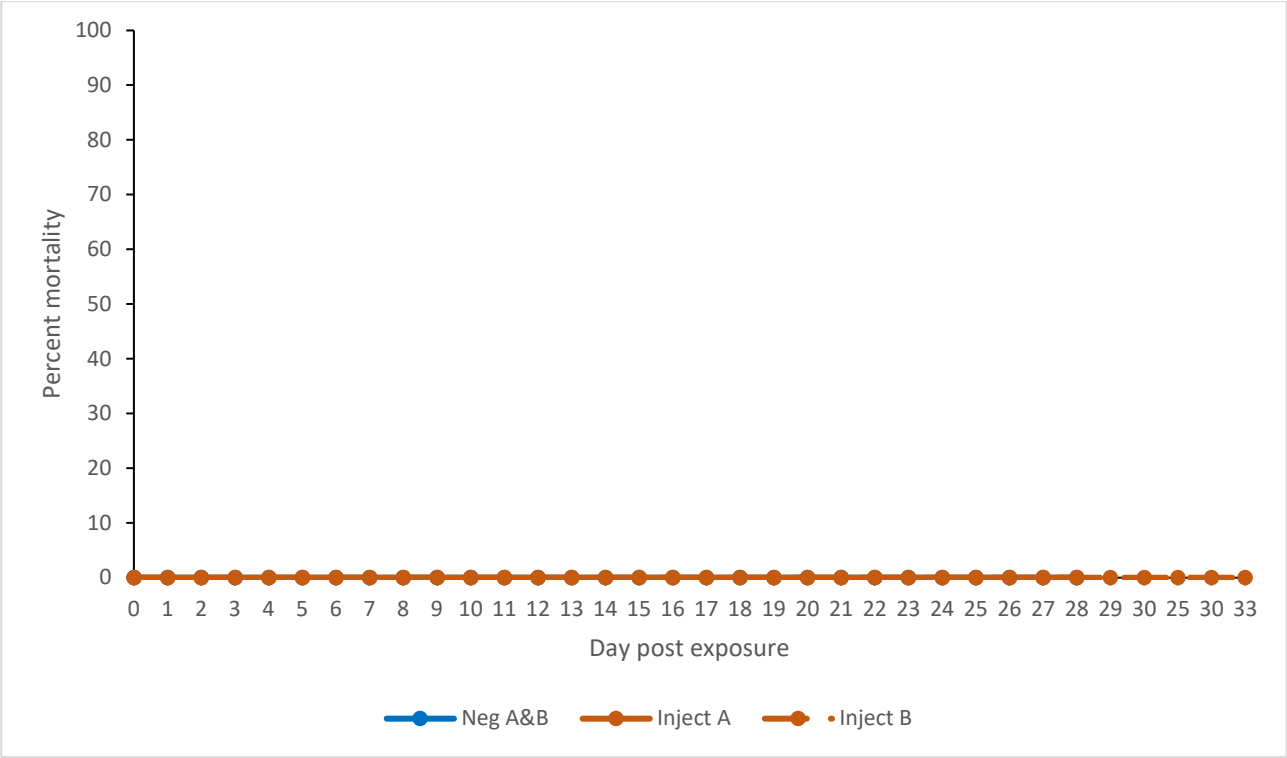


Figure 14a. Cumulative mortality in *P. monodon* after exposure to YHV10 by injection at 29°C

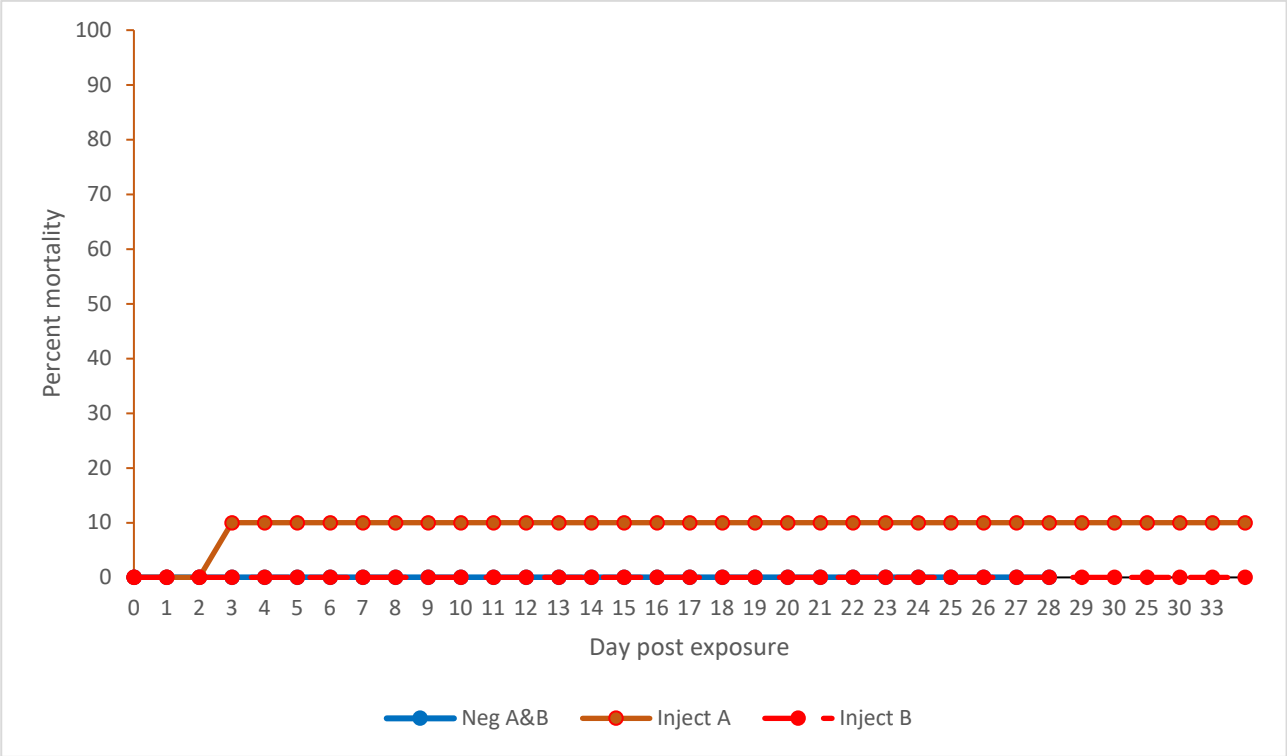


Figure 14b. Cumulative mortality in *P. merguiensis* after exposure to YHV10 by injection at 29°C

2.2.3 Molecular testing of infected prawns

Moribund, dead and sacrificed prawns were tested with assays for each of the specific YHV genotype present in the respective inoculum. All *P. monodon* were also tested for YHV2 as described previously. *P. merguiensis* inoculated with YHV7 were also tested for YHV2 as this genotype was also present in the YHV7 inoculum. Results are presented in Figures 15 to 18.

All *P. monodon* infected with YHV1 by either injection, co-habitation or feeding were test positive for YHV1. The mean C_T values for *P. monodon* exposed to YHV1 were 21.7 by injection (range of 17.0 to 33.9), 21.0 by co-habitation (range of 17.0 to 29.7) and 23.1 and 23.3 for the two replicate feeding tanks (ranges of 16.9 to 34.0 and 17.2 to 30.3), respectively. Similar to exposure of prawns with YHV1 at 24°C, exposed prawns were test positive for YHV2, but the YHV2 C_T values in prawns dying from YHV1 did not change when compared to the pre-screen and negative control YHV C_T values (Figure 15).

All *P. merguiensis* infected with YHV1 by either injection, co-habitation or feeding were test positive for YHV1. The mean C_T values for *P. monodon* exposed to YHV1 were 23.4 by injection (range of 18.4 to 29.8), 22.7 by co-habitation (range of 22.7 to 33.4) and 24.4 and 24.1 for the two replicate feeding tanks (ranges of 17.5 to 29.9 and 19.2 to 29.5), respectively. (Figure 16).

All *P. monodon* exposed to YHV7 by injection were test positive, including three sacrificed at the end of the trial on day 44, with a mean C_T value of 21.9 (range of 17.0 to 24.7). Corresponding YHV2 C_T values in these prawns had a mean C_T of 24.6 (range of 20.1 to 26.9). The YHV7 and YHV2 test results for the prawns found dead on day 1 are not included in these calculations as the mortalities are not considered to be due to YHV7. Similarly, low C_T values were observed for YHV7 and YHV2 in co-habited prawns (Figure 17) which were all test positive and had mean C_T values for YHV7 and YHV2 of 21.9 (range of 18.0 to 24.4) and 24.4 (range of 20.6 to 26.5), respectively. The majority of these prawns were alive and euthanised for testing at the end of the 44-day trial. All but one of the prawns exposed to YHV7 by feeding were test positive, with a single prawn testing Indeterminate (one replicate positive, one replicate negative). The test results for prawns fed YHV7-infected material were more variable than exposure by injection or co-habitation, with mean C_T values for YHV7 of 26.9 (range of 18.3 to 36.4) and 25.0 (range of 17.9 to 34.5) and for YHV2 of 28.4 (range of 23.0 to 38.5) and 31.2 (range of 24.5 to 36.5) across the two duplicate tanks, respectively.

After injection of *P. merguiensis* with YHV7, only three mortalities occurred on day 14 and 21, across the two tanks exposed by feeding (Figure 18). Of these prawns, testing for YHV7 resulted in one prawn testing positive with a mean C_T of 37.4, one prawn testing Indeterminate and one prawn testing negative. Five prawns were sampled and tested from each tank at the termination of the trial on Day 44. Interestingly, while none of the prawns exposed by feeding tested positive to either YHV7 or YHV2, all five prawns exposed to YHV7 by injection tested positive for YHV7 and four tested positive for YHV2. These prawns had a mean C_T value for YHV7 of 20.7 (range of 19.2 to 24.1) and for YHV2 of 32.7 (range of 31.9 to 34.0). Three of the 5 prawns from the co-habitation tank, samples on Day 44 were also test positive for YHV7 with a mean C_T of 37.1 (range of 36.1 to 37.8). These prawns were test negative for YHV2.

After injection of *P. monodon* with YHV9, 95.5% tested positive at the termination of the trial on Day 41 (1 prawn from each replicate tank tested Indeterminate) with relatively low Ct values (Figure 19). Mean C_T values for YHV9 were 26.4 (range of 22.9 to 34.5) and 28.0 (range of 24.1 to 35.6) across the two replicate tanks. The majority of these prawns also tested positive for YHV2 with mean C_T values of 32.4 (range of 24.6 to 41.4) and 30.3 (range of 21.5 to 36.2). The YHV2 C_T values were similar to those pf pre-screen and negative control *P. monodon*.

All *P. merguiensis* exposed to YHV9 tested positive even though there were only two mortalities which occurred 3-days post exposure. The remaining prawns were samples at the termination of the experiment

on Day 41. YHV9 mean C_T values were 25.5 (range of 22.0 to 30.1) and 26.8 (range of 22.0 to 40.3) across the two replicate tanks (Figure 20).

No *P. monodon* were test positive for YHV10 (Figure 19) when this experiment was terminated on Day 33. These prawns were test positive for YHV2 with C_T values similar to those pf pre-screen and negative control *P. monodon* with mean YHV2 C_T values of 30.8 (range of 21.9 to 36.7) and 30.2 (range of 20.8 to 34.7) across the two replicate tanks.

No *P. merguiensis* tested positive for YHV10 after exposure by injection when the experiment was terminated on Day 33 (Figure 20).

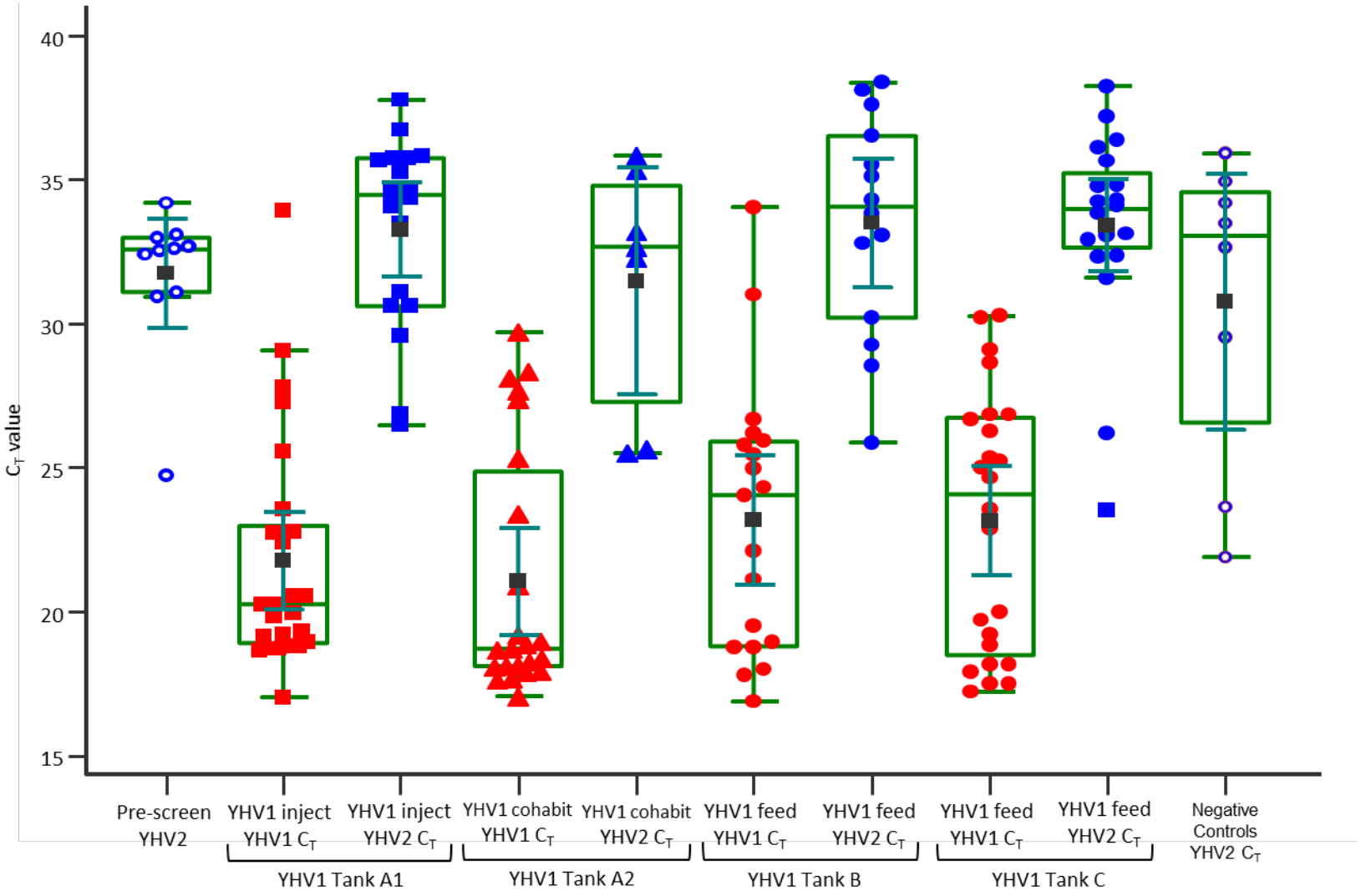


Figure 15. Molecular test results for *P. monodon* exposed to YHV1 by injection, co-habitation and feeding at 29°C

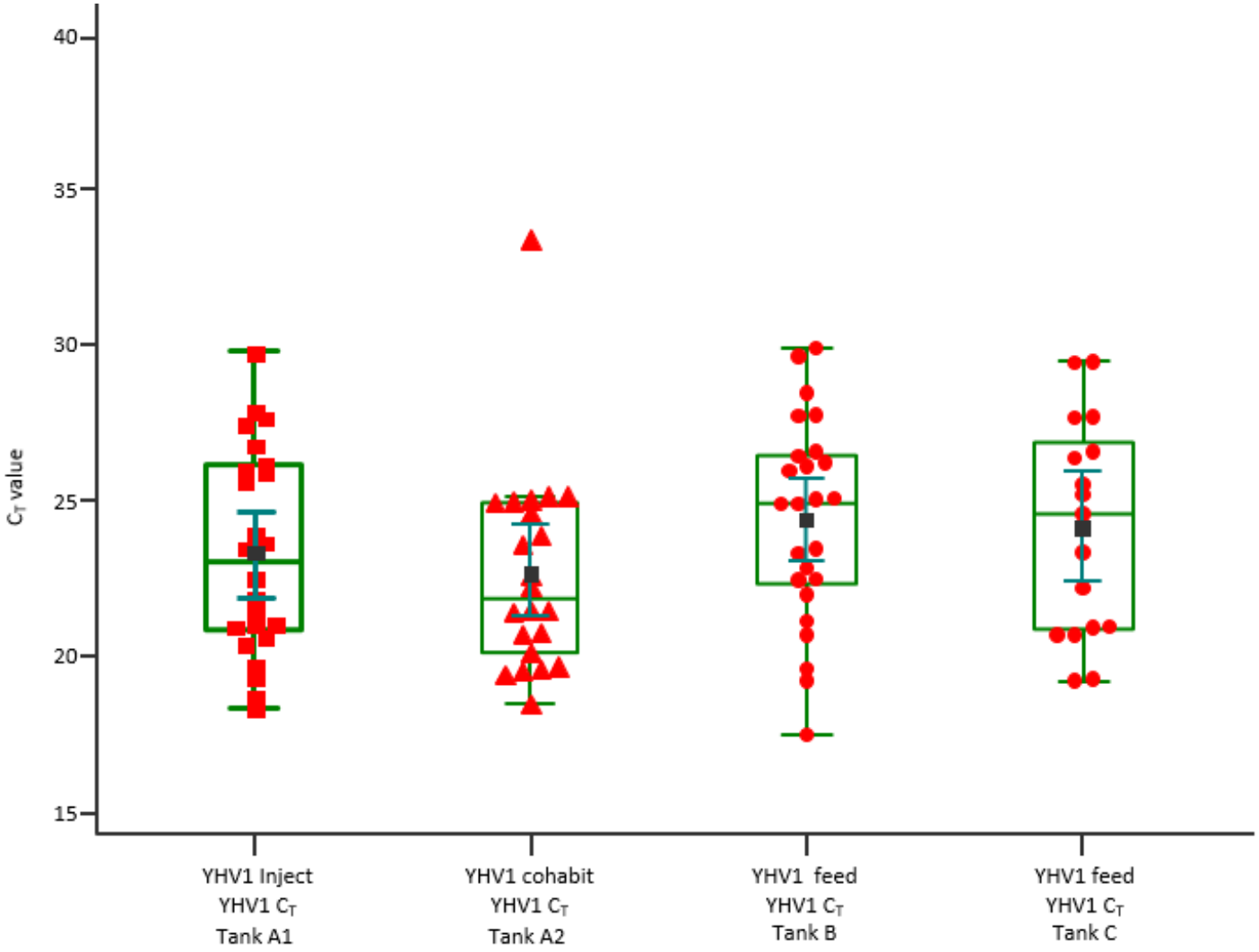


Figure 16. Molecular test results for *P. merguiensis* exposed to YHV1 injection, co-habitation and feeding at 29°C

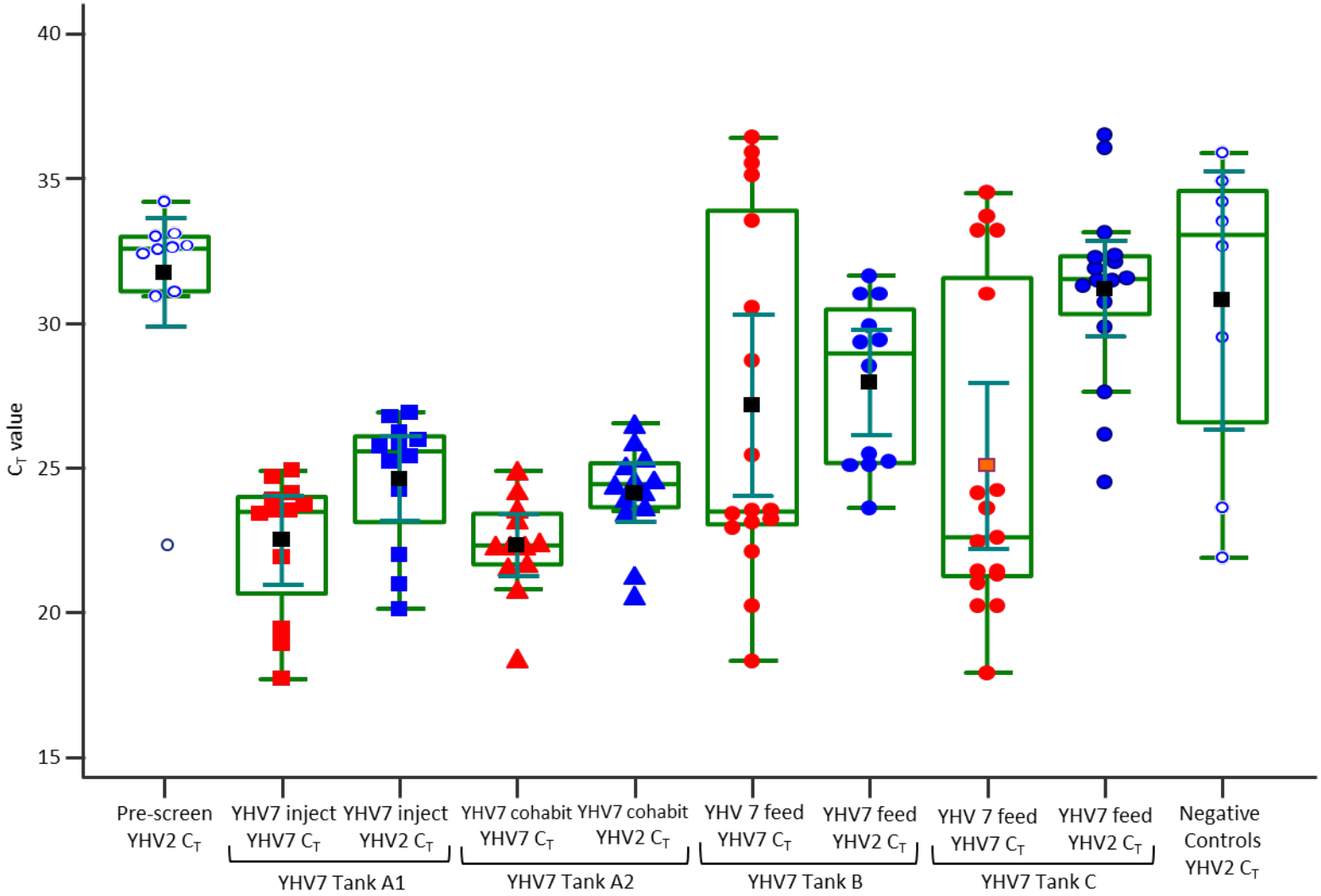


Figure 17. Molecular test results for *P. monodon* exposed to YHV7 by injection, co-habitation and feeding at 29°C

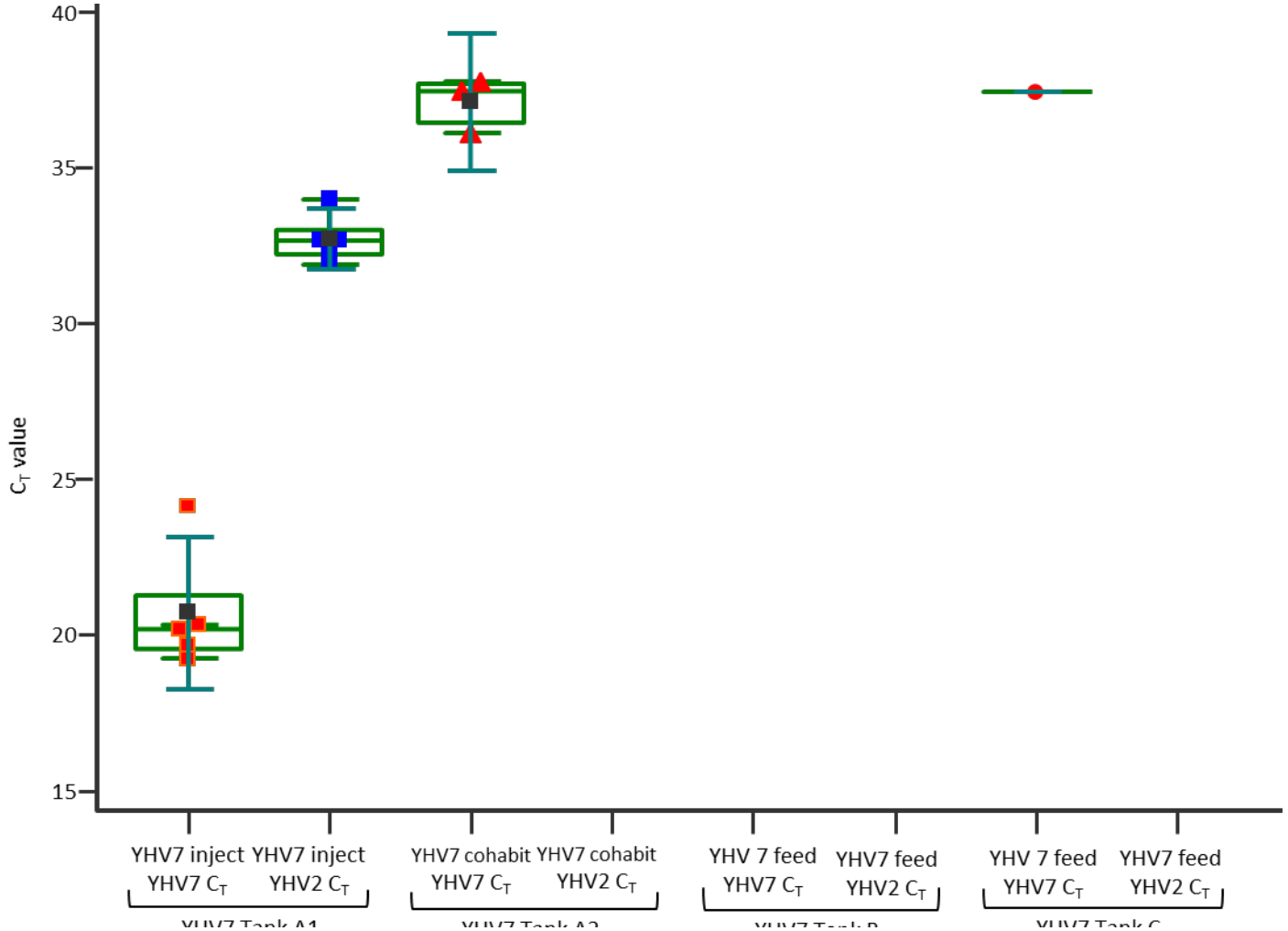


Figure 18. Molecular test results for *P. merguiensis* exposed to YHV7 by injection, co-habitation and feeding at 29°C

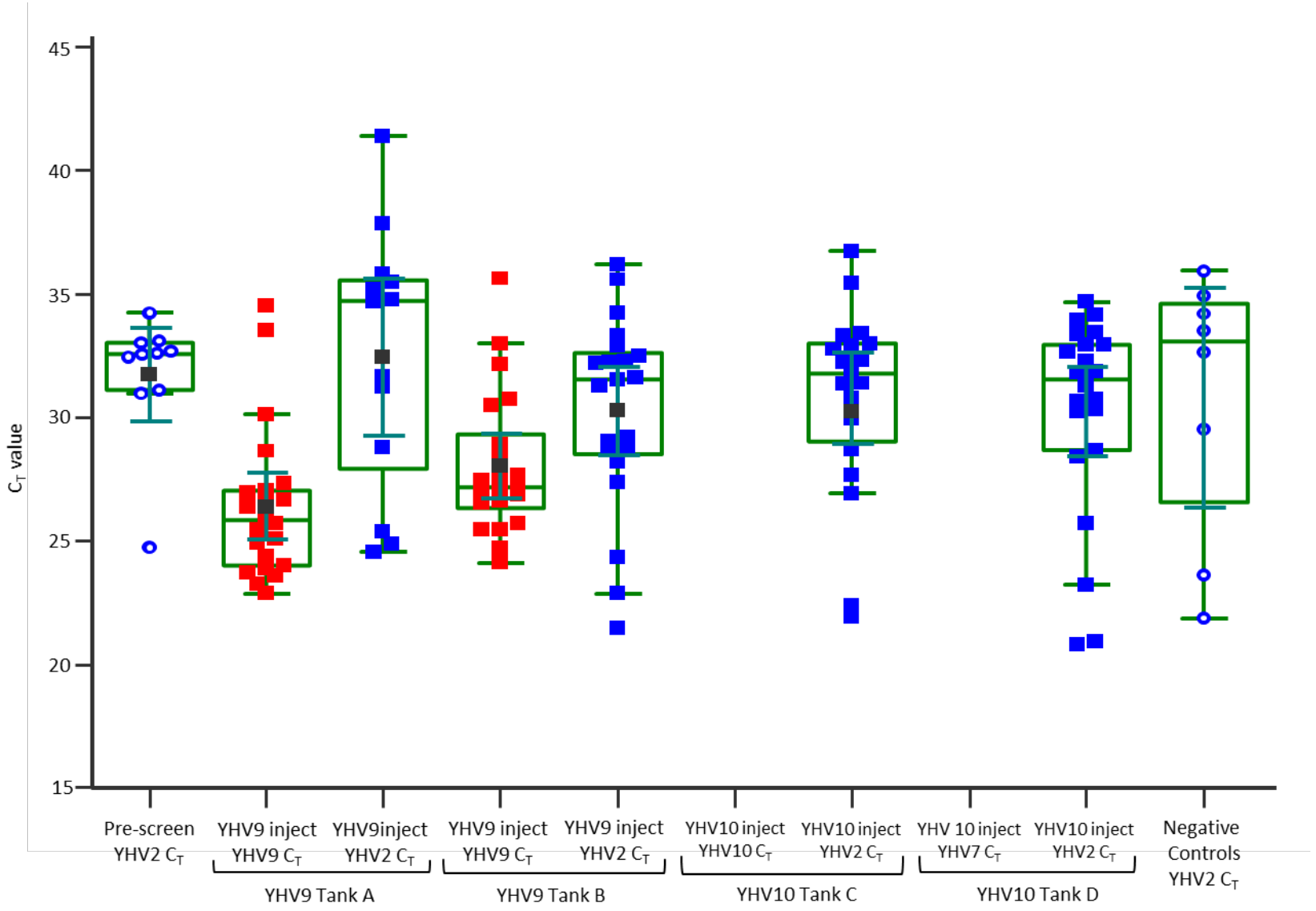


Figure 19. Molecular test results for *P. monodon* exposed to YHV9 and YHV10 by injection at 29°C

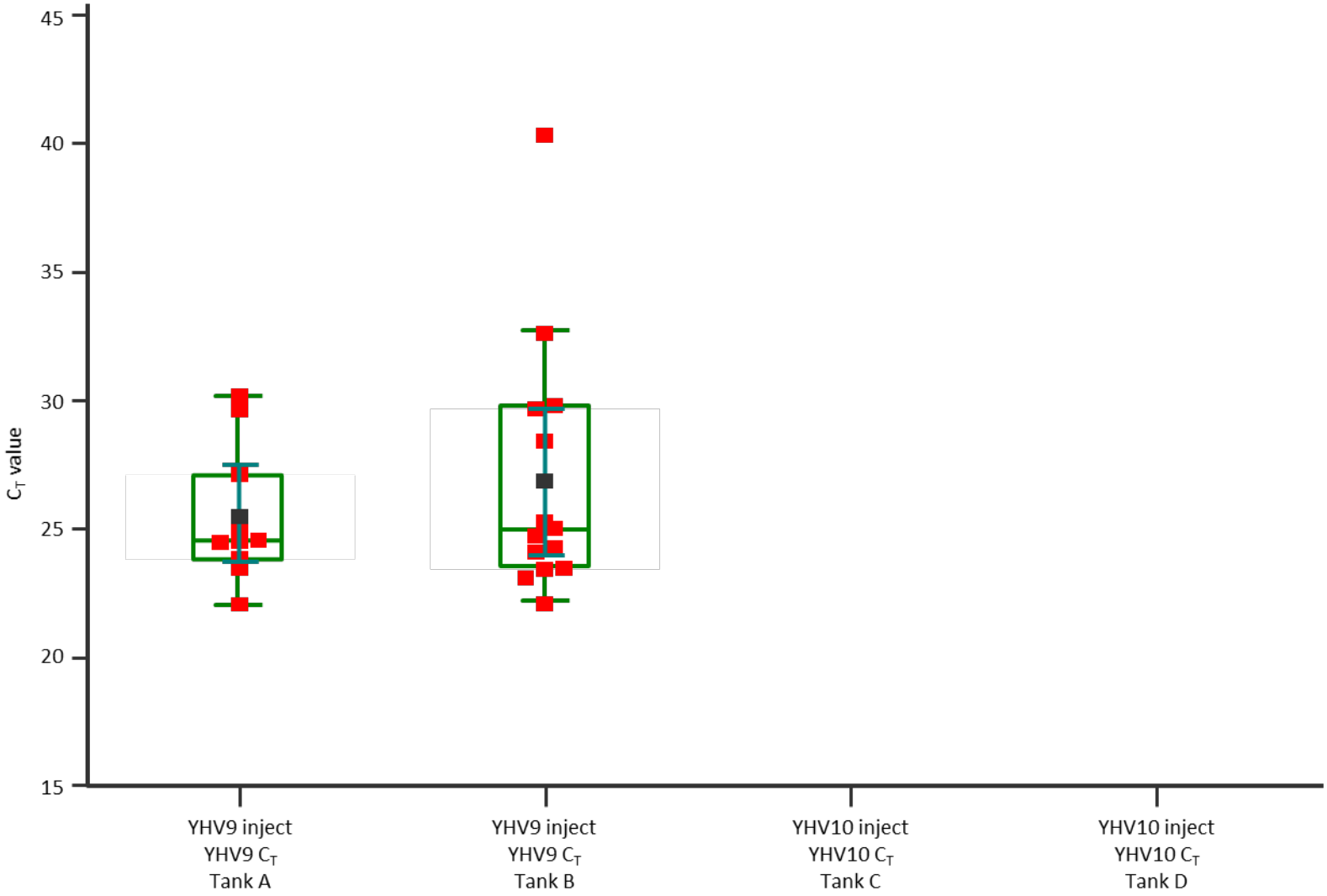


Figure 20. Molecular test results for *P. merguiensis* exposed to YHV9 and YHV10 by injection at 29°C

2.2.4 Histological examination of fixed tissues of infected prawns

2.2.4.1 Histopathology of tissues from *P. monodon* and *P. merguiensis* infected with YHV1 at 29°C

Some problems did arise with the smallest prawns where histological processing was not always successful and major organs and tissues not included in the sections and thus were not examined. Details of the specific findings from the laboratory reports and interpretation from the summary report are described in Table 5 and 6.

The histopathology supports the successful transmission of YHV1 in both *P. monodon* and *P. merguiensis*, and with feeding and co-habitation in *P. monodon*. There were insufficient specimens to demonstrate transmission by feeding and co-habitation in *P. merguiensis*.

Table 5. Histopathological description of the *P. monodon* exposed to YHV1 at 29°C

Specimen Details	Histopathology Description	Interpretation
Exposure by co-habitation Prawn No.5 Dead Day 6 YHV1 C_T = 17.6 YHV2 C_T = 32.3	• No lymphoid organ in section. • Haematopoietic tissue included some haemoblasts with large pale nuclei. Also present were some necrotic cells with karyorrhectic debris. • There was a limited storage vacuolation of hepatopancreatocytes. Some tubules contained rounded cells with degenerate nuclei; at times these were a dense mass filling the tubule. • There were occasional, suspicious necrotic haemocytes in gill filament sinusoids (dense shrunken nuclei).	No significant lesion
Exposure by co-habitation Prawn No. 10 Dead Day 9 YHV1 C_T = 18.4 YHV2 C_T = Neg	• Lymphoid organ tubules had lost all lymphoid cell definition and the walls contained degenerate, pyknotic and karyorrhectic nuclei. The interstitium contained around 11 eosinophilic, necrotic cell aggregations. • Storage vacuolation of hepatopancreatocytes was reduced. Some tubules were full of rounded cells. There were a few foci of necrotic haemocytes in the connective tissue at the edge of the hepatopancreas. • There were occasional, suspicious necrotic haemocytes in gill filament sinusoids (dense shrunken nuclei). Foregut haematopoietic tissue included some haemoblasts with large pale nuclei.	Confirms transmission of, and resulting infection by, YHV1
Exposure by co-habitation Prawn No.23 Moribund Day 9 YHV1 C_T = 19.0 YHV2 C_T = 25.6	• Some of the lymphoid organ tubules had lost lymphoid cell definition but others seemed to still contain some intact cells; but most tubules had increased number of pyknotic or karyorrhectic nuclear debris. 40% of the lymphoid organ was compromised of spheroids consisting of cells with abundant pale, purplish cytoplasm. • Two spheroids were present in ventral thoracic connective tissue. • One of the pereiopod nerves had a single area of necrosis with vacuolation of swollen nerve fibres. • There were occasional, suspicious necrotic haemocytes in gill filament sinusoids (dense shrunken nuclei). Storage vacuolation of the hepatopancreatocytes was normal.	Confirms transmission of, and resulting infection by, YHV1
Exposure by feeding Prawn No.1 Dead Day 3 YHV1 C_T = 17.8 YHV2 C_T = 26.3	• Lymphoid organ tubules had lost all lymphoid cell definition and the walls contained degenerate, pyknotic and karyorrhectic nuclei. The interstitium contained 5 eosinophilic, round necrotic cell aggregations. • Haematopoietic tissue included a few haemoblasts with large pale nuclei. Also present were some necrotic cells with karyorrhectic debris. • Eosinophilic, necrotic cell aggregations/haemocyte nests were present in the heart (>8), foregut connective tissue and other thoracic connective tissues. Some of these had an obvious early encapsulation with a layer of fibroblasts.	Confirms transmission of, and resulting infection by, YHV1

	• Storage vacuolation of the hepatopancreatocytes was normal although some of the tubules and ducts had rounded cells in the lumen; one tubule also had bacteria proliferating in the sloughed cells. • There were occasional, suspicious necrotic haemocytes in gill filament sinusoids (dense shrunken nuclei).	
Exposure by feeding <u>Prawn No.6</u> Dead Day 3 YHV1 C_T = 16.9 YHV2 C_T = Neg	• Lymphoid organ tubules had lost all lymphoid cell definition and the walls contained degenerate, pyknotic and karyorrhectic nuclei. There was one suspect spheroid in the interstitum. • Eosinophilic, necrotic cell aggregations/haemocyte nests were present in the heart (3), dorsal thoracic subcuticular connective tissue, gill cover connective tissue and in gill axis in low numbers. • Haematopoietic tissue included a few haemoblasts with large pale nuclei. Also present were necrotic cells with karyorrhectic debris. Storage vacuolation of the hepatopancreatocytes was normal although some of the tubules had rounded cells in the lumen. • There were occasional, suspicious necrotic haemocytes in gill filament sinusoids (dense shrunken nuclei).	Confirms transmission of, and resulting infection, by YHV1
Exposure by feeding <u>Prawn No.32</u> Moribund Day 4 YHV1 C_T = 18.8 YHV2 C_T = 32.9	• Lymphoid organ tubules are necrotic with pale eosinophilic material filling the stroma with occasional necrotic nuclear debris present, although the lumen lining endothelial cells remained apparent although they were degenerating. Many of the necrotic tubule walls had a proliferation of bacteria in the debris. Three spheroids were present in the interstitium. • The heart contained a haemocyte aggregation. • Haematopoietic tissue included a few haemoblasts with large pale nuclei. Also present were necrotic cells with karyorrhectic debris. There were occasional, suspicious necrotic haemocytes in gill filament sinusoids (dense shrunken nuclei). • Storage vacuolation of the hepatopancreatocytes was slightly reduced and there were tubules containing rounded, sloughed cells. There were the odd foci of proliferating bacteria in interstitium or on a tubule basement membrane.	Confirms transmission of, and resulting infection, by YHV1
Exposure by feeding 16-03582 #33 <u>Prawn No.33</u> Moribund Day 4 YHV1 C_T = 17.5 YHV2 C_T = 32.3	• Lymphoid organ tubules had lost all lymphoid cell definition and the walls contained degenerate nuclei and a few pyknotic or karyorrhectic nuclei debris. The lumen lining endothelial cells remained apparent. The interstitium contained around 9 eosinophilic , necrotic cell aggregations. A single, small proliferation of bacteria was present in the lymphoid organ. • Low numbers of eosinophilic, necrotic cell aggregation s/haemocyte nests were present in the heart (2), ventral thoracic connective tissue and in gill axis. • Haematopoietic tissue included a few haemoblasts with large pale nuclei. Also present were necrotic cells with karyorrhectic debris. There were occasional, suspicious necrotic haemocytes in gill filament sinusoids (dense shrunken nuclei). • Storage vacuolation of the hepatopancreatocytes was reduced and there were some rounded cells in tubule lumens. Eosinophilic, necrotic cell aggregation s were present in the HP interstitium .	Confirms transmission of, and resulting infection, by YHV1

Table 6. Histopathological description of the *P. merguiensis* exposed to YHV1 at 29°C

Specimen Details	Histopathology Description	Interpretation
Exposure by injection Prawn No.5 Moribund Day 2 YHV1 C_T = 22.0 YHV2 C_T = Neg	• Lymphoid organ tubules had no defined lymphoid cells in the wall although the lumen lining cells (endothelium) appeared normal. These tubule walls did contain some necrotic nuclear debris. • Haematopoietic tissue in the 3rd maxilliped had some disrupted nodules containing pyknotic nuclei and foci of karyorrhectic debris. Two small-to-moderate, melanised granulomas were present in gill axis endothelium. • Hepatopancreatocytes had a reduced storage vacuolation. Some tubules had some rounded cells in the lumen. • The eye had an area of necrotic crystalline tracts with scattered melanin pigment (from necrotic reticular cell bodies of the ommatidia). The gill cover had a haemolymph blister in the outer epidermal cells.	Confirms transmission of, and resulting infection by, YHV1
Exposure by injection Prawn No.6 Moribund Day 2 YHV1 C_T = 20.7 YHV2 C_T = Neg	• Lymphoid organ tubules had necrosis of lymphoid cells in the wall although lumen lining cells appeared normal. Necrotic cell debris were scattered through the wall. Scattered through the interstitium were large, melanised haemocyte nests. • Scattered through the maxilliped haematopoietic tissue were karyorrhectic debris. • Several small, melanised granulomas were present in gill axis endothelium. There also appear to be pyknotic nuclei or pale cells with clear nucleoplasm and a prominent nucleolus scattered through gill filaments. • Four small, melanised haemocyte nests were present in the heart siting on the surface of myocardial fibres. There were two foci of necrosis of skeletal muscle fibres in thorax and the 1st abdominal segment. • There was a little reduction of storage vacuolation in hepatopancreatocytes. There was some sloughing of cells into the lumen of tubules.	Confirms transmission of, and resulting infection by, YHV1
Exposure by injection 16-03584 #13 Prawn No.13 Moribund Day 2 YHV1 C_T = 18.8 YHV2 C_T = Neg	• Lymphoid organ tubules had necrosis of lymphoid cells in the wall although lumen lining cells appeared normal. Necrotic cell debris were scattered through the wall. • There were some necrotic cells scattered through the lumen of antenna! gland labyrinth tubules. • Two small, melanised granulomas were present in gill axis endothelium. There were some pale cells with prominent nucleoli scattered through filament epithelium. • There was a little storage vacuolation in hepatopancreatocytes. There were some unusual tubule cells blebbing or sloughing into the lumen (artefact?). • There was a necrosis of about half of the myofibrils in the dorsal band of muscle of the 1st abdominal segment. The middle of the gill cover contained a small, haemolymph filled blister.	Confirms transmission of, and resulting infection by, YHV1
Exposure by co-habitation Prawn No.47 Moribund Day 9 YHV1 C_T = 19.6 YHV2 C_T = Neg	• The lymphoid organ did not appear as necrotic as the prawns above, but the lymphoid cells were poorly defined and there were scattered necrotic nuclear debris and cells in the tubule wall. • There were some clumps of cells scattered through the lumen of antennal gland labyrinth tubules. The middle area of the eye crystalline tracts was necrotic. Scattered through the gill filaments were some pale cells with prominent nucleoli. Hepatopancreatocytes had a reduced storage vacuolation.	Inconclusive

2.2.4.2 Histopathology of tissues from *P. monodon* and *P. merguiensis* infected with YHV7 at 29°C

Details of the specific findings from the laboratory reports and interpretation from the summary report are described in Table 7 and 8. The lesions observed in the lymphoid organ and haemocyte clusters with the presence of basophilic, cytoplasmic inclusions are probably viral in origin as they represent host tissue response by the immune cells of the shrimp for both *P. monodon* and *P. merguiensis* (Figures 22 and 24). However, although the prawns have been exposed by YHV7 containing inoculums, it is unclear if these lesions are solely, directly or specifically due to YHV7. The main confounding observation is the presence (albeit at lower intensity) of similar lesions in negative control animals of both prawn species (Figures 21 and 23).

Pathologist recommendations are that additional markers are needed, specifically the development of in-situ hybridization probes specific to YHV7, supported by electron microscopy to localise the virus(es) in these lesions. Also, to be sure that histopathology assessment is more robust, whole prawns sampled at various time points along the post-challenge period, in sufficient numbers per trial, would generate more reliable data from which to make inferences about causality and role of YHV7 in shrimp disease.

Table 7. Histopathological description of the *P. monodon* exposed to YHV7 at 29°C

Specimen Details	Histopathology Description	Interpretation
Negative controls, sacrificed on Day 44 Injection x 2 YHV7 C_T = Neg YHV2 C_T = 22.8 ($\overline{x}$) Feeding x 2 YHV7 C_T = Neg YHV2 C_T = 32.6 ($\overline{x}$)	• There are relatively consistent systemic inflammatory responses in the lymphoid organ and connective tissues of carapace, cuticle, eyestalk, mouthparts, ventral nerve cord for shrimp not challenged with YHV7 inoculum. The sham-feed shrimp had fewer haemocyte clusters, and in one shrimp, many spheroids had atrophied and necrotised. • In the lymphoid organ, Type 1 spheroids can contain abundant or few cytoplasmic, basophilic inclusion bodies. Type 2 spheroids contain single cytoplasmic basophilic ovoid to fragmented inclusions with low level cell apoptosis. Occasional, Type 2 spheroids can atrophy and become necrotic. There is no frank necrosis of the lymphoid organ. • Also prominent is the systemic distribution of haemocyte clusters (HCs) as described in the histopathology results – also containing similar but much fewer cytoplasmic basophilic inclusions. These are not necrotic generally.	This pathology in negative control shrimp of the experiment suggests that the role of YHV7 in the causation of mortalities or pathology is uncertain. This pathology may be due to other unidentified virus(s) pre- existing in the experimental shrimp to give such a result.
Exposed to YHV7, sacrificed on Day 44 Injection x 3 YHV7 C_T = 20.9 ($\overline{x}$) YHV2 C_T = 24.0 ($\overline{x}$) Co-habitation x 10 YHV7 C_T = 22.1 ($\overline{x}$) YHV2 C_T = 24.8 ($\overline{x}$)	• There are relatively consistent systemic inflammatory responses in the lymphoid organ (LO) and connective tissues of pereopods, carapace, cuticle, eyestalk, nerve, ventral nerve cord, antennal gland, antenna for shrimp injection or co-habitation challenged with YHV7. If anything, the co-habitation challenged shrimp showed a wider distribution of haemocyte clusters and similar LO spheroid reactions, but with fewer numbers of basophilic, cytoplasmic inclusions in affected cells. • In the lymphoid organ, Type 1 spheroids can contain abundant or few cytoplasmic, basophilic inclusion bodies. Type 2 spheroids contain single cytoplasmic basophilic ovoid to fragmented inclusions and occasionally an eosinophilic ovoid inclusion body with low level cell apoptosis. Occasionally, Type 2 spheroids can atrophy and become necrotic. There is no frank necrosis of the lymphoid organ. • Also prominent is the systemic distribution of haemocyte clusters (HCs) as described in the histopathology results – also containing similar but much fewer cytoplasmic basophilic inclusions. These are not necrotic generally. • The suboptimal hepatopancreas lipid storage vacuolation (<70% of hepatopancreas section) suggests that the shrimp were sick (reduced	Whether the systemic responses are due specifically/only to YHV7 depends on localizing the virus to the affected cells by an *in-situ* hybridization assay using a YHV7 specific probe and electron microscopy to confirm that the inclusions are viral in nature. Exclusion of other endemic viruses (YHV2, IHHNV and MoV) from the affected cells by *in- situ* probes would also be required or that the inoculums/shrimps used in the challenges

	feeding). • Low level gill, cuticular melanisation suggest a low level of bacterial infection in the experimental shrimp.	are free of these viruses.
Exposed to YHV7, sacrificed on Day 44 <u>Feeding x 20</u> YHV7 C_T = 26.3 ($\overline{x}$) YHV2 C_T = 29.8 ($\overline{x}$)	• Of the 20 shrimp challenged, 75% showed basophilic inclusion bodies in the lymphoid cells of spheroids – mostly in type 1 and fewer in type 2 spheroids. Haemocyte clusters with a lower level of basophilic inclusions occurred in 45% of challenged shrimp. Type 2 spheroids showed low level cell apoptosis and only 1-5x necrotic spheroids with atrophied in 45% of shrimp. Interestingly occasional cells in the gills, heart and haematopoietic tissues had the basophilic, cytoplasmic inclusions. Taken together, these are relatively mild systemic viral inflammatory responses for this group of feed challenged *P. monodon.* • The suboptimal hepatopancreas lipid storage vacuolation (<70% of hepatopancreas section) of all shrimp in the group suggests that the shrimp were sick (reduced feeding) or not properly adapted to the tank conditions – given the relatively mild systemic viral reactions.	Whether the systemic responses are due specifically/only to YHV7 depends on localizing the virus to the affected cells by an *in-situ* hybridization assay using a YHV7 specific probe and electron microscopy to confirm that the inclusions are viral in nature. Exclusion of other endemic viruses (YHV2, IHHNV and MoV) from the affected cells by *in- situ* probes would also be required or that the inocula/shrimps used in the challenges are free of these viruses.

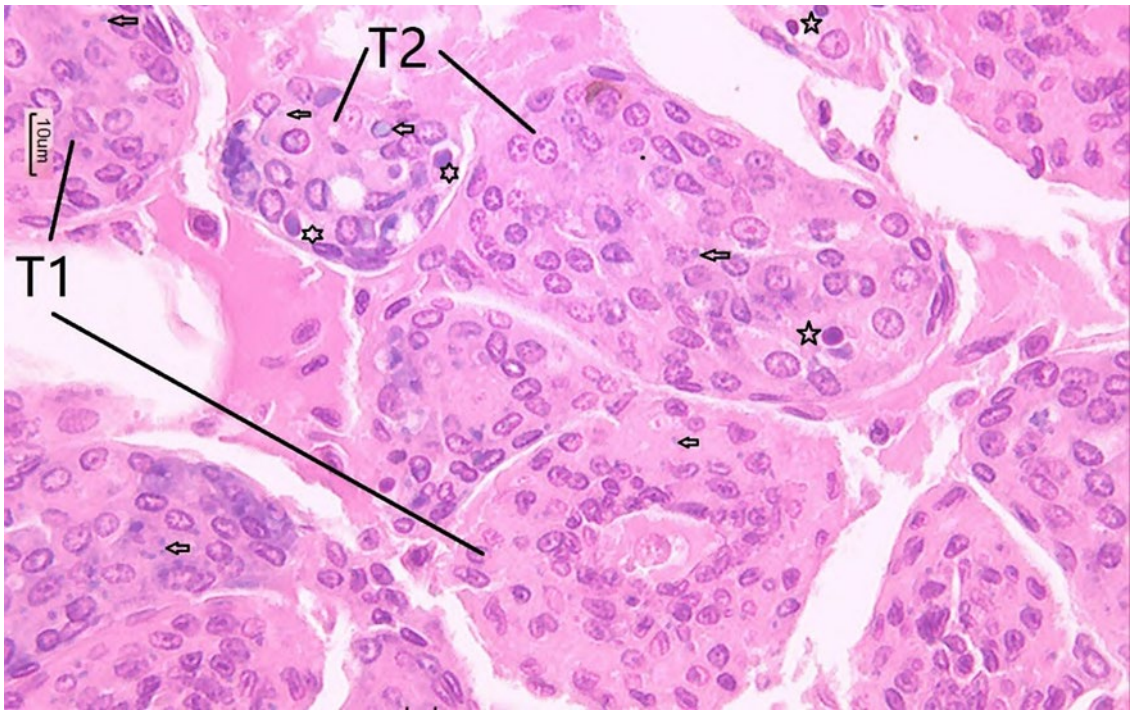


Figure 21. Histopathology of *P. monodon* negative control.

Lymphoid organ (H&E) x 400 magnification. T1 (type 1) and T2 (type 2) spheroids with small, basophilic, cytoplasmic inclusions (arrows). T2 spheroids with small and large basophilic, cytoplasmic inclusions (arrows), apoptotic lymphoid cells (stars). Scale bar 10 µm.

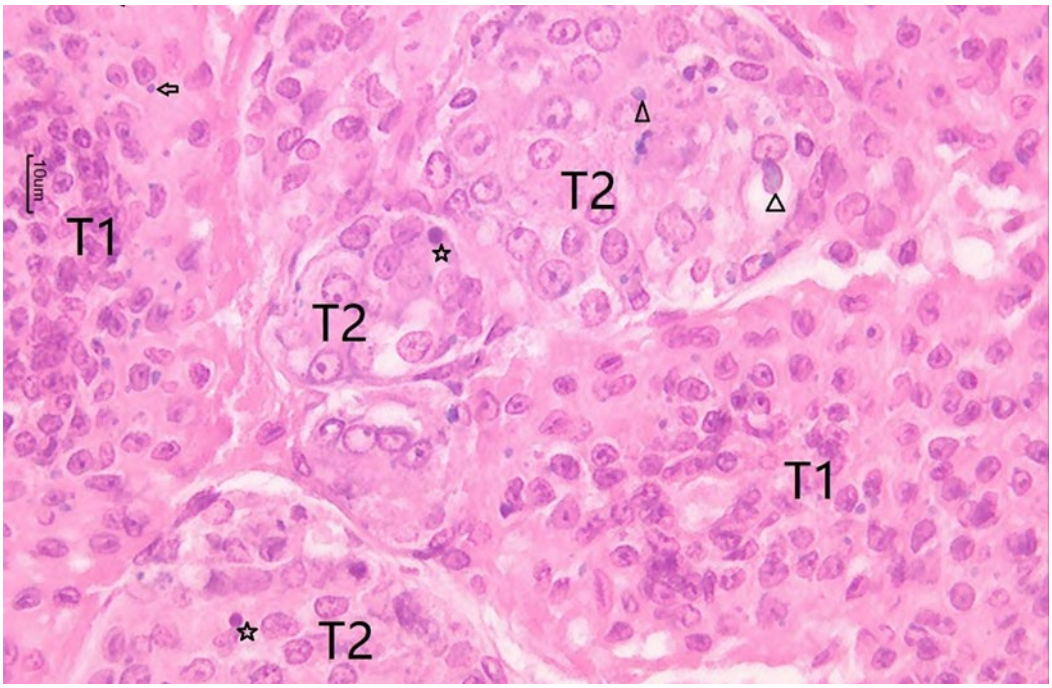


Figure 22. Histopathology of *P. monodon* exposed to YHV7

Lymphoid organ (H&E) x 400 magnification. T1 = type 1 spheroids. T2 = type 2 spheroids. Arrows point to basophilic cytoplasmic inclusions in lymphoid cells of T1 spheroids. Arrowheads point to basophilic cytoplasmic inclusions in lymphoid cells of T2 spheroid. Stars point to apoptotic lymphoid cells in T2 spheroids. Scale bar 10 µm.

Table 8. Histopathological description of the *P. merguiensis* exposed to YHV7 at 29°C

Specimen Details	Histopathology Description	Interpretation
Negative controls, sacrificed on Day 44 Injection x 4 YHV7 C_T = Neg YHV2 C_T = Neg	• It is interesting that lymphoid organ cells also contain the type of single, basophilic, ovoid or oblong inclusions but at very low levels in 50% of the unchallenged shrimp examined. Haemocyte clusters were observed in 25% of the shrimp examined.	It does pose the question as to whether these negative control shrimp already are carrying an unknown viral agent, which is not causing significant pathology for the species.
Exposed to YHV7, sacrificed on Day 44 Feeding x 10 YHV7 C_T = Neg YHV2 C_T = Neg	• No significant abnormality detected in all 10 shrimp examined as a result of the YHV7 feed challenge.	No interpretation.
Exposed to YHV7, sacrificed on Day 44 Injection x 5 YHV7 C_T = 20.7 ($\overline{x}$) YHV2 C_T = 32.7 ($\overline{x}$) Co-habitation x 5 YHV7 C_T = 37.1 ($\overline{x}$) YHV2 C_T = Neg	• There are relatively consistent systemic inflammatory responses in the lymphoid organ and connective tissues of pereopods, carapace, cuticle, eyestalk, heart, nerve and in isolated gill filament tissue for shrimp injection challenged with YHV7. The changes are localised to haemocyte cell types which is the formation of spheroid-like hypertrophy, cytoplasmic single vacuolation and within the vacuole usually basophilic ovoid to elongated inclusions (single or several) or a single intensely eosinophilic ovoid inclusion body. No frank necrosis is apparent in the affected cells. Also prominent is the systemic distribution of haemocyte clusters (HCs) as described in the histopathology results – also containing similar cytoplasmic basophilic mostly, and occasionally eosinophilic inclusions. HCs may contain degenerated cells that are vacuolated with inclusions, but not necrotic. • The co-habitation challenged shrimp had little evidence of systemic inflammatory reaction compared to the injected shrimp, but unfortunately the LO was not available for examination in the sectioned tissues. • The suboptimal hepatopancreas lipid storage vacuolation suggests that the shrimp were sick (reduced feeding). • Some hepatopancreatic granulomatous (bacterial) inflammation in 2 of the shrimp (1 each from each challenge mode group) suggests a low level of pre-existing disease in the experimental shrimp.	Whether the systemic responses are due specifically/only to YHV7 depends on localizing the virus to the affected cells by an *in-situ* hybridization assay using a YHV7 specific probe and electron microscopy to confirm that the inclusions are viral in nature. Exclusion of other endemic viruses (YHV2, IHHNV and MoV) from the affected cells by *in- situ* probes would also be required or that the inoculums/shrimps used in the challenges are free of these viruses.

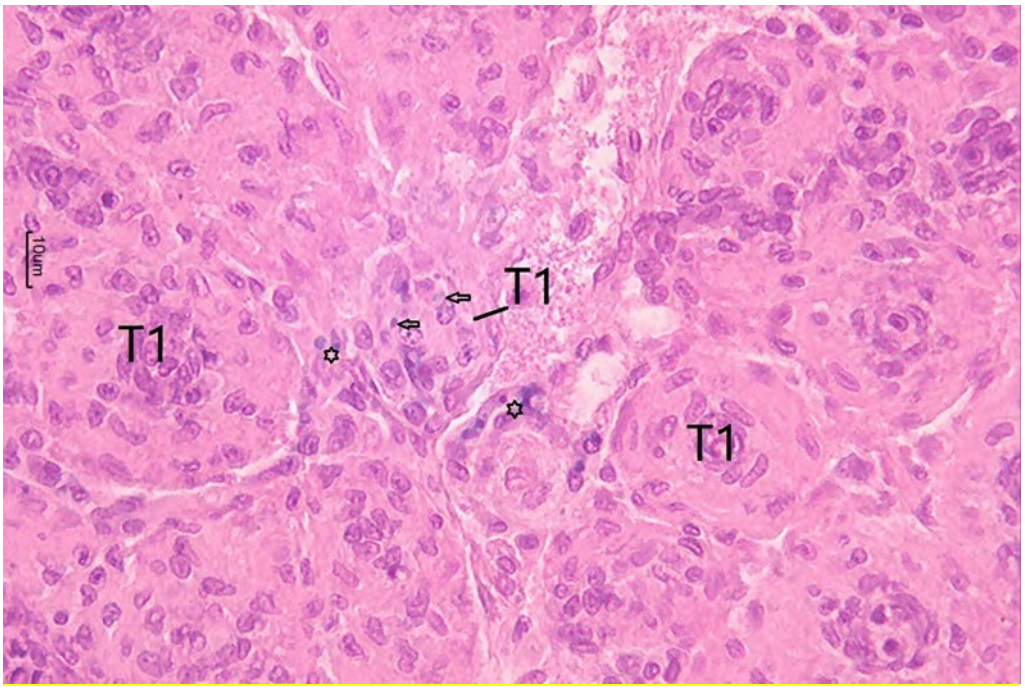


Figure 23. Histopathology of *P. merguiensis* negative control

Lymphoid organ (H&E) x 400 magnification. Arrows = cytoplasmic, basophilic inclusions. Stars = apoptotic cells. T1 = type 1 spheroids. Scale bar 10 µm.

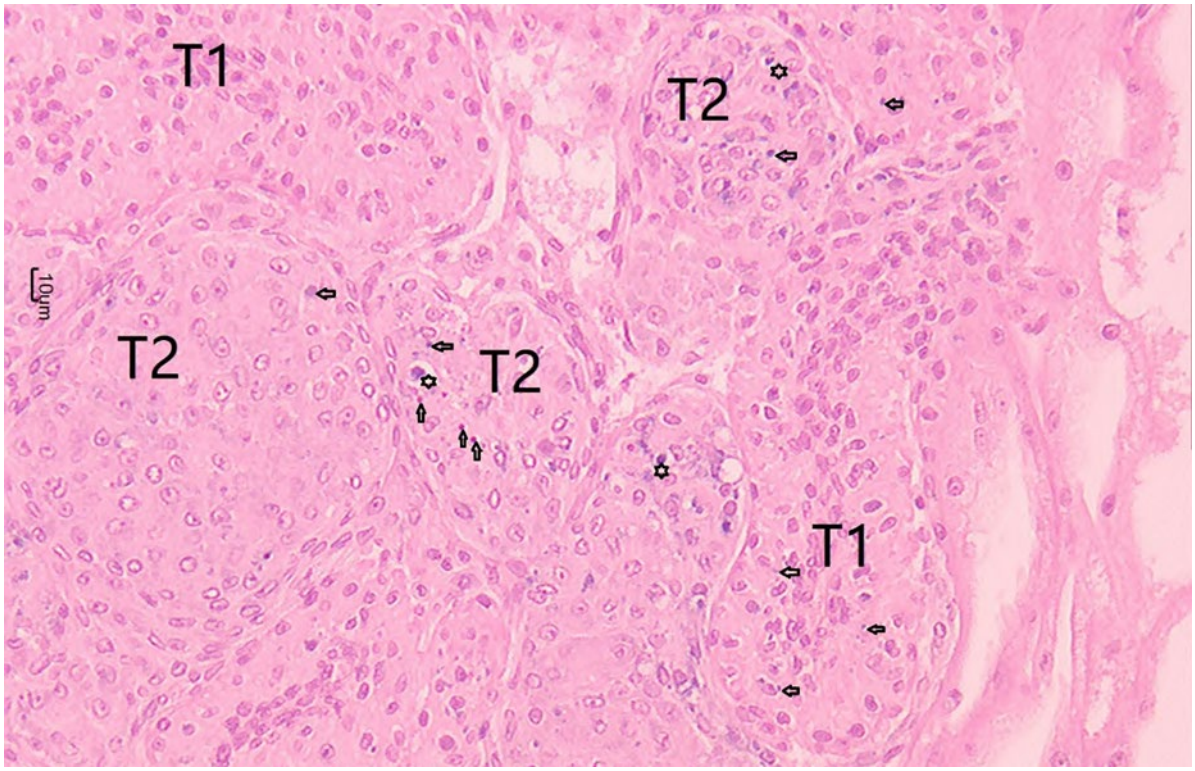


Figure 24. Histopathology of *P. merguiensis* exposed to YHV7

Lymphoid organ (H&E) x 400 magnification. T1 (type 1) spheroids with small, basophilic, cytoplasmic inclusions (horizontal arrows) T2 (type 2) spheroids with small and large basophilic, cytoplasmic inclusions (horizontal arrows), and eosinophilic inclusions (vertical arrows) and apoptotic lymphoid cells (stars). Scale bar 10 µm.

2.3 Conclusion

Results of the Experimental Trial 2, which was undertaken to investigate the pathogenicity of YHV1, and YHV7 after exposure by infection, co-habitation and feeding, and YHV9 and YHV10 after infection by injection, at 29°C, can be summarised as follows:

1. Injection of both *P. monodon* and *P. merguiensis* with YHV1 resulted in acute mortality (100% in 5-6 days) with both species having high levels of YHV1 RNA detected by RT-qPCR. Similar results, including the time to 100% mortality, were observed in both species after exposure to YHV1 by feeding. Several *P. monodon* mortalities, after exposure to YHV1 by feeding, were observed to have obviously yellow heads. This was not observed in mortalities after exposure by injection and co-habitation. While the onset of mortalities in both species exposed to YHV1 by co-habitation was delayed, once initial mortalities commenced, the time to 100% mortality was only 4 to 6 days. While histological analysis supported the successful transmission of YHV1 to both *P. monodon* and *P. merguiensis*, limited material for analysis could only allow transmission of YHV1 to be supported for feeding and co-habitation in *P. monodon*. Despite the limited histology results, mortality curves and YHV1 RT-qPCR test results indicate that both prawn species are highly susceptible to infection with YHV1 by feeding and co-habitation. The evidence generated in this report fulfil the criteria for listing *P. merguiensis* as susceptible to infection with YHV1 according to Chapter 1.5 of OIE Aquatic Animal Health Code (Aquatic Code).

2. Injection of *P. monodon* with YHV7 caused more chronic mortality compared to YHV1 (if the 20% mortality observed 1-day post infection is excluded, total cumulative mortality was 36% after the 44 days of the trial). This was a lower mortality rate than the 62% mortality observed with the same inoculum after injection of *P. monodon* with YHV7 at 24°C (Experimental Trial 1). Low levels of mortality were also observed after exposure of *P. monodon* to YHV7 by feeding (15%) and co-habitation (4%). However, given the low C_T values (mean of 21.9) observed in co-habitant *P. monodon*, evidence suggests YHV7 infection of these prawns was via waterborne and *per os* transmission. While YHV7 C_T values were on average below 25 after exposure to YHV7 by injection, YHV2 C_T values were also reduced, compared to pre-screen and negative control prawns. Combined with the ambiguous histology results, where pathology observed in prawns exposed to YHV7 could not be differentiated from pathology observed in negative control prawns, it is uncertain whether the mortalities observed in this experiment were due to YHV7 or YHV2 or an interaction between both YHV genotypes. *In situ* hybridization using YHV7- and YHV2-specific probes is required to clarify whether one, both or none of these genotypes is the cause of the pathology observed.

3. No appreciable mortalities were observed in *P. merguiensis*, exposed to YHV7 by any route of infection, although high levels of YHV7 RNA were detected in prawns exposed by injection which were sacrificed and sampled when the trial was terminated on Day 44. Interestingly, a number of these prawns also tested positive for YHV2, albeit with high C_T values. Based on mortalities (or lack thereof), molecular test results and lack of consistent pathological changes, *P. merguiensis* do not appear to be susceptible to infection with YHV7 via natural routes of infection. *In situ* hybridization using a YHV7-specific probe is required to clarify whether YHV7 is the cause of the pathology observed in prawns injected with YHV7.

4. Intra-muscular injection with YHV9 caused no mortalities in either *P. monodon* or *P. merguiensis*. However, YHV9 RNA was detected in both species of prawns sampled at the end of the 41-day trial, with relatively low mean C_T values of 22.6 for *P. monodon* and 25.5 for *P. merguiensis*. While the C_T of the YHV9 inoculum was 20.6, the low YHV9 C_T values detected in pleopods of both species, sampled 33 days later, does provide evidence that the prawns had been infected with YHV9.

5. Intra-muscular injection with YHV10 appeared to have no effect on either *P. monodon* or *P. merguiensis*. While two *P. merguiensis* died 3 days post infection, the lack of further mortalities and the fact that no prawns tested positive for YHV10 when sampled at the end of the 33-day trial indicates that neither species is susceptible to infection with YHV10, based on the inoculum made from the material available.

Based on results from Experimental Trial 1 and Experimental Trial 2, it can be concluded that YHV1 is highly pathogenic to *P. monodon* and *P. merguiensis* at 24°C and 29°C. Both prawn species appear susceptible to infection with YHV9 at both temperatures, but this YHV genotype does not appear to be pathogenic to these species. Based on the results of the experimental trials undertaken at ACDP, both species appear to be completely refractory to infection with YHV10 at 24°C and 29°C, assuming infectious virus was present in the inoculum.

However, while YHV7 does not appear to be pathogenic to *P. merguiensis*, based on the trials conducted during this project, the pathogenicity of this YHV genotype to *P. monodon* does appear to be influenced by temperature, as average mortalities after injection at 24°C were 62% compared to approximately 36% after injection at 29°C (bearing in mind the mortality of 36% is excluding 20% mortality that occurred 1-day post infection). Experimental Trial 3 will investigate the repeatability of the pathogenicity of YHV7 in *P. monodon* after exposure by injection, co-habitation and feeding at the lower temperature of 24°C.

Experimental Trial 3: Assessment of susceptibility to infection with YHV7 by natural routes at 24°C

The aim of Experimental Trial 3 was:

1. To provide an indication of pathogenicity of YHV7 to *P. monodon* after infection via a natural route (i.e. co-habitation and feeding) at 24°C.

3.1 Methods

3.1.1 Experimental animals

Sourcing and delivery of prawns was organized through the Australian Prawn Farmers Association (APFA) Research and Development Committee. Farmed *P. monodon* (~7-9 cm in length) were obtained from a prawn farm in north Queensland. On arrival, prawns were transferred to the ACDP Large Animal Facility (LAF) and placed in round (infection by feeding) or square (infection by co-habitation) polypropylene tanks containing 80 L of 100-µm-filtered seawater, a canister filter and air stones. Prawns were distributed across two rooms (negative controls and YHV7). Each tank held 22 to 25 prawns with water temperature maintained at 24±1°C using immersion heaters. At least one tank in each room was fitted with a data-logger to record water temperature. Animals were monitored and fed commercial prawn pellets daily (unless fed YHV7 positive tissue) and 30% water exchanges were undertaken every 2 days. Prior to infection, 25 prawns were sacrificed, 10 of which were fixed in 80% (v/v) EtOH for PCR testing and the other 15 fixed in Davidson's Fixative for histological analysis.

3.1.2 Preparation of inocula

Un-fixed prawn tissues, infected with YHV7 and held at -80°C at ACDP, were the sources of infected material for preparation of the inocula used in the Experimental Trial 3 (see Table 1 for original inoculum sources).

On the day of inoculation,

- YHV7: The original inoculum used during Experimental Trial 1 was used to inject prawns for co-habitation studies. Material generated by injection with YHV7 during Experimental Trial 1, used for infection by feeding exposure during Experimental Trial 2, were used for infection by feeding for Experimental Trial 3 (this trial). Pools of three or four *P. monodon* with the lowest YHV7 RT-qPCR C_T values were placed in zip-lock bags and stored at -80°C.

The inoculum was temporarily stored at 4°C and transported to the LAF rooms on wet ice. To prevent any cross-contamination, negative control animals and those infected with YHV7 were held in separate rooms in the LAF. Separate overalls and footwear were provided to personnel in each room containing animals exposed to YHV7 and a 3-minute shower was required on exiting of this room.

3.1.3 Inoculation of prawns, sampling and sample processing

3.1.3.1 Inoculation by injection

Prawns were injected as described above (Section 1.1.3.1).

3.1.3.2 Exposure by feeding

Prawns were exposed as described above (Section 2.1.3.2)

3.1.3.3 Exposure by co-habitation

Prawns were exposed as described above (Section 2.1.3.3)

3.1.4 Nucleic acid extraction

Nucleic acids were extracted as described above (Section 1.1.4)

3.1.5 Molecular tests

Molecular tests used are described above (Section 2.1.5). In addition to using Q-Beta to spike the samples and demonstrate nucleic acid extraction success and a measure of PCR inhibition, for samples tested during Experimental Trial 3, plasmid positive controls were used for each real-time assay, which contain an artificial probe insert (Moody et al., 2021). The artificial probe was added to the RT-qPCR reaction mix to determine if any positive test results were due to contamination with the plasmid positive control. Details of all assays are provided in Appendix 2.

3.1.6 Histopathology

Tissues for histology were processed for paraffin-embedding, sectioning and H&E staining using standard procedures. Sections were examined using a compound light microscope.

3.2 Results

3.2.1 Molecular testing of pre-trial prawns

All pre-trial *P. monodon* were test negative for YHV7 but the majority (9/10) were test positive for YHV2 with C_T values ranging from 26.3 to 33.0. All samples were test positive by Q-Beta RT-qPCR and the OIE Decapod PCR.

3.2.2 Mortalities in infected prawns

After exposure to YHV7 by injection, feeding and co-habitation, mortalities were observed in *P. monodon* at 24°C (Figure 25). No mortalities were observed in negative control prawns. Cumulative mortalities were highest in prawns exposed by injection (95% and 86%) followed by exposure by feeding (67% and 76%). Mortalities were lowest (61%) in prawns exposed to YHV7 by co-habitation with prawns injected with YHV7.

The time to commencement of mortalities varied slightly, depending on the route of infection, with mortalities starting 7- and 8-days post-infection in prawns injected with YHV7, and 9- to 10- days post-infection with prawns exposed by feeding and co-habitation. With the exception of prawns exposed to YHV7 by co-habitation, total mortality seemed to have plateaued by 24- or 25-days post-infection. Mortalities in prawns exposed by co-habitation were still occurring when the trial was terminated on Day 28. While it would have been useful to continue the trial past 28 days this was not possible for logistical reasons. Regardless, exposure of *P. monodon* to YHV7 by any route of infection at 24°C resulted in mortalities greater than 60% over 28 days.

While mortalities ranged from 61% to 95% there were significant differences in the mortality curves, after Kaplan-Meier survival analysis was undertaken (MedCalc), and this depended on the mode of exposure to YHV7. There was no significant difference between the two tanks of *P. monodon* exposed to YHV7 by injection ($p=0.1691$) or the two tanks exposed to YHV7 by feeding and co-habitation ($p=0.3840$). There was, however, a significant difference between the tanks of *P. monodon* exposed to YHV7 by injection and feeding ($p=0.0079$) and between the tanks exposed by injection and co-habitation ($p=0.0055$).

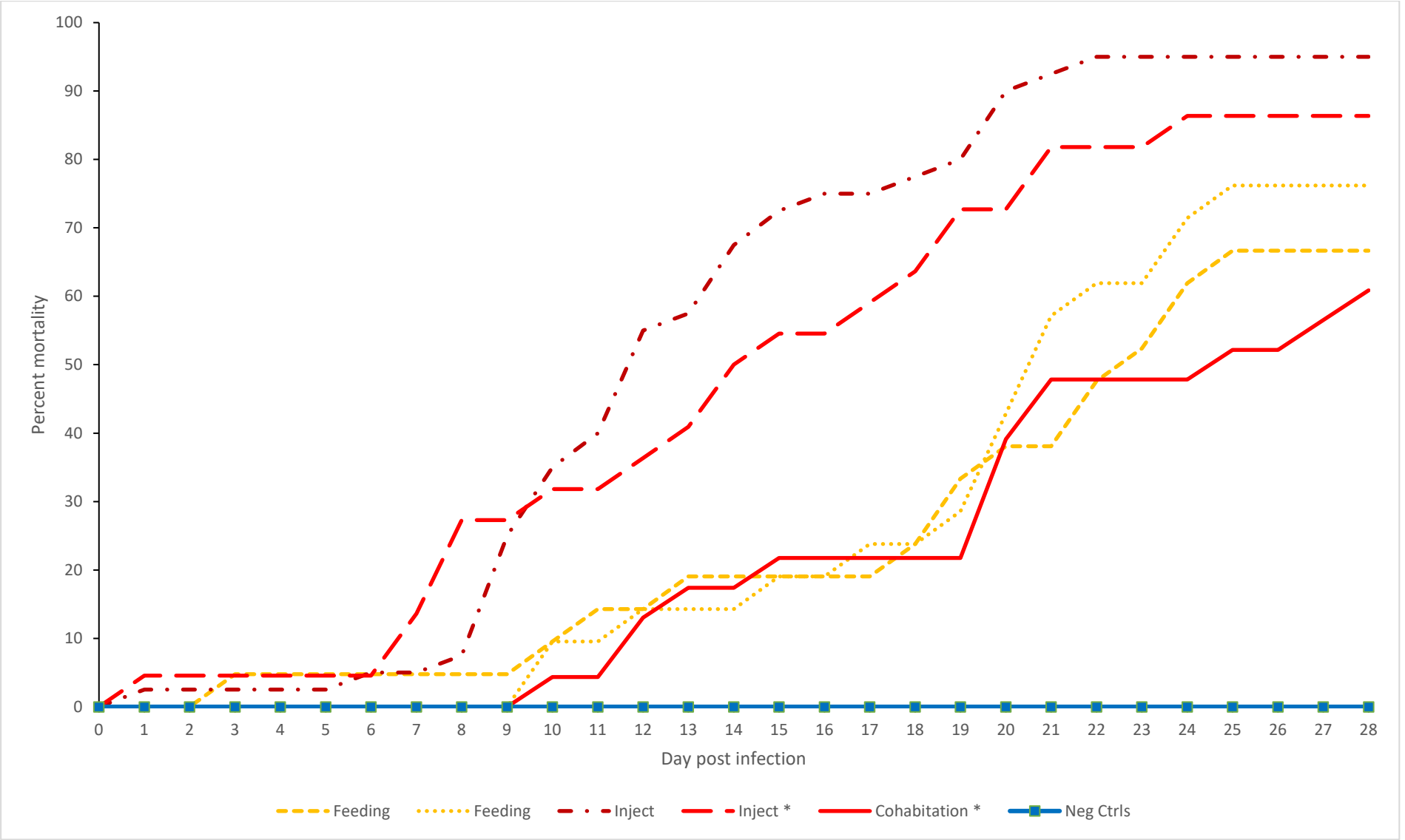


Figure 25. Cumulative mortality in *P. monodon* after exposure to YHV7 by injection, feeding and co-habitation at 24°C. (*) indicates conjoined tanks

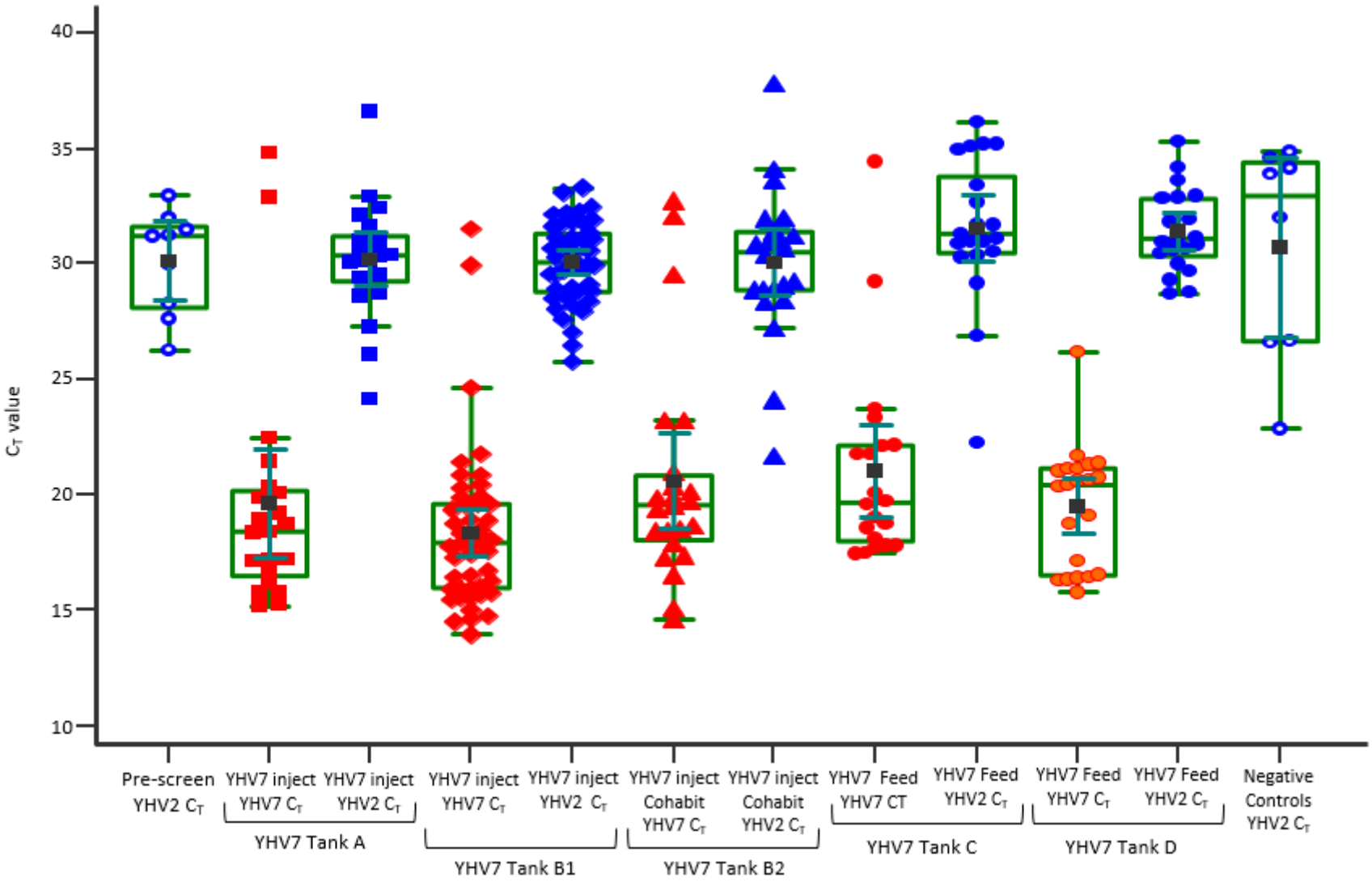


Figure 26. Molecular test results for *P. monodon* exposed to YHV7 at 24°C

3.2.3 Molecular testing of infected prawns

All *P. monodon* exposed to YHV7 by either route of infection (injection, feeding or co-habitation) were test positive, including those surviving and sacrificed at the end of the trial on day 28. Across all routes of exposure, mean C_T values were consistently low for YHV7 (range of 18.3 to 21.8) and consistently high for YHV2 (range of 30.1 to 31.6) (Figure 26, Table 9).

Table 9. Summary of YHV7 and YHV2 molecular test results for the different treatments undertaken during Trial 3.

Treatment	Percent Test Positive		YHV7 RT-qPCR C_T values		YHV2 RT-qPCR C_T values	
	YHV7	YHV2	Mean	Range	Mean	Range
YHV7 Inject	100%	100%	18.26	13.93 - 31.50	30.30	25.77 - 37.59
YHV7 inject (co-habitation)	100%	100%	19.62	15.18 - 34.81	30.22	24.15 - 36.63
YHV7 Co-habitation	100%	100%	20.59	14.56 - 32.73	30.05	21.72 - 37.83
YHV7 Fed A	100%	100%	21.77	17.47 - 36.30	31.55	22.28 - 36.19
YHV7 Fed B	100%	100%	19.50	15.77 - 26.19	31.42	28.74 - 35.35
Negative controls	0%	80%	N/A	N/A	30.72	22.88 - 34.88

3.2.4 Histological examination of lymphoid organs of infected prawns

Histological examination of fixed *P. monodon* was not informative due to either an absence of lymphoid organ in the sections examined or the presence of YHV2 in the prawns used in the bioassay. Development of *in situ* hybridisation assays, using probes specific for YHV2 and YHV7, are required to determine whether the pathological changes observed are due to infection with YHV2, YHV7, both YHV2 and YHV7 or are a general response of the prawn to viral infection.

3.3 Conclusion

Results of the Experimental Trial 3, which was undertaken to confirm the pathogenicity of YHV7 after exposure by a natural route of infection (i.e. co-habitation and feeding) at 24°C, can be summarised as follows:

1. Injection of *P. monodon* with YHV7 caused mortalities of >85% after 28 days.

2. Exposure to YHV7 by feeding with YHV7-infected prawns resulted in mortalities of >66% after 28 days.

3. Exposure to YHV7 by co-habitation with YHV7-injected prawns resulted in mortalities of >60% after 28 days.

4. All *P. monodon* exposed to YHV7 by any route of infection tested positive for YHV7 by RT-qPCR with mean C_T values <22. All prawns also tested positive for YHV2 with mean C_T values >30.

Discussion

The objectives of these experiments were to determine the susceptibility of *P. monodon* and *P. merguiensis* to newly detected enzootic YHV7 and two exotic YHV genotypes, YHV9 and YHV10, that had been detected in imported commodity prawns. The susceptibility of both species to exotic, OIE-listed YHV1 was also undertaken as a positive infection control, to confirm both *P. monodon* and *P. merguiensis* were susceptible to infection by co-habitation and feeding, and to assess the virulence of YHV1 to both species. Where warranted by the virulence of the different YHV genotypes, the material generated would be used to continue assay validation with YHV genotype-specific assays, provide positive control material and allow transfer of assay performance data to state diagnostic laboratories at the completion of the project. The experimental infection trials resulted in a wide range of observed susceptibilities and virulences, depending on the YHV genotype. The outcomes for *P. monodon* after exposure to the different YHV genotypes at both temperatures are summarised in Figure 27, and for *P. merguiensis* are in Figure 28.

Susceptibility of *P. monodon* and *P. merguiensis* to YHV-complex Genotype 1 (YHV1)

Both *P. monodon* and *P. merguiensis* were highly susceptible to infection with YHV1. After infection by either injection, co-habitation or feeding, at either 24°C or 29°C, acute disease was observed with 100% mortality occurring in less than 7 days. Interestingly, 100% mortality occurred in the same short period of time when exposure was by either injection or feeding of YHV1-infected prawns. In *P. monodon* the only clinical sign observed, aside from the occasional moribund prawn observed lying on its side, was observation of prawns with bright yellow heads. However, this was only observed in *P. monodon* exposed by feeding (not after infection by injection and co-habitation) and was not observed in *P. merguiensis*. Disease caused by YHV1 was confirmed by observation of mortalities after infection, positive YHV1 RT-qPCR test results in all prawns (mean C_T values across all routes of exposure of 17.01 and 18.39 for *P. monodon* and *P. merguiensis*, respectively) and the presence of lesions after histological analysis. The evidence generated in this report fulfil the criteria for listing *P. merguiensis* as susceptible to infection with YHV1 according to Chapter 1.5 of the OIE Aquatic Animal Health Code (Aquatic Code).

Objective 1: Determine the susceptibility of *P. monodon* and *P. merguiensis* to YHV-complex Genotype 7 (YHV7)

P. monodon were susceptible to infection with YHV7, with the infection resulting in mortalities. Cumulative mortalities were lower and less acute than those observed after infection with YHV1; initial mortalities were not observed until approximately 6 to 11 days post exposure, depending on the route of infection, and mortalities continued to occur for at least a further 15 to 19 days, when the trials were terminated on Day 28. Exposure of *P. monodon* with YHV7 at 24°C resulted in higher cumulative mortalities (>85% by injection, >66% by feeding and <60% by co-habitation) than exposure to YHV7 at 29°C (mortalities of <57% by injection, <16% by feeding and <5% by co-habitation) by all routes of infection.

Infection by YHV7 in *P. monodon* at either temperature was demonstrated by positive YHV7 RT-qPCR test results in all prawns tested during the trial and prawns sacrificed and sampled when the trials were terminated (mean C_T values across all routes of exposure were 20.0 and 23.9 at 24°C and 29°C, respectively). Disease caused was confirmed by observation of mortalities after infection combined with the positive YHV7 RT-qPCR test results. Histological analysis was equivocal as no unique lesions were observed in prawns sampled when mortalities were occurring – the lesions seen were similar to those described in pre-screen and negative control prawn which were PCR test positive for YHV2.

P. merguiensis appeared to be susceptible to infected after exposure to YHV7 by injection, at 24°C and 29°C, but not after exposure by co-habitation and feeding at 29°C. While mortalities were observed after

infection at both temperatures, the levels of mortality were not significantly different to those observed in negative control prawns. As high levels of YHV7 RNA (indicated by low C_T values) were detected in prawns which were sacrificed and sampled when the trial was terminated, after exposure by injection, *P. merguiensis* appear to be susceptible to infection with YHV7 but the infection did not cause disease. *P. merguiensis* did not appear to be susceptible to infection with YHV7 after exposure by natural routes of infection (i.e. feeding and co-habitation with infected prawns) at 29°C.

In both species, *in situ* hybridization using YHV2- and YHV7-specific probes is required to confirm whether the lesions observed, in particularly the spheroids in the lymphoid organ, were caused by YHV2 or YHV7 or both YHV genotypes. A high number of samples that were test positive for YHV7 were also test positive for YHV2, including samples of *P. merguiensis* after injection with the YHV7 inoculum derived from prawn tissue. This was thought to be caused by YHV2 being present in the YHV7 inoculum (Table 3). YHV2 positive results generally only occurred where YHV7 RT-qPCR C_T values were relatively low (<22) with YHV2 RT-qPCR C_T values from the same samples being relatively high (>29). The exception to this was *P. monodon* exposed by injection and co-habitation at 29°C where the YHV2 C_T values were markedly lower in prawns exposed to YHV7 than pre-screen (Figure 29) and negative control prawns. This contrasted with results for the same routes of exposure at 24°C, where YHV2 C_T values did not markedly change compared to prescreen and negative control prawns, but mortalities were much higher. It appears that, at 29°C, infection of *P. monodon* with YHV7 resulted in an increase in replication of YHV2, resulting in lower YHV2 C_T values.

There is also a possibility that the high YHV2 RT-qPCR C_T values are due to a lack of specificity of the YHV2 assay, where non-specific amplification of YHV7 is occurring at high YHV7 template loads. This is important to consider for results observed after injection of *P. merguiensis*, where positive results using the YHV2 RT-qPCR were observed. As there was YHV2 present in the YHV7 inoculum, it cannot be stated with certainty that the positive results for YHV2 are due to actual infection of *P. merguiensis* with YHV2, or due cross-reactivity with YHV7, given the high levels of YHV detected in these prawns. While this is a specificity issue with the YHV2 RT-qPCR, leading to potentially lower C_T values than may be actually correct, the YHV7 RT-qPCR does not detect YHV2, so concurrent infections with YHV2 would not result in erroneous results for the detection of YHV7 This lack of specificity may have led to inaccurate result interpretations with the YHV2 RT-qPCR throughout all three experiments, where high C_T values attributed to infection with YHV2 may be due to concurrent high template loads of YHV7. Given the non-specific nature of histological changes observed after infection with YHV7, histology could not be used to clarify the cause of disease observed. There are also specificity issues with the OIE YHV/GAV RT-nPCR, where non-specific amplification of YHV7 in the primary PCR led to the identification of this new YHV genotype. Unfortunately, this also results in the absence of a conventional assay of known specificity for YHV2 which could be used to investigate specificity issues with the YHV2 RT-qPCR. Additional work, outside the scope of this project, is underway to confirm the specificity of the YHV2 RT-qPCR.

Objective 2: Determine the susceptibility of *P. monodon* and *P. merguiensis* to YHV-complex Genotype 9 (YHV9)

After exposure to YHV9, both *P. monodon* and *P. merguiensis* appeared to have been infected, based on positive YHV9 RT-qPCR test results obtained from prawns sacrificed and sampled when the Trial 2 was terminated on Day 41. Therefore, both species appear susceptible to infection but refractory to disease, after exposure by injection, particularly when infected at 29°C. As no mortalities were observed after infection by injection at either temperature with material amplified during Trial 1, no further experimental work investigating different routes of infection was undertaken with YHV9.

Objective 3: Determine the susceptibility of *P. monodon* and *P. merguiensis* to YHV-complex Genotype 10 (YHV10)

After exposure to YHV10, based on absence of mortalities (or mortalities at similarly low levels to those seen in negative control prawns) and negative molecular test results in prawns sacrificed and sampled when Trial 2 was terminated on Day 31, both *P. monodon* and *P. merguiensis* did not appear to be susceptible to infection. As no mortalities were observed after infection by injection at either temperature, particularly at 29°C, no further experimental work investigating different routes of infection was undertaken with YHV10. Consideration should be given to further bioassays if additional material infected with YHV10, and with lower YHV10 RT-qPCR C_T values, becomes available.

Objective 4: Transfer protocols and controls for diagnostic tests to state diagnostic laboratories

A positive control plasmid, specific for the YHV7 RT-qPCR, has been prepared and quality control checks undertaken as described in Moody et al. (2021). Test protocols and positive control material have been provided to Elizabeth Macarthur Agriculture Institute, NSW and Berrimah Veterinary Laboratory, NT. Testing to confirm/exclude YHV7 has been undertaken on samples submitted from several jurisdictions as has advice on differential detection of YHV7 and YHV2. YHV1 positive control material had been transferred to state government and private laboratories previously. Real-time assays for YHV9 and YHV10 are also available.

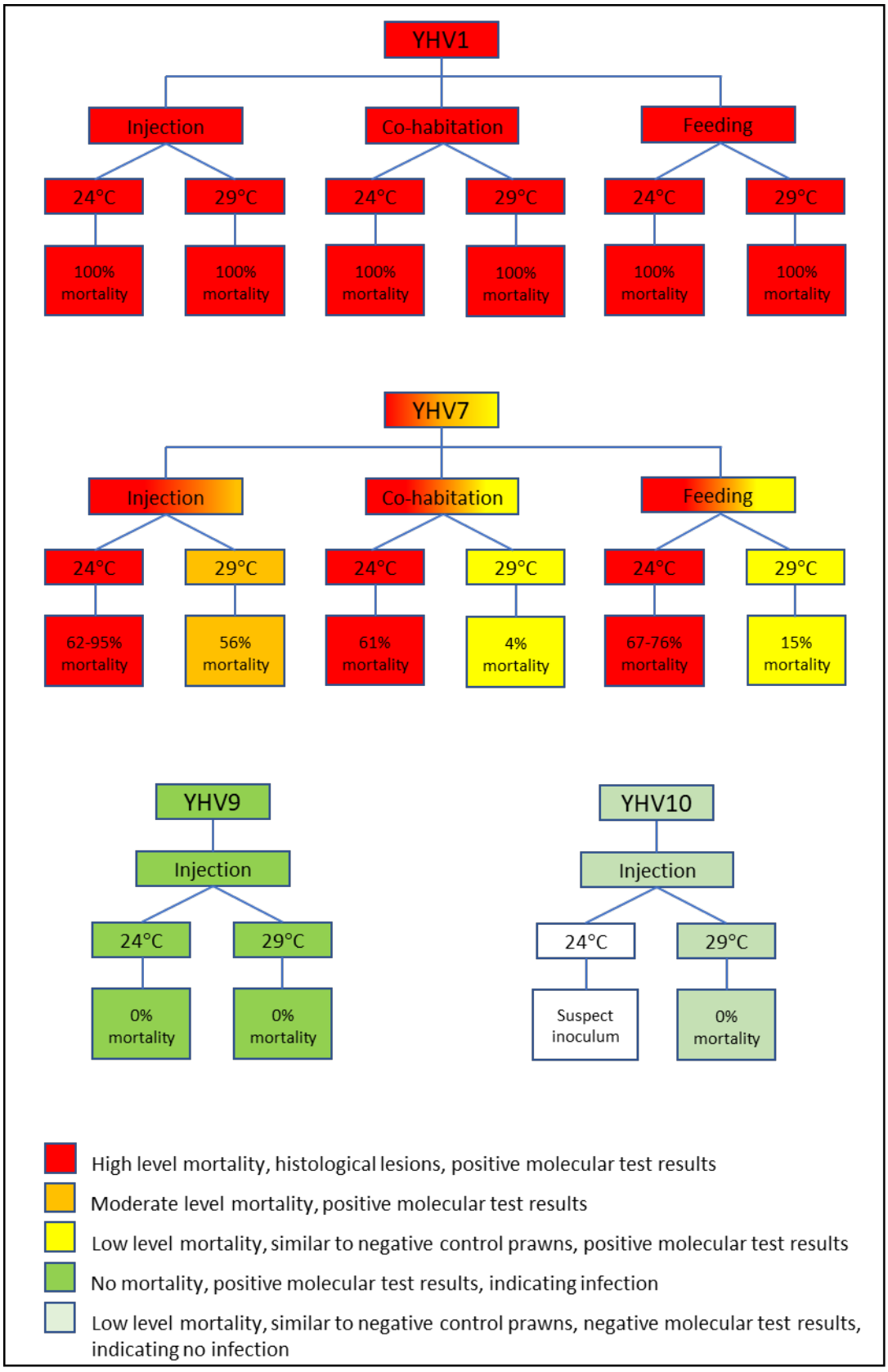


Figure 27. Outcomes of susceptibility studies in *P. monodon* with YHV1, YHV7, YHV9 and YHV10

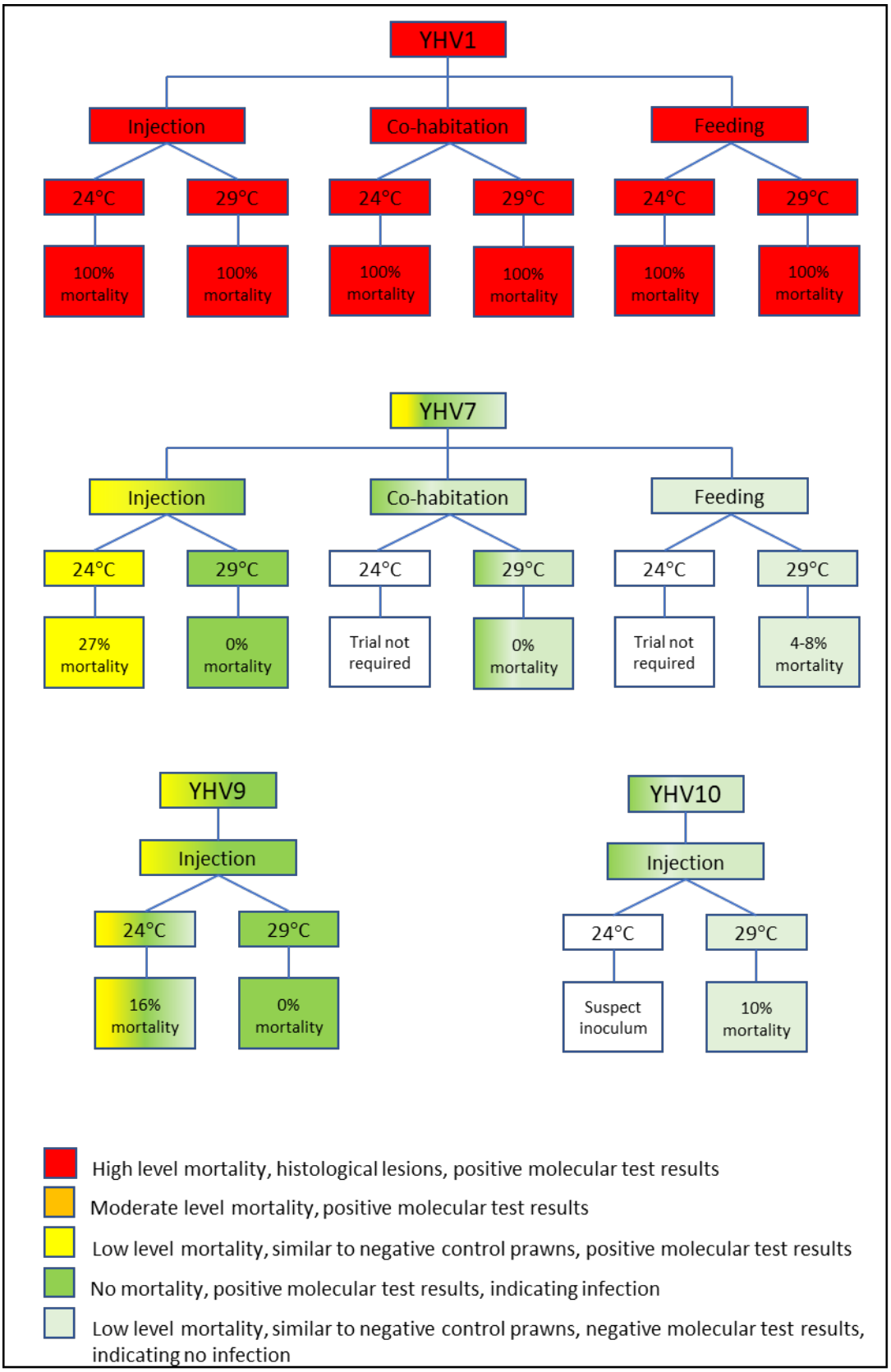


Figure 28. Outcomes of susceptibility studies in *P. merguiensis* with YHV1, YHV7, YHV9 and YHV10.

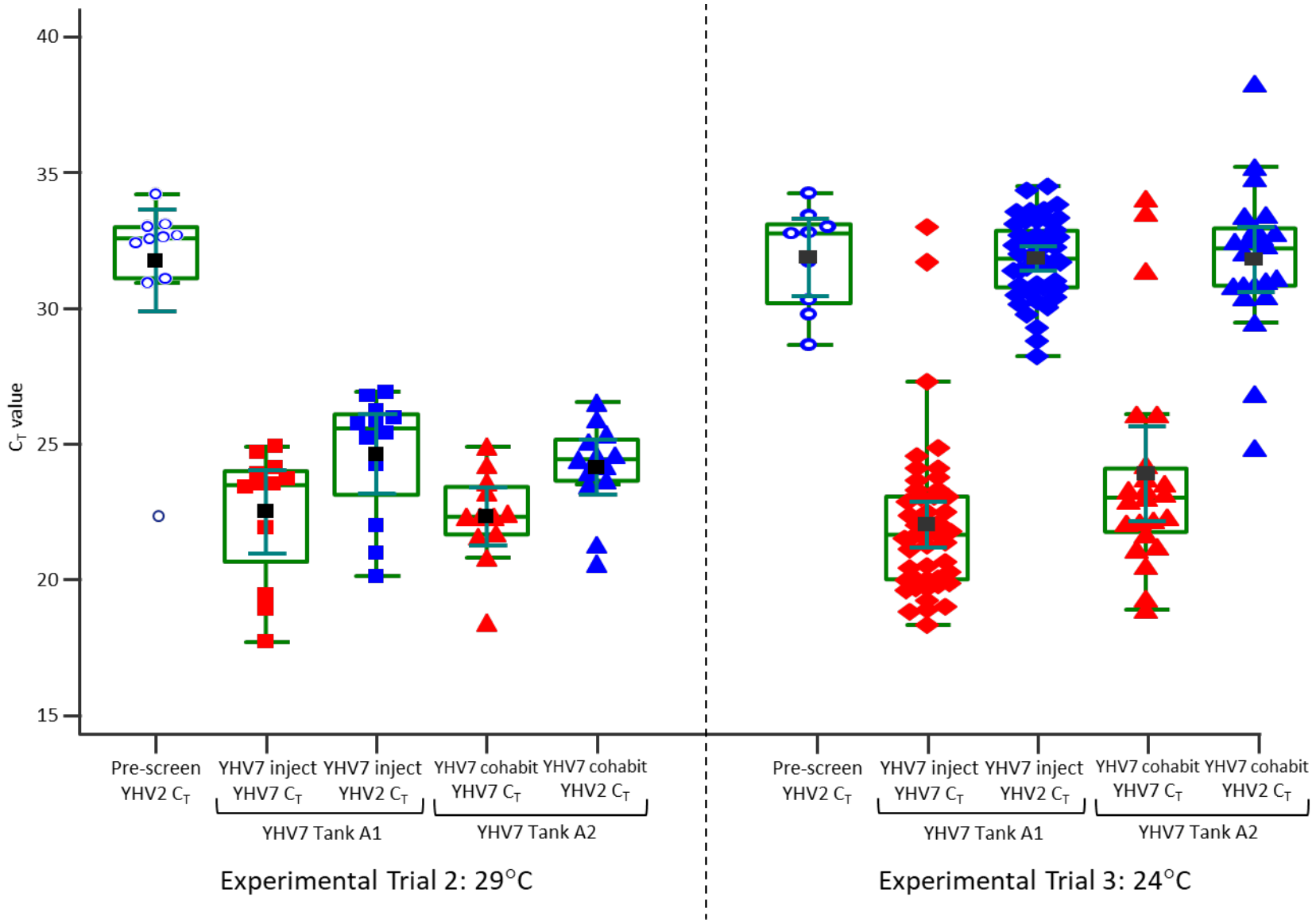


Figure 29. Comparison of YHV7 and YHV2 C_T values in prawns exposed to YHV7 at 29°C and 24°C.

Conclusions

All Objectives of the project were met.

1. *P. monodon* and *P. merguiensis* are highly susceptible to infection with YHV1. The evidence generated in this report fulfil the criteria for listing *P. merguiensis* as susceptible to infection with YHV1 according to Chapter 1.5 of OIE Aquatic Animal Health Code (Aquatic Code). Acute mortalities of 100% were observed after 4 to 6 days after exposure to YHV1 by injection and feeding and 9 to 10 days after exposure by co-habitation.

2. *P. monodon* were susceptible to infection with YHV7 with infection resulting in mortalities, although these the time to total mortality was less acute than after infection with YHV1. Infection of *P. monodon* with YHV7 at 24°C resulted in higher mortalities than infection at 29°C. Mortalities occurred in *P. monodon* after exposure to YHV7 by injection, co-habitation and feeding.

3. Disease in *P. merguiensis* after infection with YHV7, particularly when exposed by co-habitation or feeding, was not observed. While mortalities were observed after infection at both temperatures, the levels of mortality were not significantly different to those observed in negative control animals. As high levels of YHV7 RNA were detected in prawns which were sacrificed and sampled when the trials were terminated, after exposure by injection, *P. merguiensis* appear to be susceptible to infection with YHV7 but refractory to disease.

4. For both species, *in situ* hybridization using YHV2- and YHV7-specific probes is required to confirm whether the lesions observed, in particularly the spheroids in the lymphoid organ, were caused by either YHV2 or YHV7 or both YHV genotypes.

5. *P. monodon* and *P. merguiensis* are susceptible to infection with YHV9, but refractory to disease, after exposure by injection based on the trials undertaken at ACDP. No additional bioassays were undertaken due to the absence of disease observed after exposure by injection.

6. *P. monodon* and *P. merguiensis* are not susceptible to infection with YHV10 based on the trials undertaken at ACDP. No additional bioassays were undertaken due to the absence of infection observed after exposure by injection. It cannot be determined whether the absence of infection was due to non-susceptibility of either species or lack of infectious material in the limited material available to generate the inocula.

7. YHV7 was identified during an investigation into a disease event in domestic *P. monodon* where atypical RT-PCR results were obtained. The identification of YHV9 and YHV10 in imported commodity prawns occurred through an awareness of specificity issues for tests detecting YHV1 and evaluation of new candidate assays specific for YHV1. These detections highlight the need to fully investigate anomalous test results and confirm detections of pathogens by real-time PCR screening methods, particularly those associated with disease, by conventional PCR and sequence analysis. This will also contribute to continuing monitoring of test performance and accuracy of test results.

Implications

The results presented in this report are based on experimental infections in an artificial environment. However, the disease observed (mortalities, molecular test results and histological analysis) confirms the highly pathogenic nature of the exotic YHV1 to both *P. monodon* and *P. merguiensis*. Materials generated from the bioassays have contributed to ongoing generation pf performance data for sensitive and specific real-time and conventional molecular assays for the rapid detection and confirmation of infection with YHV1.

While based on experimental infections, the mortalities generated after exposure to *P. monodon,* by all routes of infection and with the observed low C_T values in dead prawns, indicates that YHV7 is pathogenic to *P. monodon*. Mortalities observed after exposure were more severe at 24°C than at 29°C. Screening for YHV7 should be included in the suite of pathogens tested for as part of the disease investigation into increased mortalities in Australian prawn farms, including samples where high C_T results are obtained with the YHV2 RT-qPCR. Testing for YHV7 would be particularly important when there has been an event (e.g. torrential rainfall) resulting in a rapid reduction in water temperature to 24°C. As the distribution of YHV7 in farmed and wild prawn populations is unknown, the utility of the YHV7 RT-qPCR demonstrated through its us during this project will enable surveillance activities to be undertaken and to determine the distribution of YHV7 and, if required, enable sensitive and specific screening of prawns prior to translocation among and between jurisdictions, for regulatory purposes. Routine testing for YHV7 from field material will increase our knowledge of the distribution and impact of this pathogen.

The differences in host specificities and pathogenicity and pathology produced, combined with sequence differences over a number of regions provided additional evidence that YHV1 and YHV7 are distinctly different viruses.

Form the experimental infections undertaken during this project, the YHV genotypes detected in the imported commodity prawns (YHV9 and YHV10) did not appear to be pathogenic to either *P. monodon* or *P. merguiensis*. However, these YHV genotypes were only detected due to a lack of specificity of the YHV1 RT-qPCR screening test used at the time and they may be non-pathogenic YHV genotypes, similar to YHV4, YHV5 and YHV6.

While test specificity is a critical element of assay validation, YHV7, YHV9 and YHV10 were only detected due to a lack of specificity of assays targeting YHV1 and YHV2. These detections were unexpected and highlight the need to consider unknown pathogens when undertaking disease investigations and where the use of pathogen-specific assays results in unusual test results. Confirmation of positive real-time test results, by conventional PCR and sequence analysis of amplicons, should be undertaken to ensure that the real-time test results are accurate. Ongoing assessment of assay performance, particularly with regard to analytical specificity, is critical to ensure assays remain fit for purpose.

Recommendations

Australian stocks of *P. monodon* and *P. merguiensis* are highly susceptible to infection with YHV1. The results reported here support inclusion of YHV1 on Australia's National List of Reportable Diseases of Aquatic Animals and biosecurity protocols must include YHV1 as a significant pathogen to be excluded.

As YHV7 has been added to a number of prawn translocation and health screening protocols, with an increasing number of laboratories undertaking testing for YHV7, consideration should be given to including YHV7 as a pathogen within the crustacean panel of the DAWE-funded National Aquatic Proficiency Testing Program.

Further development

YHV7 appears more virulent at 24°C than 29°C. As 24°C is generally lower than typical husbandry temperatures, additional research to determine the effect of rapid temperature decreases (which may occur during a significant rainfall event) on the virulence of YHV7. This would involve infecting replicated tanks of *P. monodon* at 29°C for 7 days then rapidly reducing the temperature to 24°C in half the replicates and comparing the disease observed.

If cold-stress experiments resulted in the expression of more significant disease at 24°C, development of a pond-side chromatographic strip test would be justified. This could be used if mortalities were observed after a rain event to enable producers to implement potential harvest strategies as over a 3-week period, mortalities of up to 70% may occur.

It is unusual for a pathogen causing the mortalities observed after infection with YHV7 to not observe pathological changes. Targeting of the lymphoid organ for histology, based on experience with YHV1 and YHV2, may not have been ideal. A detailed structured sampling regime to comprehensively document histological lesions and disease caused by infection with YHV7 is required. To improve the aquatic animal histology capability at ACDP, training in fixed prawn sectioning, embedding processing would be sought from Dr Ian Anderson, BSL Queensland.

As the spheroids observed in the prawns exposed to YHV7 could not be distinguished from lesions caused by the concurrent infection with YHV2, development of *in-situ* hybridization probes specific to YHV2 and YHV7 are required. Use of these probes would enable identification of YHV2 and/or YHV7 RNA in association with the spheroids observed. YHV7 *in-situ* hybridization capability would also enable confirmation of the presence of YHV7 in any lesions identified during the structured histological sampling work described above.

The YHV2 RT-qPCR assay may lack analytical specificity, as this assay also appears to detect YHV7, at least when high template levels of YHV7 are present. Additional work is underway to investigate this and in the interim mortalities in *P. monodon*, particularly where there is histological evidence of spheroids in the lymphoid organ, should be tested for both YHV2 and YHV7.

The whole genome sequence of YHV7 will be determined to enable more detailed genetic comparisons with YHV1 and YHV2, to complement the virulence and pathogenicity results presented in the report and provide additional evidence that, while closely related, the different genotypes within the *Okavirus* genus are in fact, distinctly different viruses.

Extension and Adoption

Presentations regarding the project have been provided to industry at the Australian Prawns Farmers Association symposia:

- Nick Moody (2016) FRDC 2015-015: Aquatic Animal Health Subprogram: Determining the susceptibility of Australian *Penaeus monodon* and *P. merguiensis* to newly identified enzootic (YHV7) and exotic (YHV8 and YHV10) Yellow head virus (YHV) genotypes. Ridley Australian Prawn & Barramundi Farmers Symposium, 2-3 August 2016, Townsville, Queensland.
- Moody NJG, Hoad J, Cummins DM, Williams LM, Slater J, Mohr PG, and Crane MStJ (2017) FRDC 2015-005 and 2016-013 Project Update: Pathogenicity trials involving newly-detected Yellow head complex viruses and bacterial strains associated with hepatopancreatitis (PMMS) in prawns. Ridley Australian Prawn Farmers Symposium - A New Horizon, 1-2 August 2017, Gold Coast, Queensland.
- Moody NJG, Hoad J, Cummins DM, Williams LM, Slater J, Mohr PG, Valdeter S and Crane MStJ (2018) AFDL Project updates: FRDC 2015-005 Pathogenicity trials involving newly-detected Yellow head complex viruses and bacterial strains associated with hepatopancreatitis (PMMS) in prawns and WSSV Projects. Ridley Australian Prawn Farmers Association Symposium, 13-18 August 2018, Gold Coast, Queensland.

Presentations regarding the project have been provided to national and international colleagues;

- NJG Moody, PG Mohr, J Hoad, LM Williams, RO Bowater, DM Cummins, JA Cowley and MStJ Crane (2015) The Yellowhead Disease Complex: it's complex. FRDC AAHBS 3rd Australasian Scientific Conference on Aquatic Animal Health, Cairns 6-10 July 2015
- NJG Moody, PG Mohr, J Hoad, LM Williams, DM Cummins, J Slater and M Crane (2017) Detection of new Yellow head virus genotypes and assessment of pathogenicity. European Association of Fish Pathologists 18th International Conference on Diseases of Fish and Shellfish, 4-8 September 2017, Belfast United Kingdom.
- Moody NJG, Mohr PG, Hoad J, Williams LM, Cummins DM, Slater J and Crane MStJ (2019). Determining the susceptibility of Australian *Penaeus monodon* and *P. merguiensis* to newly identified enzootic (YHV7) and exotic (YHV9 and YHV10) yellow head virus (YHV) genotypes. FRDC AAHBS 5th Australasian Scientific Conference on Aquatic Animal Health and Biosecurity, 8-12 July, Cairns, Queensland
- Nick Moody (2019) OIE Reference Laboratory Report. Second meeting of the ad hoc Steering Committee Members of the Regional Collaboration Framework on Aquatic Animal Health, 3-4 December 2020 (zoom)

An AQUAPLAN Webinar is planned when the Final Report has been approved and results will be published in a peer-reviewed journal.

References

ABARES (2018) Australian fisheries and aquaculture statistics 2017. https://www.agriculture.gov.au/SiteCollectionDocuments/abares/publications/AustFishAquacStats_2017_v1.2.0.pdf

BAA 2009/25 (2009) Generic Import Risk Analysis Report for Prawns and Prawn Products. https://www.agriculture.gov.au/sites/default/files/sitecollectiondocuments/ba/memos/2009/Final_prawn_IRA_report_7_Oct_09.pdf

Boonyaratpalin S, Supamattaya K, Kasornchandra J, Direkbusaracom S, Aekpanithanpong U and Chantanachooklin C. (1993) Non-Occluded Bacula-like Virus, the Causative Agent of Yellow Head Disease in the Black Tiger Shrimp (*Penaeus monodon*). Gyobyo Kenkyu 28:103-109

Castro-Longoria R, Quintero-Arredondo N, Grijalva-Chon JM and Ramos-Paredes J. (2008) Detection of the yellow-head virus (YHV) in wild blue shrimp, *Penaeus stylirostris*, from the Gulf of California and its experimental transmission to the Pacific white shrimp, *Penaeus vannamei*. J Fish Dis 31:953-956

Cowley JA, Cadogan LC, Wongteerasupaya C, Hodgson RAJ, Spann KM, Boonsaeng V and Walker PJ. (2004) Differential detection of gill-associated virus (GAV) from Australia and yellow head virus (YHV) from Thailand by multiplex RT-nested PCR. J Virol Methods 117:49–59

Cowley JA, Dimmock CM, Spann KM and Walker PJ. (2000) Detection of Australian gill-associated virus (GAV) and lymphoid organ virus (LOV) of *Penaeus monodon* by RTnested PCR. Dis Aquat Org 39:159–167

Cowley JA, Hall MR, Cadogan LC, Spann KM and Walker PJ. (2002) Vertical transmission of gill-associated virus (GAV) in the black tiger prawn *Penaeus monodon*. Dis Aquat Org 50:95–104

Cowley JA, McCulloch RJ, Rajendran KV, Cadogan LC, Spann KM and Walker PJ. (2005) RT-nested PCR detection of Mourilyan virus in Australian *Penaeus monodon* and its tissue distribution in healthy and moribund prawns. Dis Aquat Org 66:91-104

Cowley JA, Moody NJG, Mohr PG, Rao M, Williams LM, Sellars MJ and Crane MStJ. (2015) FRDC 2013/036 Tactical Research Fund: Aquatic Animal Health Subprogram: Viral presence, prevalence and disease management in wild populations of the Australian Black Tiger prawn (*Penaeus monodon*). Final Report. Fisheries Research and Development Corporation, Canberra.

Cowley JA, Rao M, Mohr P, Moody N, Sellars MJ and Crane MStJ (2019) TaqMan real-time and conventional nested PCR tests specific to yellow head virus genotype 7 (YHV7) identified in giant tiger shrimp in Australia. J Virol Methods https://doi.org/10.1016/j.jviromet.2019.113689.

de la Vega E, Degnan BM, Hall MR, Cowley JA and Wilson KJ. (2004) Quantitative real-time RT-PCR demonstrates that handling stress can lead to rapid increases of gill-associated virus (GAV) infection levels in *Penaeus monodon*. Dis Aquat Org 59:195-203

Gudkovs N, Slater J and Crane M. 2015. Tactical Research Fund Aquatic Animal Health Subprogram: Determining the susceptibility of Australian species of prawns to infectious myonecrosis. Fisheries Research and Development Corporation Project No. 2011/048. http://frdc.com.au/research/final-reports/Pages/2011-048-DLD.aspx

Han JE, Tang KFJ, Pantoja CR, White BL and Lightner DV. (2015) qPCR assay for detecting and quantifying a virulence plasmid in acute hepatopancreatic necrosis disease (AHPND) due to pathogenic *Vibrio parahaemolyticus*. Aquaculture 442:12-15

Hasson KW, Lightner DV, Mohney LL, Redman R and White BM. (1999) Role of lymphoid organ spheroids in chronic Taura syndrome virus (TSV) infections in *Penaeus vannamei*. Dis Aquat Org 38:93-105

Kumar S, Stecher G, Li M, Knyaz C, and Tamura K (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35:1547-1549.

Liu Q, Huang J, Yang H-L, Yang GB, Want H-L, Want Q-T, Liu F and Zhang Q-L. (2014) Detection of a new genotype of yellow-head virus in farmed shrimp suspicious of EMS/AHPNS infection. Oceanol Limnol Sin 45:703–709

Lo CF, Leu JH, Ho CH, Chen CH, Peng SE, Chen YT, Chou CM, Yeh PY, Huang CJ, Chou HY, Wang CH and Kou GH. (1996) Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Dis Aquat Org 25:133-141

Mohr PG, Moody NJG, Hoad J, Williams LM, Cummins DM, Cowley JA and Crane MStJ. (2015) Molecular discovery of a unique genotype of Yellow head virus (YHV7) in Giant Tiger shrimp (*Penaeus monodon*) indigenous to Northern Australia. Dis Aquat Org 115:263-268

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

OIE (2015) Report of the first meeting of the OIE ad hoc group on susceptibility of crustacean species to infection with OIE listed diseases
https://www.oie.int/en/standard-setting/specialists-commissions-working-ad-hoc-groups/ad-hoc-groups-reports/

OIE (2019) Infection with Yellow Head Virus genotype 1. Chapter 2.2.9. In: The Manual of Diagnostic Tests for Aquatic Animals, OIE, Paris.

Poulos BT and Lightner DV (2006) Detection of infectious myonecrosis virus (IMNV) of penaeid shrimp by reverse-transcriptase polymerase chain reaction (RT-PCR). Diseases of Aquatic Organisms 73, 69-72.

Senapin S, Thaowbut Y, Gangnonngiw W, Chuchird N, Sriurairatana S and Flegel TW. (2010) Impact of yellow head virus outbreaks in the whiteleg shrimp, *Penaeus vannamei* (Boone), in Thailand. J Fish Dis 33:421–430

Sritunyalucksana K, Srisala J, McColl K, Nielsen L and Flegel TW. (2006) Comparison of PCR methods for white spot syndrome virus (WSSV) infections in penaeid shrimp. Aquaculture 255:95-104

Spann KM, Vickers JE and Lester RJG. (1995) Lymphoid organ virus of *Penaeus monodon* from Australia. Dis Aquat Org 23:127-134

Spann KM, Cowley JA, Walker PJ and Lester RJG. (1997) A yellow-head-like virus from *Penaeus monodon* cultured in Australia. Dis Aquat Org 31:169–179

Surachetpong W, Poulos BT, Tang KFJ and Lightner DV. 2005. Improvement of PCR method for the detection of monodon baculovirus (MBV) in penaeid shrimp. Aquaculture 249, 69-75.

Tamura K. and Nei M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10:512-526.

Tang KFJ, Wang J and Lightner DV. (2004) Quantitation of Taura syndrome virus by real-time RT-PCR with a TaqMan assay. J Virol Methods 115:109-114

Tang KFJ, Navarro SA and Lightner DV. (2007) PCR assay for discriminating between infectious hypodermal and haematopoietic necrosis virus (IHHNV) and virus-related sequences in the genome of *Penaeus monodon*. Dis Aquat Org 74:165-170

Walker PJ and Winton JR. (2010) Emerging diseases of fish and shrimp. Vet Res 41:51-74

Walker, PJ, Cowley JA, Dong X, Moody N and Ziebuhr (2021) ICTV Virus Taxonomy Profile: *Roniviridae*, J Gen Virol, 102 (1): 001514

Wijegoonawardane PKM, Cowley JA, Phan T, Hodgson RAJ, Nielsen L, Kiatpathomchai W and Walker PJ. (2008) Genetic diversity in the yellow head nidovirus complex. Virology 380:213–225

Appendices

Appendix 1. Summary of detections of YHV genotypes (YHV1 to YHV10) and their phylogenetic relationship.

Appendix 2. Details of assays used during this project

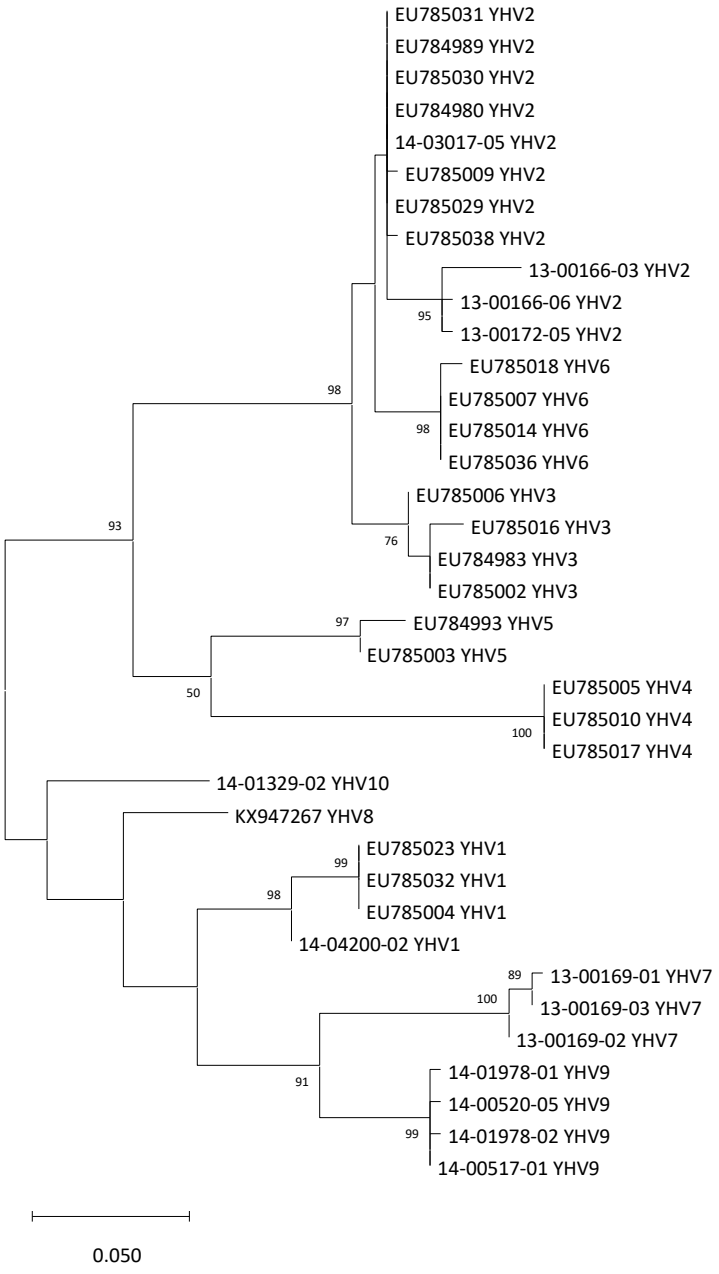
Appendix 3. List of project staff

Appendix 1. Summary of detections of YHV genotypes (YHV1 to YHV10) and their phylogenetic relationship.

Appendix 1.1. Summary of detections of YHV genotypes (YHV1 to YHV10)

Genotype	Location	Year	Sample source	Reference
YHV1	Thailand	1992	Diseased	Boonyaratpalin et al., 1993
	Thailand	2000	Diseased	Wijegoonawardane et al., 2008
	Taiwan	2000	Diseased	Wijegoonawardane et al., 2008
	Thailand	2001	Diseased	Wijegoonawardane et al., 2008
	Thailand	2002	Diseased	Wijegoonawardane et al., 2008
	Thailand	2003	Diseased	Wijegoonawardane et al., 2008
	Gulf of California, Mexico	2003	Healthy	Castro-Longoria et al., 2008
	Thailand	2007	Diseased	Senapin et al., 2010
	Thailand	2008	Diseased	Senapin et al., 2010
YHV2	Queensland, Australia	1997	Diseased (MCMS)	Wijegoonawardane et al., 2008
	Queensland, Australia	2000	Healthy	Wijegoonawardane et al., 2008
	Vietnam	2001	Healthy	Wijegoonawardane et al., 2008
	Vietnam	2002	Healthy	Wijegoonawardane et al., 2008
	Thailand	2003	Healthy	Wijegoonawardane et al., 2008
	Thailand	2004	Healthy	Wijegoonawardane et al., 2008
	North Queensland, Australia	2013	Healthy	Cowley et al., 2015
	Guld of Carpentaria, Australia	2013	Healthy	Cowley et al., 2015
YHV3	Thailand	2000	Healthy	Wijegoonawardane et al., 2008
	Taiwan	2000	Healthy	Wijegoonawardane et al., 2008
	Vietnam	2001	Healthy	Wijegoonawardane et al., 2008
	Vietnam	2002	Healthy	Wijegoonawardane et al., 2008
	Malaysia	2003	Healthy	Wijegoonawardane et al., 2008
	Taiwan	2003	Healthy	Wijegoonawardane et al., 2008
	Vietnam	2003	Healthy	Wijegoonawardane et al., 2008
	Indonesia	2004	Healthy	Wijegoonawardane et al., 2008
	Shandong, China	2016	Diseased	Chen et al., 2018
YHV4	Nellore, India	2002	Healthy	Wijegoonawardane et al., 2008
YHV5	Malaysia	2003	Healthy	Wijegoonawardane et al., 2008
	Iloilo, Philippines	2003	Healthy	Wijegoonawardane et al., 2008
	Thailand	2003	Slow growth	Wijegoonawardane et al., 2008
YHV6	Mozambique	2004	Healthy	Wijegoonawardane et al., 2008
	North Queensland, Australia	2013	Healthy	Cowley et al., 2015
YHV7	North Queensland, Australia	2012	Diseased	Mohr et al., 2015
	North Queensland, Australia	2013	Healthy	Cowley et al., 2015
	Joseph Bonaparte Gulf	2013	Healthy	Cowley et al., 2019
	North Queensland, Australia	2017	Diseased (MCMS)	Cowley et al., 2019
YHV8	Hebei Province, China	2012	Diseased (AHPND)	Liu et al., 2014
	Korea	2019	Healthy	Kim et al., 2020
YHV9	China	2014	Imported Commodity	AFDL, pers. obs.
YHV10	China	2014	Imported commodity	AFDL, pers. obs.
	Unknown	2016	Imported commodity	AFDL, pers. obs.
YHV11	Indonesia	2016	Imported commodity	AFDL, pers. obs

Appendix 1.2 Phylogenetic relationship of YHV genotypes (YHV1 to YHV10) based on sequence of the genome targeted by the OIE Protocol 3 RT-nPCR. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 37 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 284 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).



Appendix 2. List of assays used during this project

CSIRO YHV RT-qPCR

Primer	Sequence	Reference
YHV-QPF1	5'- CAA AGG ATC CAA ACA TTG TGA ATG T -3'	Jeff Cowley, personal communication
YHV-QPR1	5'- ATG GAT TGC CCT GGT GCA T -3'	
YHV probe	5'-**6FAM** CTC AGT CGT TTC CGC TTC CAG TGT ATC TG **TAMRA**-3'	
Reaction conditions; each 25 µl reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl 2× RT-PCR buffer, 1 µl 25× RT-PCR enzyme mix (AgPath-ID™ One-Step RT-PCR, Life Technologies), a final concentration of 900 nM for each primer, 250 nM for the probe and molecular grade water. The RT-qPCR assays are performed in a 7500 Fast Real-Time PCR System or QuantStudio 5 Real-Time PCR System (Life Technologies) and analysed with the 7500 software or QuantStudio design and analysis software respectively. PCR amplifications are programmed as follows: 30 min reverse transcription at 48°C followed by an initial 10 min denaturation at 95°C followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A threshold of 0.1 is used to determine C_T value		

AFDL YHV1 RT-qPCR

Primer	Sequence	Reference
YHV1-12-qF	5'- AGT CTA CAG TGC TCT GAT CT -3'	AFDL, in-house
YHV1-12-qR	5'- GAT TCT TGA AGC GCA TGA GT -3'	
YHV1-12-qPr ZEN	5'-**6FAM** TCT CAT GTG **/ZEN/** TCA TGA TAT TCT CAA GCG AGT **IABkFQ**-3'	
Reaction conditions; each 25 µl reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl 2× RT-PCR buffer, 1 µl 25× RT-PCR enzyme mix (AgPath-ID™ One-Step RT-PCR, Life Technologies), a final concentration of 900 nM for each primer, 250 nM for the probe and molecular grade water. The RT-qPCR assays are performed in a 7500 Fast Real-Time PCR System or QuantStudio 5 Real-Time PCR System (Life Technologies) and analysed with the 7500 software or QuantStudio design and analysis software respectively. PCR amplifications are programmed as follows: 30 min reverse transcription at 48°C followed by an initial 10 min denaturation at 95°C followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A threshold of 0.1 is used to determine C_T value.		

CSIRO YHV7 RT-qPCR

Primer	Sequence	Reference
qYHV7-F1	5'- CAT CCA ACC TAT CGC CTA CA -3'	Cowley et al (2019)
qYHV7-R1	5'- TGT GAA GTC CAT GTG AAC GA -3'	
qYHV7-Pr1	5'- **6FAM -** CAA CGA CAG ACA CCT CAT CCG TGA **- TAMRA** -3'	
Reaction conditions; each 25 µl reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl 2× RT-PCR buffer, 1 µl 25× RT-PCR enzyme mix (AgPath-ID™ One-Step RT-PCR, Life Technologies), a final concentration of 900 nM for each primer, 250 nM for the probe and molecular grade water. The RT-qPCR assays are performed in a 7500 Fast Real-Time PCR System or QuantStudio 5 Real-Time PCR System (Life Technologies) and analysed with the 7500 software or QuantStudio design and analysis software respectively. PCR amplifications are programmed as follows: 30 min reverse transcription at 48°C followed by an initial 10 min denaturation at 95°C followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A threshold of 0.1 is used to determine C_T value.		

De la Vega YHV2 RT-qPCR

Primer	Sequence	Reference
GAVQPF1	5’- GGG ATC CTA ACA TCG TCA ACG T -3’	de la Vega et al. (2004)
GAVQPR1	5’- AGT AGT ATG GAT TAC CCT GGT GCA T -3’	
GAVprobe1	5’-**6FAM** TCA GCC GCT TCC GCT TCC AAT G **TAMRA**-3’	
Reaction conditions; each 25 µl reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl 2× RT-PCR buffer, 1 µl 25× RT-PCR enzyme mix (AgPath-ID™ One-Step RT-PCR, Life Technologies), a final concentration of 900 nM for each primer, 250 nM for the probe and molecular grade water. The RT-qPCR assays are performed in a 7500 Fast Real-Time PCR System or QuantStudio 5 Real-Time PCR System (Life Technologies) and analysed with the 7500 software or QuantStudio design and analysis software respectively. PCR amplifications are programmed as follows: 30 min reverse transcription at 48°C followed by an initial 10 min denaturation at 95°C followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A threshold of 0.1 is used to determine C_T value.		

AFDL YHV9 RT-qPCR

Primer	Sequence	Reference
YHV9qFor2	5'- AGG TTC AAG AAT CCT CTC TC-3'	AFDL, in-house
YHV9qRev2	5'- CTC TTG ACC TGA TGT GAA GT -3'	
YHV9qProbe	5'-**6FAM** ACA CAG CTC TAA ATC CAG AAC GCC AAC T **TAMRA**-3'	
Reaction conditions; each 25 µl reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl 2× RT-PCR buffer, 1 µl 25× RT-PCR enzyme mix (AgPath-ID™ One-Step RT-PCR, Life Technologies), a final concentration of 900 nM for each primer, 250 nM for the probe and molecular grade water. The RT-qPCR assays are performed in a 7500 Fast Real-Time PCR System or QuantStudio 5 Real-Time PCR System (Life Technologies) and analysed with the 7500 software or QuantStudio design and analysis software respectively. PCR amplifications are programmed as follows: 30 min reverse transcription at 50°C followed by an initial 10 min denaturation at 95°C followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A threshold of 0.1 is used to determine C_T value.		

AFDL YHV10 RT-qPCR

Primer	Sequence	Reference
YHV10qFor	5'- GCC CTG ATA TCC ATC CAG AAA T-3'	AFDL, in-house
YHV10qRev	5'- GAG TGA GTT GCC GTG AGA TT -3'	
YHV10qProbe	5'-**6FAM** TCT GAA AAG GGT CTT CAA TCA CTG CAA AGA **TAMRA**-3'	
Reaction conditions; each 25 µl reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl 2× RT-PCR buffer, 1 µl 25× RT-PCR enzyme mix (AgPath-ID™ One-Step RT-PCR, Life Technologies), a final concentration of 900 nM for each primer, 250 nM for the probe and molecular grade water. The RT-qPCR assays are performed in a 7500 Fast Real-Time PCR System or QuantStudio 5 Real-Time PCR System (Life Technologies) and analysed with the 7500 software or QuantStudio design and analysis software respectively. PCR amplifications are programmed as follows: 30 min reverse transcription at 50°C followed by an initial 10 min denaturation at 95°C followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A threshold of 0.1 is used to determine C_T value.		

OIE AHPND PirA qPCR

Primer	Sequence	Reference
VpPirA-F	5’-TTG GAC TGT CGA ACC AAA CG-3’	Han et al. (2015)
VpPirA-R	5’-GCA CCC CAT TGG TAT TGA ATG-3’	
VpPirA-Probe	5’-**6FAM** AGA CAG CAA ACA TAC ACC TAT CAT CCC GGA **TAMRA**-3’	
Reaction conditions: each 25 µl reaction mix is made up of 2 µl extracted nucleic acid template, 12.5 µl TaqMan Fast Universal PCR Master Mix (Life Technologies), a final concentration of 900 nM for each primer, 250 nM for the probe and molecular-grade water. The qPCR assays are performed in a 7500 Fast Real-Time PCR System or QuantStudio 5 Real-Time PCR System (Life Technologies) and analysed with the 7500 software or QuantStudio design and analysis software respectively. PCR amplifications are programmed as follows: 1 cycle of 95°C for 59 seconds followed by 45 cycles of 95°C for 3 seconds and 60°C for 30 seconds. A threshold of 0.1 is used to determine C_T value.		

OIE TSV RT-qPCR

Primer	Sequence	Reference
TSV 1004F	5’- TTG GGC ACC AAA CGA CAT T -3’	Tang et al. (2007)
TSV 1075R	5’- GGG AGC TTA AAC TGG ACA CAC TGT -3’	
TSV-P1	5’- **6FAM** CAG CAC TGA CGC ACA ATA TTC GAG CAT C **TAMRA** -3’	
Reaction conditions: each 25 µl reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl 2× RT-PCR buffer, 1 µl 25× RT-PCR enzyme mix (AgPath-ID™ One-Step RT-PCR, Life Technologies), a final concentration of 900 nM for each primer, 250 nM for the probe and molecular grade water. The RT-qPCR assays are performed in a 7500 Fast Real-Time PCR System or QuantStudio 5 Real-Time PCR System (Life Technologies) and analysed with the 7500 software or QuantStudio design and analysis software respectively. PCR amplifications are programmed as follows: 30 min reverse transcription at 48°C followed by an initial 10 min denaturation at 95°C followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A threshold of 0.1 is used to determine C_T value.		

CSIRO WSSV qPCR

Primer	Sequence	Reference
WSSV-F	5’- CCG ACG CCA AGG GAA CT -3’	Sritunyalucksana et al. (2006)
WSSV-R	5’- TTC AGA TTC GTT ACC GTT TCC A -3’	
WSSV probe	5’- **6FAM** CGC TTC AGC CAT GCC AGC CG **TAMRA** -3’	
Reaction conditions: each 25 µl reaction mix is made up of 2 µl extracted nucleic acid template, 12.5 µl TaqMan Universal PCR Master Mix (Life Technologies), a final concentration of 900 nM for each primer, 250 nM for the probe and molecular-grade water. The qPCR assays are performed in a 7500 Fast Real-Time PCR System or QuantStudio 5 Real-Time PCR System (Life Technologies) and analysed with the 7500 software or QuantStudio design and analysis software respectively. PCR amplifications are programmed as follows: 1 cycle of 50°C for 2 minutes, 1 cycle of 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A threshold of 0.1 is used to determine C_T value.		

AFDL YHV9 RT-nPCR

Primer	Sequence	Reference
YHV9 For	5'- TGC GCA TCT ATC TAT CCT AAT GAA G -3'	AFDL, in-house
YHV9 Rev	5'- AAT GAG TTG GCG TTC TGG ATT -3'	
621bp	50°C for 30 minutes, 94°C for 2 minutes, 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 68°C for 45 seconds, 68°C for 7 minutes, hold at 4°C	
YHV9 For	5'- TGC GCA TCT ATC TAT CCT AAT GAA G -3'	
YHV9 Rev2	5'- TGT GAA TTT CTG GAT AGA TGT TAG T -3'	
450bp	95°C for 15 minutes, 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, 72°C for 7 minutes, hold at 4°C	
Reaction conditions: each 25 µl RT-PCR reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl 2× reaction mix and 1 µl Superscript III/Platinum Taq mix (Invitrogen) and a final concentration of 180 nM of each primer. Each 25 µl nPCR reaction mix is made up of 2 µl primary RT-PCR, 12.5 µl HotStarTaq Master Mix (Qiagen) and 360 nM of each primer. Amplicons of the expected size are observed by agarose gel electrophoresis and nucleotide sequence generated by Sanger sequencing.		

OIE 390 IHHNV PCR

Primer	Sequence	Reference
IHHNV309F	5'-TCC AAC ACT TAG TCA AAA CCA A -3'	Tang et al. (2007)
IHHNV309R	5'-TGT CTG CTA CGA TGA TTA TCC A -3'	
309bp	95°C for 15 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 90 seconds, 72°C for 7 minutes, hold at 4°C	
Reaction conditions: each 25 µl reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl HotStarTaq Master Mix (Qiagen) and 360 nM of each primer. Amplicons of the expected size are observed by agarose gel electrophoresis and nucleotide sequence generated by Sanger sequencing.		

OIE IMNV RT-nPCR

Primer	Sequence	Reference
IMNV 4587F	5'- CGA CGC TGC TAA CCA TAC AA-3'	Poulos and Lightner (2006)
IMNV 4914R	5'- ACT CGG CTG TTC GAT CAA GT-3'	
328bp	50°C for 30 minutes, 95°C for 2 minutes, 40 cycles of 94°C for 45 seconds, 60°C for 45 seconds, 68°C for 45 seconds, 68°C for 7 minutes, hold at 4°C	
IMNV 4725 NF	5'- GGC ACA TGC TCA GAG ACA-3'	
IMNV 4863 NR	5'- AGC GCT GAG TCC AGT CTT G-3'	
139bp	95°C for 15 minutes, 40 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds, 72°C for 7 minutes, hold at 4°C	
Reaction conditions: each 25 µl RT-PCR reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl 2× reaction mix and 1 µl Superscript III/Platinum Taq mix (Invitrogen) and a final concentration of 180 nM of each primer. Each 25 µl nPCR reaction mix is made up of 2 µl primary RT-PCR, 12.5 µl HotStarTaq Master Mix (Qiagen) and 360 nM of each primer. Amplicons of the expected size are observed by agarose gel electrophoresis and nucleotide sequence generated by Sanger sequencing.		

MBV PCR

Primer	Sequence	Reference
MBV261F	5'- AAT CCT AGG CGA TCT TAC CA -3'	Surachetpong et al. (2005)
MBV261R	5'- CGT TCG TTG ATG AAC ATC TC -3'	
261bp	95°C for 15 minutes, 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, 72°C for 7 minutes, hold at 4°C	
Reaction conditions: each 25 µl reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl HotStarTaq Master Mix (Qiagen) and 360 nM of each primer. Amplicons of the expected size are observed by agarose gel electrophoresis and nucleotide sequence generated by Sanger sequencing.		

MoV RT-nPCR

Primer	Sequence	Reference
MoV24F	5'- GGG ATG GTG TTG CCA TAC AAA GG -3'	Cowley et al. (2005)
MoV25R	5'- GTC ATT AGC TGG TCT TAG TTT TCA C -3'	
610bp	50°C for 30 minutes, 94°C for 2 minutes, 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 68°C for 45 seconds, 68°C for 7 minutes, hold at 4°C	
MoV148F	5'- ACA GTT TGT CAA GCT CAC AGG ATG -3'	
MoV149R	5'- AGA AGC GCC ATT CTG ATG AAC ATC -3'	
322bp	95°C for 15 minutes, 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, 72°C for 7 minutes, hold at 4°C	
Reaction conditions: each 25 µl RT-PCR reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl 2× reaction mix and 1 µl Superscript III/Platinum Taq mix (Invitrogen) and a final concentration of 180 nM of each primer. Each 25 µl nPCR reaction mix is made up of 2 µl primary RT-PCR, 12.5 µl HotStarTaq Master Mix (Qiagen) and 360 nM of each primer. Amplicons of the expected size are observed by agarose gel electrophoresis and nucleotide sequence generated by Sanger sequencing.		

HPV nPCR

Primer	Sequence	Reference
HPV-409F	5'- ATG AAY CAY YTD CAG GCA TTY GAR ATG -3'	Waqairatu (unpubl.) Cowley et al. (2015)
HPV-661R	5'- CCT CKY TGT GTR TGR RCR TAW GGC AT -3'	
782bp	95°C for 15 minutes, 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds, 72°C for 40 seconds, 72°C for 7 minutes, hold at 4°C	
HPV-552F	5'- AGG AAR GAR ATH CAR TCR GGY ATG GA -3'	
HPV-661R	5'- CCT CKY TGT GTR TGR RCR TAW GGC AT-3'	
353bp	95°C for 15 minutes, 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds, 72°C for 7 minutes, hold at 4°C	
Reaction conditions: each 25 µl reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl HotStarTaq Master Mix (Qiagen) and 360 nM of each primer. Two microlitres of primary PCR is used as template for nested PCR. Amplicons of the expected size are observed by agarose gel electrophoresis and nucleotide sequence generated by Sanger sequencing.		

OIE Decapod PCR

Primer	Sequence	Reference
143F	5'-TGC CTT ATC AGC TNT CGA TTG TAG-3'	Lo et al. (1996)
145R	5'-TTC AGN TTT GCA ACC ATA CTT CCC-3'	
848bp	95°C for 15 minutes, 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 90 seconds, 72°C for 7 minutes, hold at 4°C	
Reaction conditions: each 25 µl reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl HotStarTaq Master Mix (Qiagen) and 360 nM of each primer. Amplicons of the expected size are observed by agarose gel electrophoresis and nucleotide sequence generated by Sanger sequencing.		

Appendix 3. List of Project staff

Name	Position	Organisation
Dr Nick Moody	Group Leader – AFDL	CSIRO ACDP Fish Diseases Laboratory
Dr Peter Mohr	Team Leader – Aquatic Diagnostic Capability	CSIRO ACDP Fish Diseases Laboratory
Dr Mark Crane	Senior Principal Research Scientist	CSIRO ACDP Fish Diseases Laboratory
Dr David Cummins	Team Leader – Aquatic Research Capability	CSIRO ACDP Fish Diseases Laboratory
Ms Lynette Williams	Research Technician	CSIRO ACDP Fish Diseases Laboratory
Ms Joanne Slater	Research Technician	CSIRO ACDP Fish Diseases Laboratory
Mr John Hoad	Senior Research Technician	CSIRO ACDP Fish Diseases Laboratory
Ms Stacey Valdeter	Research Technician	CSIRO ACDP Fish Diseases Laboratory