Surveillance testing of imported commodity prawns for DIV1, CMNV and EHP

AUSTRALIAN CENTRE FOR DISEASE PREPAREDNESS (ACDP)
www.csiro.au

Dr Nick Moody | Research Group Leader, ACDP Fish Diseases Laboratory
Proaqua Australian Prawn Farmers Association (APFA) Symposium 2023

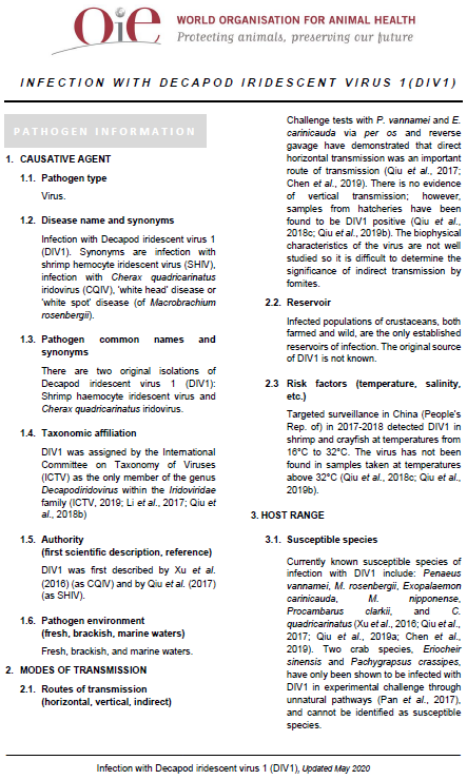
Purpose

To inform the "Review of the biosecurity risks of prawns imported from all countries for human consumption (the Prawn Review)", the Department of Agriculture, Fisheries and Forestry (DAFF) contracted the ACDP Fish Diseases Laboratory (AFDL) to undertake targeted surveillance.

- Ninety batches of imported commodity prawns
  - each batch is 65 prawns, collected as 13 bags of 5 prawns (n=1170)
- Tested for:
  - Decapod iridescent virus 1 (DIV1)
  - Covert mortality nodavirus (CMNV)
  - *Enterocytozoon hepatopenaei* (EHP)

DIV1 listed by WOAH, CMNV and EHP are not

- DIV1 listed in the WOAH *Aquatic Code* but no *Aquatic Manual* chapter (draft for Member Country comment in WOAH AAHSC September 2023 meeting report



WORLD ORGANISATION FOR ANIMAL HEALTH
Protecting animals, preserving our future

INFECTION WITH DECAPOD IRIDESCENT VIRUS 1 (DIV1)

PATHOGEN INFORMATION

1. CAUSATIVE AGENT

1.1. Pathogen type

Virus.

1.2. Disease name and synonyms

Infection with Decapod iridescent virus 1 (DIV1). Synonyms are infection with shrimp hemocyte iridescent virus (SHIV), infection with *Cherax quadricarinatus* iridovirus (CQIV), 'white head' disease or 'white spot' disease (of *Macrobrachium rosenbergii*).

1.3. Pathogen common names and synonyms

There are two original isolations of Decapod iridescent virus 1 (DIV1): Shrimp haemocyte iridescent virus and *Cherax quadricarinatus* iridovirus.

1.4. Taxonomic affiliation

DIV1 was assigned by the International Committee on Taxonomy of Viruses (ICTV) as the only member of the genus *Decapodiridovirus* within the *Iridoviridae* family (ICTV, 2019; Li *et al.*, 2017; Qiu *et al.*, 2018b)

1.5. Authority (first scientific description, reference)

DIV1 was first described by Xu *et al.* (2016) (as CQIV) and by Qiu *et al.* (2017) (as SHIV).

1.6. Pathogen environment (fresh, brackish, marine waters)

Fresh, brackish, and marine waters.

2. MODES OF TRANSMISSION

2.1. Routes of transmission (horizontal, vertical, indirect)

Challenge tests with *P. vannamei* and *E. carinicauda* via *per os* and reverse gavage have demonstrated that direct horizontal transmission was an important route of transmission (Qiu *et al.*, 2017; Chen *et al.*, 2019). There is no evidence of vertical transmission; however, samples from hatcheries have been found to be DIV1 positive (Qiu *et al.*, 2018c; Qiu *et al.*, 2019b). The biophysical characteristics of the virus are not well studied so it is difficult to determine the significance of indirect transmission by fomites.

2.2. Reservoir

Infected populations of crustaceans, both farmed and wild, are the only established reservoirs of infection. The original source of DIV1 is not known.

2.3 Risk factors (temperature, salinity, etc.)

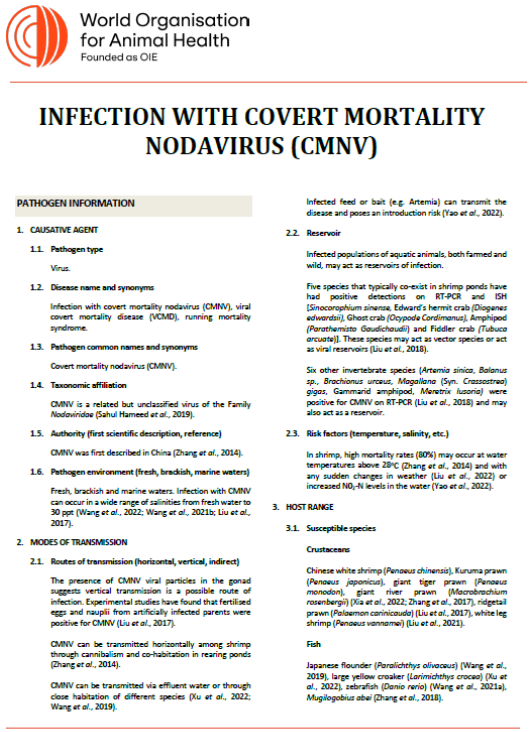
Targeted surveillance in China (People's Rep. of) in 2017-2018 detected DIV1 in shrimp and crayfish at temperatures from 16°C to 32°C. The virus has not been found in samples taken at temperatures above 32°C (Qiu *et al.*, 2018c; Qiu *et al.*, 2019b).

3. HOST RANGE

3.1. Susceptible species

Currently known susceptible species of infection with DIV1 include: *Penaeus vannamei, M. rosenbergii, Exopalaemon carinicauda, M. nipponense, Procambarus clarkii,* and *C. quadricarinatus* (Xu *et al.*, 2016; Qiu *et al.*, 2017; Qiu *et al.*, 2019a; Chen *et al.*, 2019). Two crab species, *Eriocheir sinensis* and *Pachygrapsus crassipes*, have only been shown to be infected with DIV1 in experimental challenge through unnatural pathways (Pan *et al.*, 2017), and cannot be identified as susceptible species.

Infection with Decapod iridescent virus 1 (DIV1), *Updated May 2020*



World Organisation for Animal Health
Founded as OIE

INFECTION WITH COVERT MORTALITY NODAVIRUS (CMNV)

PATHOGEN INFORMATION

1. CAUSATIVE AGENT

1.1. Pathogen type

Virus.

1.2. Disease name and synonyms

Infection with covert mortality nodavirus (CMNV), viral covert mortality disease (VCMD), running mortality syndrome.

1.3. Pathogen common names and synonyms

Covert mortality nodavirus (CMNV).

1.4. Taxonomic affiliation

CMNV is a related but unclassified virus of the Family *Nodaviridae* (Sahul Hameed *et al.*, 2019).

1.5. Authority (first scientific description, reference)

CMNV was first described in China (Zhang *et al.*, 2014).

1.6. Pathogen environment (fresh, brackish, marine waters)

Fresh, brackish and marine waters. Infection with CMNV can occur in a wide range of salinities from fresh water to 30 ppt (Wang *et al.*, 2022; Wang *et al.*, 2021b; Liu *et al.*, 2017).

2. MODES OF TRANSMISSION

2.1. Routes of transmission (horizontal, vertical, indirect)

The presence of CMNV viral particles in the gonad suggests vertical transmission is a possible route of infection. Experimental studies have found that fertilised eggs and nauplii from artificially infected parents were positive for CMNV (Liu *et al.*, 2017).

CMNV can be transmitted horizontally among shrimp through cannibalism and co-habitation in rearing ponds (Zhang *et al.*, 2014).

CMNV can be transmitted via effluent water or through close habitation of different species (Xu *et al.*, 2022; Wang *et al.*, 2019).

Infected feed or bait (e.g. Artemia) can transmit the disease and poses an introduction risk (Yao *et al.*, 2022).

2.2. Reservoir

Infected populations of aquatic animals, both farmed and wild, may act as reservoirs of infection.

Five species that typically co-exist in shrimp ponds have had positive detections on RT-PCR and ISH (*Sinocarophium sinense*, Edward's hermit crab (*Diogenes edwardsii*), Ghost crab (*Ocypode Cordimanus*), Amphipod (*Parathemisto Gaudichaudii*) and Fiddler crab (*Tubuca arcuata*)). These species may act as vector species or act as viral reservoirs (Liu *et al.*, 2018).

Six other invertebrate species (*Artemia sinica, Balanus sp., Brachionus urceus, Magallana* (Syn. *Crassostrea*) *gigas*, Gammarid amphipod, *Meratrix lusoria*) were positive for CMNV on RT-PCR (Liu *et al.*, 2018) and may also act as a reservoir.

2.3. Risk factors (temperature, salinity, etc.)

In shrimp, high mortality rates (80%) may occur at water temperatures above 28°C (Zhang *et al.*, 2014) and with any sudden changes in weather (Liu *et al.*, 2022) or increased NO_2-N levels in the water (Yao *et al.*, 2022).

3. HOST RANGE

3.1. Susceptible species

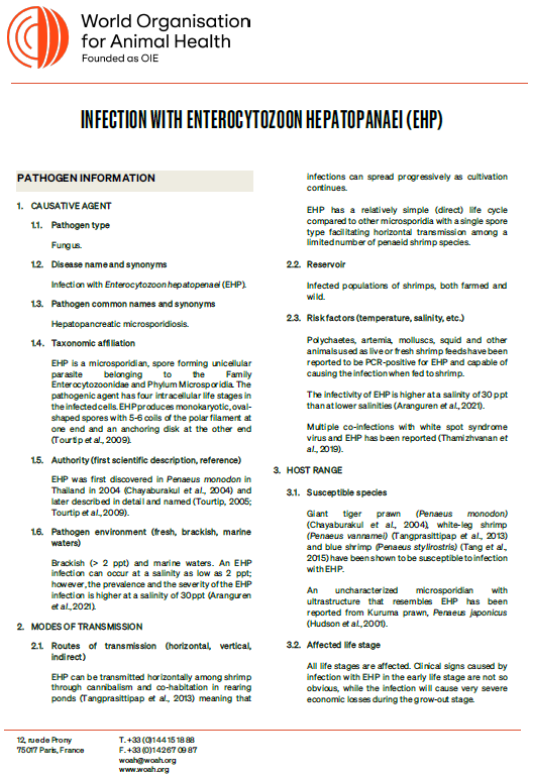
Crustaceans

Chinese white shrimp (*Penaeus chinensis*), Kuruma prawn (*Penaeus japonicus*), giant tiger prawn (*Penaeus monodon*), giant river prawn (*Macrobrachium rosenbergii*) (Xia *et al.*, 2022; Zhang *et al.*, 2017), ridgetail prawn (*Palaemon carinicauda*) (Liu *et al.*, 2017), white leg shrimp (*Penaeus vannamei*) (Liu *et al.*, 2021).

Fish

Japanese flounder (*Paralichthys olivaceus*) (Wang *et al.*, 2019), large yellow croaker (*Larimichthys crocea*) (Xu *et al.*, 2022), zebrafish (*Danio rerio*) (Wang *et al.*, 2021a), *Mugilogobius abei* (Zhang *et al.*, 2018).

12, rue de Prony
75017 Paris, France
T. +33 (0)1 44 15 18 88
F. +33 (0)1 42 67 09 87
woah@woah.org
www.woah.org



World Organisation for Animal Health
Founded as OIE

INFECTION WITH ENTEROCYTOZOON HEPATOPANAEI (EHP)

PATHOGEN INFORMATION

1. CAUSATIVE AGENT

1.1. Pathogen type

Fungus.

1.2. Disease name and synonyms

Infection with *Enterocytozoon hepatopenaei* (EHP).

1.3. Pathogen common names and synonyms

Hepatopancreatic microsporidiosis.

1.4. Taxonomic affiliation

EHP is a microsporidian, spore forming unicellular parasite belonging to the Family Enterocytozoonidae and Phylum Microsporidia. The pathogenic agent has four intracellular life stages in the infected cells. EHP produces monokaryotic, oval-shaped spores with 5-6 coils of the polar filament at one end and an anchoring disk at the other end (Tourtip *et al.*, 2009).

1.5. Authority (first scientific description, reference)

EHP was first discovered in *Penaeus monodon* in Thailand in 2004 (Chayaburakul *et al.*, 2004) and later described in detail and named (Tourtip, 2005; Tourtip *et al.*, 2009).

1.6. Pathogen environment (fresh, brackish, marine waters)

Brackish (> 2 ppt) and marine waters. An EHP infection can occur at a salinity as low as 2 ppt; however, the prevalence and the severity of the EHP infection is higher at a salinity of 30 ppt (Aranguren *et al.*, 2021).

2. MODES OF TRANSMISSION

2.1. Routes of transmission (horizontal, vertical, indirect)

EHP can be transmitted horizontally among shrimp through cannibalism and co-habitation in rearing ponds (Tangprasittipap *et al.*, 2013) meaning that infections can spread progressively as cultivation continues.

EHP has a relatively simple (direct) life cycle compared to other microsporidia with a single spore type facilitating horizontal transmission among a limited number of penaeid shrimp species.

2.2. Reservoir

Infected populations of shrimps, both farmed and wild.

2.3. Risk factors (temperature, salinity, etc.)

Polychaetes, artemia, molluscs, squid and other animals used as live or fresh shrimp feeds have been reported to be PCR-positive for EHP and capable of causing the infection when fed to shrimp.

The infectivity of EHP is higher at a salinity of 30 ppt than at lower salinities (Aranguren *et al.*, 2021).

Multiple co-infections with white spot syndrome virus and EHP has been reported (Thamizhvanan *et al.*, 2019).

3. HOST RANGE

3.1. Susceptible species

Giant tiger prawn (*Penaeus monodon*) (Chayaburakul *et al.*, 2004), white-leg shrimp (*Penaeus vannamei*) (Tangprasittipap *et al.*, 2013) and blue shrimp (*Penaeus stylirostris*) (Tang *et al.*, 2015) have been shown to be susceptible to infection with EHP.

An uncharacterized microsporidian with ultrastructure that resembles EHP has been reported from Kuruma prawn, *Penaeus japonicus* (Hudson *et al.*, 2001).

3.2. Affected life stage

All life stages are affected. Clinical signs caused by infection with EHP in the early life stage are not so obvious, while the infection will cause very severe economic losses during the grow-out stage.

12, rue de Prony
75017 Paris, France
T. +33 (0)1 44 15 18 88
F. +33 (0)1 42 67 09 87
woah@woah.org
www.woah.org

Screening molecular tests used

- Decapod iridescent virus 1 (DIV1)
  - DIV1 Qiu et al. (2018) qPCR targeting the ATPase gene
  - DIV1 Qiu et al. (2020) qPCR targeting the major capsid protein gene

- Covert mortality nodavirus:
  - CMNV Pooljun et al. (2016) qPCR assay, targeting the RNA-dependent RNA polymerase (RdRp) gene

- *Enterocytozoon hepatopenaei* (EHP)
  - EHP Liu et al. (2018) qPCR targeting the 18S SSU ribosomal RNA gene
  - AFDL EHP SWP qPCR (ACDP unpublished) targeting the SWP gene

Control tests used

- Plasmid positive controls (heterologous)
  - Artificial probe (positive control contamination)
  - FRDC 2014-002 (Moody et al. 2021)
- Housekeeping genes
  - CSIRO Shrimp EF1 qPCR (Cowley et al. 2018)
  - WOAH Decapod PCR (Lo et al. 1996)
- Negative extraction control
  - Extraction reagents only
- No template control
  - qPCR reagents only

Testing undertaken

- **90 batches** of prawns received between 15/12/2020 and 03/03/2021.
- **5850 prawns** received with **1170 pooled tissue samples** collected by biopsy punch over 3 months (starting in February).
- Nucleic acid was extracted in **24 plates** after bead homogenisation of the samples.
- **DIV1** testing consisted of **>7000 PCR reactions** (2 assays) on >110 PCR plates (February to May).
- **EHP/CMNV** testing consisting of **>8500 PCR reactions** (3 assays) (June to August).
- Two **EHP conventional nested PCR assays** were optimised and used to test **192 samples** (September to October).
  - **>300 amplicons** were purified for ~700 nucleotide sequencing reactions (October).
- **>7000** test results were entered and authorised in Sample Manager.
- Reporting: **164 interim reports** and **90 final reports**.

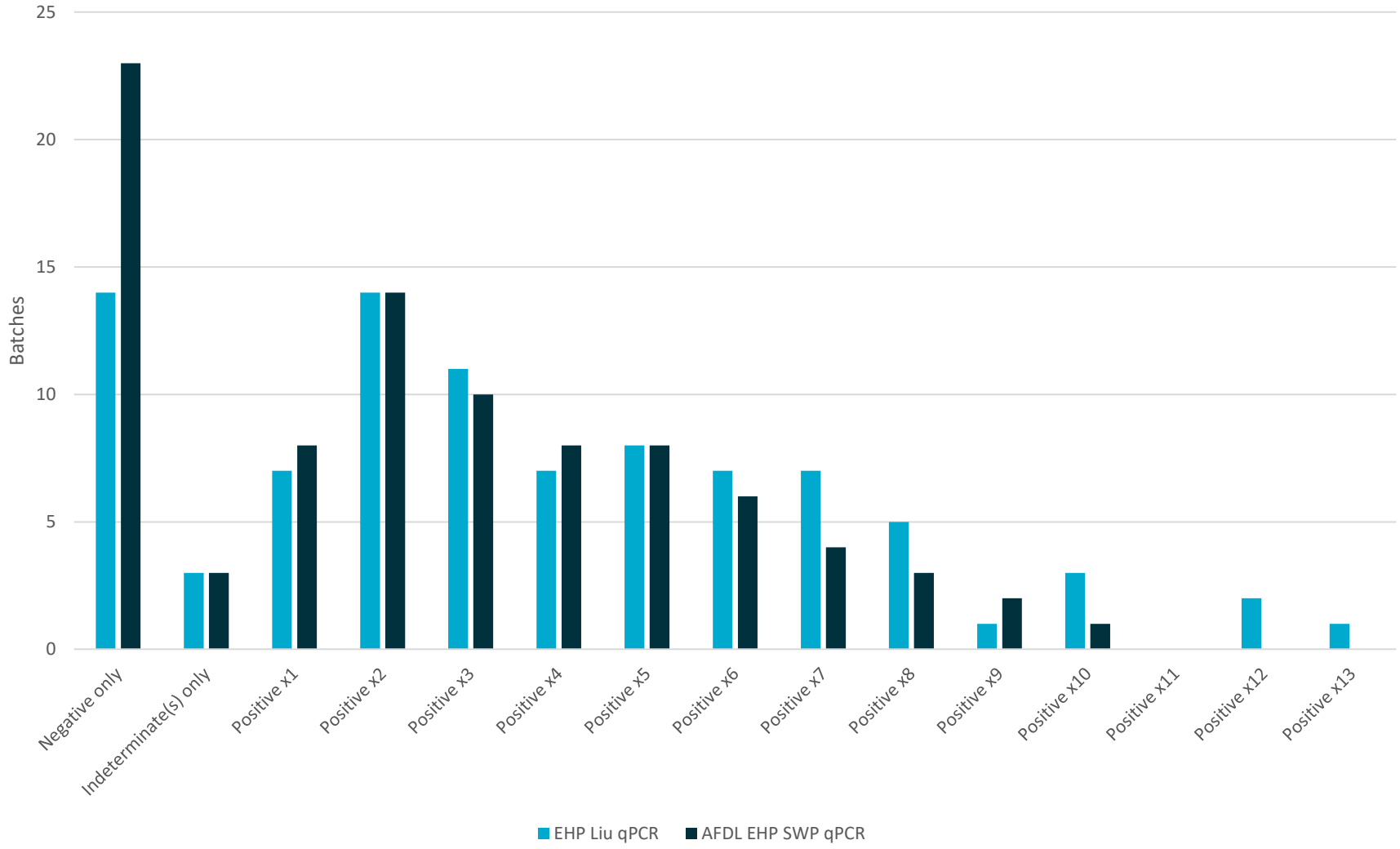
Results – screening tests

- Decapod iridescent virus 1 (DIV1)
  - All pools test negative (by both assays)

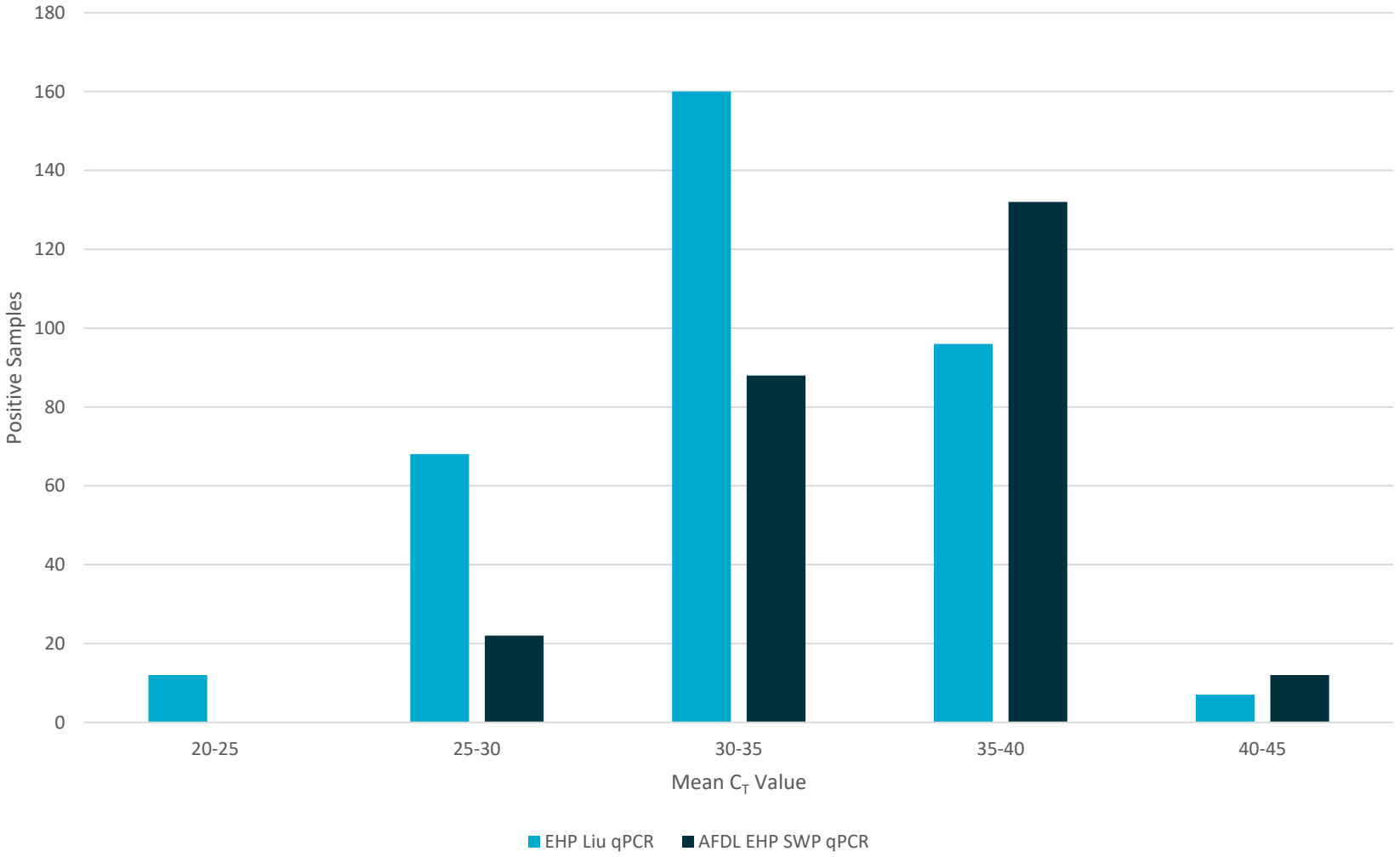
- Covert mortality nodavirus (CMNV)
  - All pools test negative (by single assay)

- *Enterocytozoon hepatopenaei* (EHP)
  - EHP Liu qPCR: 73 batches had at least one sample test positive and 3 batches had samples test indeterminate
  - AFDL qPCR (ACDP unpublished): 64 batches had at least one sample test positive and 3 batches had samples test indeterminate
  - Only 14 batches had all samples test negative with both EHP Liu qPCR and AFDL qPCR assays
  - We knew there were specificity issues with the EHP Liu qPCR, which was why we developed the AFDL EHP qPCR (targets an EHP single gene region so specificity is increased but sensitivity is decreased)

Results – EHP


25
20
15
10
5
0
Batches
Negative only
Indeterminate(s) only
Positive x1
Positive x2
Positive x3
Positive x4
Positive x5
Positive x6
Positive x7
Positive x8
Positive x9
Positive x10
Positive x11
Positive x12
Positive x13
EHP Liu qPCR
AFDL EHP SWP qPCR

Results – EHP


180
160
140
120
100
80
60
40
20
0
Positive Samples
20-25
25-30
30-35
35-40
40-45
Mean C_T Value
EHP Liu qPCR
AFDL EHP SWP qPCR

Results – EHP confirmatory tests

- 192 samples from 74 batches were tested by conventional PCR
  - Tangprasittipap et al. (2013) nPCR: 178 amplicons
  - Jaroenlak et al. (2016) nPCR: 161 amplicons

- Tangprasittipap et al. (2013) nPCR
  - Of 178 amplicons, 168 shared 100% identity with each other and the EHP SE Asia reference sequence.
  - The identity of the remaining 10 sequences from four batches could not be confirmed
  - Assay known to have specificity issues

- Jaroenlak et al. (2016) nPCR
  - All 161 amplicon sequences shared 100% identity with each other and the EHP SE Asia reference sequence

Results – EHP confirmatory tests

Conventional PCR	Species	% Identity (NCBI GenBank MEGABLAST Match)	Samples Confirmed	Batches Confirmed
EHP Tangprasittipap nPCR	EHP (SE Asia)	• 100% (i.e. KF362130.1 18S SSU rRNA, Thailand 2011, *Penaeus vannamei*)	168	65
	Unknown 1	• 91.74% with *Nucleospora* sp. (i.e. MN702766.1, 18S SSU rRNA, China 2019, *Hippocampus erectus*) • 91.68% with *Enterospora nucleophila* (i.e. KF135645.1, 18S SSU rRNA, Spain, *Sparus aurata*) • 90.91% with *Nucleospora salmonis* (i.e. AF185992.1, 18S SSU rRNA, Canada, *Oncorhynchus tshawytscha*) • 90.83% with EHP Southeast Asia (i.e. KF362130.1 18S SSU rRNA, Thailand 2011, *Penaeus vannamei*)	9	4
	Unknown 2	• 88.89% with *Enterospora nucleophila* (i.e. KF135645.1, 18S SSU rRNA, Spain, *Sparus aurata*) • 87.31% with EHP Southeast Asia (i.e. KF362130.1 18S SSU rRNA, Thailand 2011, *Penaeus vannamei*)	1	1
EHP Jaroenlak SWP nPCR	EHP (SE Asia)	• 100% (i.e. KX258197.1 Spore Wall Protein, Thailand 2015, *Penaeus vannamei*)	160	65

Summary

- Tested 90 batches of prawns (n=1170) imported for human consumption
- All were test negative for DIV1
- All were test negative for CMNV
- 74 batches screened positive for EHP, (4 batches were not EHP)
- Results contributed to the “Review of the biosecurity risks of prawns imported from all countries for human consumption (the Prawn Review)”
- Testing contributed to improved capability at ACDP

Thank you

Nick Moody
Senior Research Scientist
Research Group Leader
ACDP Fish Diseases Laboratory

t +61 3 5227 5749
e nick.moody@csiro.au
w www.csiro.au