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Biosecurity in Northern Australia prawn aquaculture

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1. Acknowledgments

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- compliance with ethical guidelines
- conclusions against results
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and provided constructive feedback which was considered and addressed by the author(s).

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5 Acronyms

AHPND	Acute Hepatopancreatic Necrosis Disease
APFA	Australian Prawn Farmers Association
Ct	Cycle Threshold
DNA	Deoxyribonucleic Acid
DIV-1	Decapod Iridescent Virus-1 (exotic to Australia)
ECWM	East Coast sourced wild *P.monodon*
GAV	Gill Associated Virus (endemic to Australia)
GBO	General Biosecurity Obligation (required under the QLD Biosecurity Act 2014)
HDV/HPV	Hepandensovirus/ Hepatopancreatic Parvovirus Virus (endemic to Australia)
hemo	Hemolysin D gene
HTS	High throughput sequencing
IHHNV/PstDV	Infectious hypodermal hematopoietic necrosis virus/Penaeus stylirostris Densovirus
IMNV	Infectious myonecrosis virus (exotic to Australia)
MBV	Monodon Baculovirus (endemic to Australia)
nd	Not detected
NTWM	Northern Territory sourced wild *P.monodon*
Pir-A	Photorhabdus insect-related toxin gene-component A
PL	Post larval stage prawns
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic Acid
rt	Reverse transcriptase (a reaction for detecting RNA targets)
Rtx	Repeats in toxin gene
TNA	Total nucleic acid
TSV	Taura syndrome Virus (exotic to Australia)
YHV	Yellow head Virus (Strain 1 exotic to Australia; Strains 2,6,7 endemic to Australia)
When-2	Whenzhou Prawn Virus (detected in Australia by HTS, unknown a percentage of positive detections)
WSSV	Whitespot syndrome Virus
SD	Standard Deviation
Zon	Zonula occludens toxin gene

6 Project Participants

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1 Executive Summary

The objectives of the Cooperative Research Centre for Developing Northern Australia (CRCNA) Improving Biosecurity in Northern Australian prawn farms (A.3.1718113) were to obtain an overview of the pathogens and the level of protection provided by the current biosecurity practises that occur in prawn farms in Australia. Effective farm biosecurity relies on knowledge of the prevalence and quantity of pathogens present in the production line to formulate management strategies that reduce the likelihood of introduction or impact of prioritised pathogens. This project completed scheduled sampling to investigate the percentage of positive detections and calculated quantity of pathogens that were previously known to occur (endemic) in Australian prawn farms from the hatchery; broodstock (n = 967) and post larval stages (n = 411); and grow out farm: pond prawns (n = 666). The study significantly increased the volume of pathogen data available to Australian prawn farmers to prepare risk assessment and biosecurity management plans to meet their General Biosecurity Obligations (GBOs) as is required under the QLD Biosecurity Act (2014).

Prior to this study the most extensive survey of endemic pathogens in Australian broodstock analysed 493 broodstock for the presence of Gill Associated Virus (GAV) and Yellowhead Virus-7 (YHV-7) and 337 broodstock for the presence of Infectious Hypodermal Haematopoietic Necrosis Virus (IHHNV), Hepatopancreatic Parvovirus (HPV) and Monodon Baculovirus (MBV) (Cowley *et al.*, 2015). An IHHNV specific surveillance conducted on prawn farms in Queensland detected IHHNV prevalence between 1.4% and 5.3% from a total of 1298 samples (Moody *et al.*, 2011). This study represents the most extensive investigation into the distribution and quantity of multiple pathogens collected from multiple stages of production to be conducted on Australian farmed prawns.

The analysis of broodstock samples within the hatchery component of the study indicated the practice of commencing production with wild broodstock collected from Northern Territory and East Coast QLD fisheries presents a high likelihood of introduction of viral pathogens to hatchery production systems. Although all samples (n=967) were negative for the detection of WSSV and YHV-1, 93% of broodstock samples were positive for the detection of at least one endemic pathogen target. Only 71/967 (7%) of samples were negative for all of the targeted pathogens. Gill Associated Virus (GAV) (89%), Infectious Hypodermal Hematopoietic Necrosis Virus (IHHNV) (47%) and Wenzhou Shrimp Virus-2 (When-2) (32%) were the most frequently detected pathogen targets. The A component (Pir-A) of the *Photorhabdus* insect related-Ab (Pir-AB) toxin gene was detected in a low percentage (8%) directly from pleopod samples.

The pathogen targets detected in the wild-captured broodstock were also detected in a high percentage of the post larval (PL) samples. A total of 365/411 (88%) of post larvae samples were positive for the detection of an endemic pathogen target. GAV (88%) and IHHNV (61%) were the most frequently detected pathogen targets. Pir-A was detected with a low percentage of positive detections (2%) and low quantity of target in PL stages. When-2 was not detected in any PL sample.

In the grow out stage, the pathogen targets detected in broodstock and post-larval samples were also detected in a high percentage of samples. There were no detections of WSSV, YHV-1 or Pir-A from any pond collected prawn samples obtained from the 55 ponds across 4 farms sampled in this study. The majority, (665/666, 99%) of samples collected from grow-out ponds were positive for at least one pathogen target. GAV (98%), IHHNV (62%), and When-2 (22%) were the most frequently detected pathogen targets. There was considerable variation in the maximum calculated quantity of each pathogen target detected across ponds within and between farms. Survival at harvest ranged from 26% to 100%.

Investigating potential reservoirs of pathogens in prawn farms, Glass prawn, *Acetes*, spp. (n= 59 pools of 10) cohabiting the prawn ponds were positive for the detection of When-2 (49%), IHHNV (20%), GAV (24%), and Hepatopancreatic

Necrosis Virus/Hepandensovirus (HDV) (20%). A potential role of glass prawn (*Acetes* spp.) and jelly prawn (*Palaemon* spp.) as sources of introduction of pathogens was indicated by the detection of IHHNV (23%), HDV (20%), GAV (1%), WSSV (9%), Pir-A (1%) and When-2 (1%) in specimens (n= 191 pooled samples) collected in intake areas external to prawn ponds.

Analysis of samples submitted for ad hoc investigations into sub-optimal productivity, associated with a mass mortality event at a hatchery, was conducted. Using a high throughput sequencing (HTS) approach, bacterial toxin genes including Zonular occludens toxin (Zon *syn.* Zona occludens toxin or ZON), Repeats in toxin (RtX) and, hemolysin D (hemo) were identified. Targeted analysis on the schedule collected samples indicated the toxin genes were present across industry with variable rates of positive detection (%) and generally in low quantity in the absence of reported sub-optimal productivity. While the significance of the toxin genes requires further investigation, preliminary studies have highlighted a need to consider the role played by bacterial species as co-contributors to disease incidents and reduced production efficiency. The HTS approach was also applied to cases of pond collected prawn samples exhibiting reduced growth. Nucleopolyhedrovirus (MBV), Hepandensovirus (HDV) and a variant strain of IHHNV was detected. Genetic sequence of a circovirus, Iflavirus and insect polyhedrovirus and endogenous nimarvirus were also identified and require further investigation.

Opportunistic analysis was conducted on a single 1 kg package of imported uncooked retail purchased prawns, applying all of the qPCR targets used within the project. There was 100% detection of When-2 and 57% detection of IHHNV from the imported prawn samples. Notably, WSSV and YHV-1 were not detected from any of the imported prawn samples.

In addition to presenting a broad overview of the percentage of positive detections of seven pathogens within a typical prawn production season, under typical biosecurity management protocols, the project also identified trends, or lack thereof, in the transfer and amplification of pathogens through the production cycle. Trends identified included; increased percentages of positive detection and calculated quantity of the detection of IHHNV, GAV and Pir-A and, an unexpected decrease in When-2 from pre- to post-spawned broodstock samples. Despite relatively high percentages of positive detection of Pir-A in broodstock samples, only a small percentage of PL tanks (n=2) and no pond samples were positive for the detection of Pir-A.

While the whole of industry actively attempts to manage the risk of introduction of WSSV and YHV-1 by exclusion, current operational biosecurity practises are not uniformly directed towards the control of endemic pathogens. The annual intake of wild captured broodstock facilitates the entry of endemic pathogens into the prawn production facilities and the pathogens are detected in variable percentages and quantity in subsequent stages of production. Some pathogens such as YHV-7, When-2 and Pir-A were positively detected in only a small proportion of samples and could potentially be excluded from production systems through culling, treatment or hatchery disinfection protocols. IHHNV and GAV, however, were detected in a high percentage of wild captured broodstock and post larval samples indicating current, standard targeted protocols are not effective in excluding or eradicating either pathogen from production. Comparison between hatcheries that actively adopt a reduction approach to IHHNV and bacterial toxin genes and those that do not conduct any targeted management indicated reduction, but not elimination, in the proportion of positive detections in the pond samples derived from hatcheries that perform targeted management. A reduction in When-2 may have also been achieved through serendipitous removal of individuals positive for high quantity of IHHNV.

In overseas prawn farming, the impacts of IHHNV, GAV, WSSV, YHV-1, *Enterocytozoan hepatopenaei* (EHP), Infectious Myonecrosis Virus (IMNV), HPV and Monodon Baculovirus (MBV) have been reduced from the production system through the domestication of *L.vannamei* based on animals that were naturally free of the pathogens and subsequently selectively bred for increased tolerance. Such lines are termed specific pathogen free/pathogen tolerant and are not

commercially available in Australia. The Australian prawn farming industry needs to pursue domestication of *P. monodon* as a strategy to reduce biosecurity risk. Domestication would allow industry to remove the biosecurity risk posed by the annual intake of wild-captured broodstock and enable genetic selection and selective breeding approaches to culture stock with beneficial traits. However, the results of this study indicate attempts by the Australian prawn farming industry to develop SPF lines from wild-captured *P. monodon* could be inhibited by the scarcity (7% in this study) of broodstock free of pathogens. The detection of WSSV, Pir-A, IHHNV, GAV, HPV, and When-2 in glass shrimp external to prawn farms also casts doubt on the ability of farms to maintain pathogen-free status of ponds stocked with SPF animals. Management protocols to reduce the risk of disease incidents, when pathogens are present in a large percentage of stock and cannot be excluded from culture systems, may consider asset-based protection approaches.

Effective asset-based protection approaches require the management of the interaction between pathogens, prawn health and the environment. There is a need to improve the understanding of pathogens, prawn health and the influence of environmental factors in instigating disease outbreaks. Although this study has provided the most extensive multi-pathogen analysis conducted in Australian prawn farming, there remains ambiguity in the factors that contribute to disease outbreaks on farm and what the real costs to productivity are. This study demonstrates that the presence of a pathogen does not invariably lead to catastrophic disease outbreaks. However, historically, many disease studies that describe the acute and chronic impacts of pathogen loading on productivity in prawn farming have not been experimentally demonstrated. Thus, current knowledge and management strategies are based on the findings of associative studies that do not consider a number of variables which could significantly alter management approaches. The industry would benefit from purification and characterisation of the viral strains that occur in Australian prawns and studies that consider both the contribution of pathogen-environmental and prawn interactions to initiate disease incidents and the actual effects on survival and growth.

The ubiquitous presence of pathogens in cultured prawns allows for incubation within a host that will invariably undergo stress during the production cycle. Such scenarios cater for the recombination and transfer of genetic material which drives the emergence of more virulent pathogen strains. Whilst the Australian industry has experienced relatively few disease incidents and an absence of the pandemic outbreaks that have been reported overseas, the past 9 years of production volumes have been variable and coincided with the detection of some novel pathogens. For instance, in 55 ponds examined for pathogens in the current project there was considerable variability in survival (26-100%) and FCR (1.70 -2.34). The increased seawater temperatures and more extreme weather conditions forecast to prevail with global climate change, continued import of frozen commodity prawns with the potential to transfer presently exotic pathogens into Australia, and intensification and expansion of the industry all bode to present a climate of increased biosecurity risk. If the current model of production, which is highly exposed to environmental conditions, is retained in future expansions of industry, improved understanding of the host-pathogen-environment interaction will be required to prevent disease outbreaks. A strengthening in the frozen prawn IRA to prevent the introduction of exotic pathogens and policy support and investment to strengthen regional diagnostic and research capability in health is required to support the long-term biosecurity needs of the prawn aquaculture industry and aquaculture sector more generally in Northern Australia.

2 Strategic recommendations

Key priority actions for sector development	Action owner and key partners	Pathways to implementation and timeline	Intended industry impacts
1. Strengthen National Border Biosecurity to keep exotic pathogens exotic.	Australian Government: Department of Agriculture Australian Prawn Farmers Association Australian Seafood Association Queensland Government Emerging aquaculture crustacean species	Short-term: Strengthen the current prawn IRA to prevent entry of viable exotic pathogens into the uncontrolled Australian retail market. Short-term: Implement a frequent and scheduled review of the frozen prawn IRA, including a mandatory review when the OIE Aquatic Manual is updated; to enable the inclusion of current scientific knowledge of risk and mitigation strategies. Short-medium term: leveraging of industry translocation testing into a surveillance program for exotic pathogens to demonstrate proof of freedom and detection of emerging native strains with homology to exotic pathogens.	Protection of a current industry production circa $80 Million (4 630T) and reduce risk to the forecast expansion of industry $432 Million; (19 915 T; 825 direct FTE) in regional Northern Australia and continued economic activity of commercial and recreational fishers. Reduced reliance on import of frozen commodity prawns from overseas to support domestic demand (2018 import value $484.5 Million) Reduced risk to threaten expansion of aquaculture of emerging crustacean species e.g. *P. ornatus, T. orientalis, C. quadricarinatus* which are also susceptible to exotic prawn pathogens. Prevention of repeated direct losses from exotic disease outbreaks e.g. WSSV industry loss of $23 M; QLD and Australian Government response costs. Cost effective-collection of data to support claims of freedom from pathogens considered within the frozen commodity prawn IRA. Collection of baseline data to support identification of emergent native pathogen strains Secure present and future consumer demand: Maintain image of Australian clean green product brand in National and International markets

2. Strengthen regional capability and capacity in prawn health research and aquatic biosecurity response:	APFA ABFA Queensland crayfish farmers Association CRC NA FRDC Australian Government Queensland Government Research Institutes Commercial investors Individual farms CRC Food agility JCU, Charles Darwin University and other Northern Australia located research institutes	Short-medium term: Permit NATA accredited laboratories to perform testing for exotic pathogens beyond strict application to translocation protocols. Short-medium term: Include Northern Australian stakeholders within the biosecurity planning and response training such as AquaPLAN and disease simulation training. Form Northern Australian node of emergency aquatic animal disease response. Short-medium-long term: Develop experimental disease research facilities in Northern Australia: Co-funded model between industry/ government /research or commercial providers. Include provision for experimental disease challenge and testing efficacy of treatments. This initiative benefits multiple aquaculture industries.	Reduced response time to detection of an exotic pathogen and facilitation of rapid containment and eradication of an exotic disease in Northern Australian prawn farms. Improved likelihood of successful and cost-effective emergency disease biosecurity response through the provision of trained, industry-invested, regionally located workforce. Increased regional resilience to disruption of access to metropolitan located providers of exotic disease diagnosis and biosecurity response. Provision of critical infrastructure at regional location which facilitates flow on benefits to competitively access FRDC and other such funding to expand on tropical aquaculture scientific capability in regional Northern Australia. Reduce the risk of Northern Australian aquaculture industry priority research being delayed due to COVID activities in regions in southern Australia. Positioning of regional facility with most efficient access to Australian farmed prawns and reduce inefficiencies in research resources being diverted to extremely costly freight costs and other inefficiencies to conduct research positioned at considerable distance from industry. Capacity to conduct tropical research under tropical conditions (especially incorporating tropical marine bacterial species and role of tropical microbiomes in health).

			Reduced reliance on scientific challenge experiments being conducted from the Australian Centre for Disease Preparedness (formerly AAHL which now also performs a major role in human emergency disease response). Reduce delay in characterisation of new and emerging prawn pathogens due to AAHL hosted activities being postponed to enable national exotic disease emergency response and nationally prioritised research. Completion of selective breeding experiments to support industry development of resistant/tolerant lines. Regional access to facilities to complete hands on biosecurity training programs Regional access to facilities to develop and test efficacy of novel therapeutics and treatments to prevent disease in the Australian setting.
3. Domestication of *P.monodon*	Australian Prawn Farmers Association CRC NA FRDC ARC CRC-P Private business including current prawn farming operations	Short-term-medium term:	Reduced risk of annual introduction of pathogens into farm production line Increase farm profits by 200-300% through selective breeding or genetic selection of: o Disease resistant lines o Lines tolerant to impacts of climate change o Favourable traits. Production of new market as a global supplier of domesticated *P. monodon.*
4. Increase research focus on the interaction of host-pathogen-environment in the expression of disease	APFA FRDC CRC NA ARC CRC-P and Research providers, preferably those with strong regional presence in Northern Australia	Short-Long term: Policy shift and increased research investment into multi-disciplinary approach to prawn health and disease management Targeted research to characterise endemic pathogens (IHHNV, When-	Research that increases understanding of the factors critical to prawn health could reduce the variability in pond survival which, in this project, ranged from 26-100% equating to gross farm gate ~ $78 000 v $300 000 per hectare. Research that increases the factors critical to prawn health could reduce the variability in food conversion ratio which, in this project, ranged from 1.7 to 2.34 which equates to feed purchase of 25 500 kg per hectare v 35 700 kg per hectare or feed cost of $76 500 v $107 100 per hectare.

		2, GAV) and elucidate effects on production and factors that contribute to increased impact on production.	Target research towards the specific strains of pathogen with highest propensity to cause disease outbreaks and impact on production. Focus resource and effort to those pathogens with robustly demonstrated impact.
5. Increase Education and training of workforce in prawn health and biosecurity response procedures	APFA Queensland Government NT Government WA Government Universities, TAFEs and other education providers	Medium- Long term: develop specific training in prawn health and management.	Training of an adequately skilled workforce that can manage the host-pathogen-environment parameters and prevent aquatic disease incidents. Training of an adequately skilled workforce that can rapidly identify and effectively respond to aquatic disease incidents. Reduced reliance of industry on government resources to conduct eradication and containment activities in logistically difficult regional locations.

3 Introduction

3.1 Global Prawn Industry Overview

The global production of prawns in marine and brackish water aquaculture has increased from 2.9 Million Tonnes (US$ 14B) in 2010 to 5.3 Million Tonnes (US$ 32B) in 2018 (FAO.org, accessed 21/6/20). Global prawn aquaculture is dominated by the production of the Pacific white-legged shrimp (*Litopenaeus vannamei*) which accounts for approximately 80% of production, while the black tiger prawn (*Penaeus monodon*) accounts for approximately 13.5% of global production (Zhang *et al.*, 2019; FAO.org, accessed 21/6/20). Although the sector has experienced gains in production, disease is estimated to cause losses in productivity circa 40% and is one of the major impediments to sustainable prawn aquaculture production (Stentiford, 2016). Whitespot syndrome Virus (WSSV) has historically been the most detrimental viral pathogen to prawn aquaculture, however, numerous other pathogens have impacted on global prawn production including, amongst others, Infectious hypodermal haematopoietic necrosis virus (IHHNV: 1981), Hepatopancreatic Parvovirus (HPV: 1982), *Penaeus monodon* baculovirus (MBV: 1988), Yellowhead virus (YHV: 1990), Taura Syndrome Virus (TSV: 1993), Infectious Myonecrosis Virus (IMNV: 2004); Acute Hepatopancreatic Necrosis Disease (APHND: 2009), *Enterocytozoan hepatopenaei* (EHP: 2016) (Shinn et al., 2018) and recently, Decapod Iridescent Virus-1 (DIV-1: 2014). The emergence of a new virus in a region has typically initially involved severe production losses followed by a gradual recovery and return to production volumes over successive years of aquaculture (Glanville et al., 2017). Despite the presence of many pathogens, global prawn production outside of Australia has continued to expand through three central approaches, namely:

1. The domestication of specific pathogen-free (SPF) broodstock.

2. Screening of broodstock and PLs to ensure the production of virus-free stock for grow-out.

3. Selective breeding of pathogen resistant/tolerant strains.

Domestication of the white-legged prawn has facilitated significant disease risk mitigation (FAO, 2018) and refers to the ability to culture the complete lifecycle of an organism within captivity (i.e. broodstock are spawned to produce progeny which grow into broodstock which are subsequently spawned to produce the next generation). Domestication, supported by molecular detection assays including quantitative polymerase chain reaction (qPCR), has facilitated the selective breeding of domesticated *L. vannamei* for the supply of specific pathogen free (SPF), specific pathogen resistant (SPR), or specific pathogen tolerant (SPT) stock for grow out production. Access to domesticated lines has led to many countries converting prawn aquaculture from local and endemic species such as *P. monodon, P. stylirostris, P. chinensis and P. indicus,* to that *L. vannamei.* Some countries have further reduced the risk of disease by converting from semi-closed pond to totally enclosed, biosecure facilities for prawn grow-out. Despite the benefits of domestication, disease remains a threat to production and aquatic animal health specialists have recognised the need to better understand the prawn-pathogen-environment interaction to effectively reduce the risk of disease outbreaks.

3.2 Australian Prawn Industry Overview

Australian prawn aquaculture is dominated by production of the black tiger prawn, *Penaeus monodon,* and to a smaller degree that of banana prawn, *Fenneropenaeus merguiensis.* The majority of prawn farming occurs in tropical regions of Queensland, although approximately one third of industry production is also derived from temperate locations in South East Queensland and Northern New South Wales. Prawns are the second largest sector of aquaculture production in

Northern Australia by value and volume (Cobcroft *et al.,* 2020). Figure 1 presents an overview of production of the northern Australian prawn farming industry. Despite research efforts into domestication of *P. monodon* since the 1980s, the Australian *P. monodon* farming industry does not have access to domesticated stock and the industry relies on the annual intake of thousands of wild-captured broodstock from the East Coast Queensland (EC) and Northern Territory (NT) fisheries. In 2018-2019 the wild-captured broodstock were transferred to the five commercial hatcheries that produced the 388 million post larvae ($0.9M) stocked across 20 farms to produce a total volume of 4630 T of prawns valued at $80.4M in the 2018-2019 season (Schofield, 2020) (Figure 1).
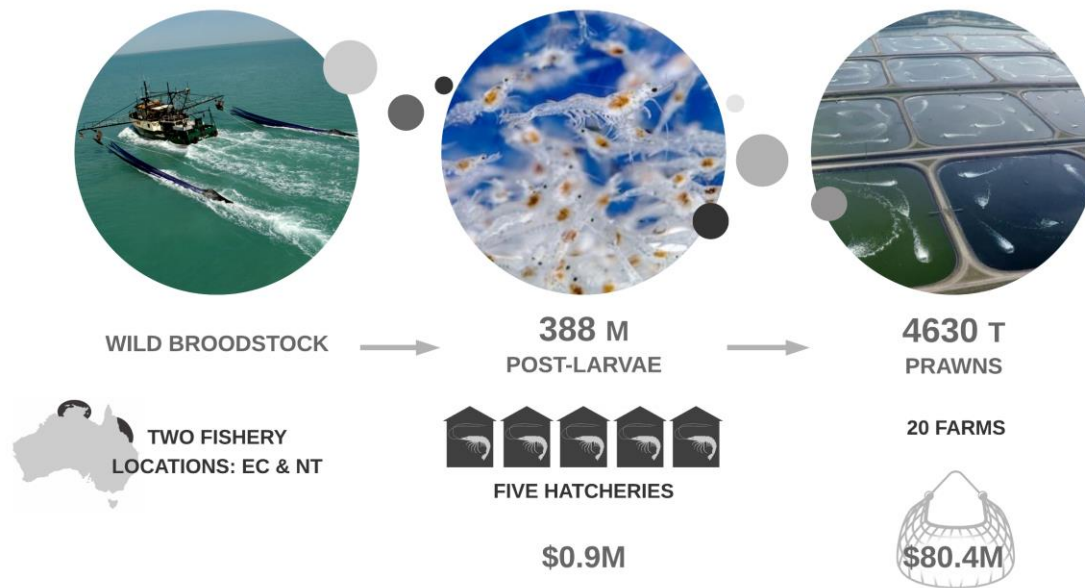
Figure 1 Overview of the Australian *P. monodon* production cycle 2018-2019. Data sourced from Schofield (2020).

During the 2018-2019 season, Australian prawn aquaculture production increased in value by 7.6 % (+ $5.7 M) (Schofield, 2020). Under currently planned expansions, the industry is forecast to produce a 4-fold increase to over 19 000 T per annum in coming years (Cobcroft *et al.*, 2020). There is consumer demand to support the expansion of the industry as domestic demand for prawns exceeds domestic supply. The import value of prawns during 2017-2018 was $484.6 M (https://www.agriculture.gov.au/abares/research-topics/fisheries/fisheries-and-aquaculture-statistics/trade-2018#imports-by-origin). The Australian prawn farming industry growth is forecast to occur despite suffering from a Whitespot syndrome virus (WSSV) disease outbreak for the first time in November, 2016 (OIE, 2016).

Until Summer 2016, Australia was one of the last remaining prawn-aquaculture producing countries to be free of WSSV (Glanville, 2017). WSSV causes Whitespot disease (WSD), which is one of the most serious diseases impacting global prawn aquaculture. Between 1992 -2012, the global economic cost of WSD was estimated to total US$15 Billion, accounting for annual losses of approximately 10% of global prawn production (Stentiford *et al.*, 2012). The WSD outbreak during the 2016/2017 season on prawn farms in South East QLD (SE QLD) affected seven prawn producing enterprises in the Logan River catchment and caused the destruction of prawn crops with an estimated value of ~$43 M (Scott-Orr *et al.*, 2017). The virus has since been detected in wild captured crustaceans from a region isolated to SE QLD in 2018 and 2020, and from two prawn farms in April, 2020 (OIE, 2020 & Oakey *et al.*, 2019). Ominously for the

Australian industry, in every prawn growing country following initial incursions of WSSV, the virus has typically spread and persisted to be an ever-present pathogen.

3.3   Biosecurity Obligations in Australian prawn farming

Biosecurity aims to prevent or mitigate losses incurred by disease outbreaks. Effective farm biosecurity will be required if the forecast industry expansions are to be successful. The majority of prawn farming conducted in Northern Australia occurs in Queensland and must comply with the QLD Biosecurity Act (2014). Under the Act, all Queenslanders have a general biosecurity obligation (GBO) for managing biosecurity risks that are under their control or that they should "reasonably be expected" to know about. All farmers, including prawn farmers are "expected to stay informed about pests and diseases that could affect or be carried by their animals and to manage those pests and diseases appropriately". Steps that are considered "reasonable and practical" vary but key considerations include:

- How likely an activity is to pose a risk: the more likely the risk, the more action is expected to be taken.

- How harmful the activity could be: cause human deaths or extensive productivity or economic losses.

- How much the person managing the activity knows or should reasonably be expected to know: about how dangerous it is and how it is spread.

- What methods are available to minimise the risk.

Sourced from: Queensland Government. Department of Agriculture and Fisheries website. Refer to the attached link: https://www.daf.qld.gov.au/business-priorities/biosecurity/policy-legislation-regulation/biosecurity-act-2014/general-biosecurity-obligation (accessed 21 June 2020).

3.3.1   Biosecurity guidelines to the Australian prawn farming industry

The Australian Prawn Farmers Association (APFA) commissioned the preparation of "National Biosecurity Guidelines for Australian Prawn Farms". The guidelines support individual farms to prepare systems to meet their GBO's and represent "best industry practice". The guidelines include basic principles in biosecurity; template documents for the preparation of farm biosecurity plans, and risk analysis matrices with recommended management actions aligning to risk assessment. The steps to completing a risk assessment and developing a biosecurity plan are illustrated in Figure 2.
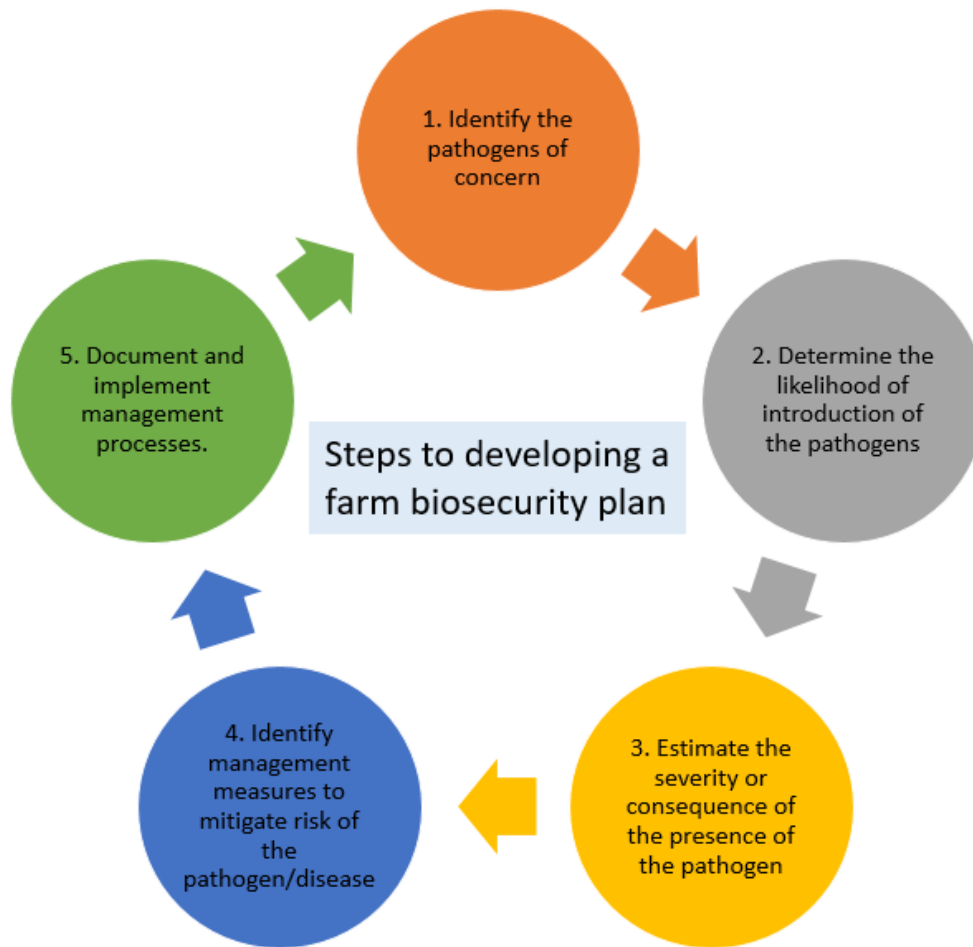
Figure 2 Diagram indicating the steps to develop a farm biosecurity plan based on determination of likelihood and consequence of the introduction of a pathogen into the farm system.

Currently, there is insufficient data available for Australian prawn farmers to:

1. Identify the pathogens of concern;

2. Assess the likelihood of introduction of a pathogen;

3. Determine the consequence of the presence of the pathogen;

4. Identify and implement processes to mitigate the risk of the pathogen.

There is a need to improve the understanding of pathogen presence, distribution, impact and management in prawn farming to support the sustainable production and expansion of the Australian prawn farming industry and enable Australian prawn farmers in meeting their GBOs.

Pre-existing data to support the formulation of evidence-based biosecurity plans in Australian prawn farms

3.4.1 Likelihood of introduction of Endemic Pathogens in Australian prawn aquaculture

2. Determine the likelihood of introduction of the pathogens

Compared to global prawn aquaculture, prior to the WSSV disease events, the Australian prawn farming industry had reported few disease issues. Australia is free of the majority of pathogens that cause substantial impact on global prawn production including YHV-1, TSV, IMNV, EHP and DIV-1 (Australian Government, 2020). Detection by genetic analysis, applying PCR and/or quantitative PCR (qPCR), is recommended for the surveillance and diagnosis of prawn pathogens and diseases by The World Organisation for Animal Health (OIE) (OIE, 2019). However, only a small number of reports have applied genetic analysis to determine the relative presence of endemic prawn pathogens from Australian-sourced penaeids, in the absence of disease (OIE 2008; Moody *et al.*, 2011; Condon and Bochow, 2015; Cowley *et al.*, 2015) (Table 1). The detection of IHHNV at a prevalence ranging from 3.3% to 5.3% from pond-collected samples at two QLD farms (n= 150 samples each site), in the absence of disease, was first reported to the OIE in 2008 (OIE notification reference 7166). An IHHNV specific surveillance conducted on prawn farms in Queensland detected IHHNV at 1.39% and 5.33% prevalence from a total of 1298 samples (Moody *et al.*, 2011). In 2015, the prevalence of GAV (43%) and YHV-7 (6.5%) was reported from wild-sourced broodstock collected from Northern Territory, Gulf of Carpentaria and North Eastern Queensland (n=493) (Cowley *et al.* 2015). Applying non-quantitative PCR analysis, the same study also detected IHHNV (6%), MBV (1%) and HDV (7.9%) in a low percentage of samples (n=337) (Cowley *et al.*, 2015). Following Hepatopancreatitis disease incidents reported to the OIE, associated with an AHPND-like Pir-AB detection (OIE, 2016), a survey applying qPCR to pond-collected samples (n=980) from 13 Queensland prawn farms did not detect Pir-A (Condon & Bochow, 2016).

Table 1 Summary of positive detections of viral targets applying PCR from Australian sampled *P. monodon*

Pathogen/Disease	Summary of Source of sample: Positive detection (%) and number of samples (n=)	Reference
Monodon Baculovirus (MBV)	Broodstock: 0.8% detection (n=337) noting pleopod samples are not target tissue for MBV	Cowley et al., 2015
IHHNV	Ponds: 1.39% to 5.3% in ponds (n=1298 samples) no disease Broodstock: 6% positive (n=337)	Moody et al., 2008 Cowley et al., 2015
Spawner Mortality Virus (SMV)	Broodstock: 1.1% (n=337).	Cowley et al., 2015
Hepatopancreatic Necrosis Virus (HPV)	Broodstock: 11 % (n=33)7 noting pleopod samples are not target tissue for HPV/HDV	Cowley et al., 2015
Gill Associated Virus (GAV)	Broodstock 43% (n= 493)	Cowley et al., 2015
Yellowhead Virus-7 (YHV-7)	Broodstock 6.5% (n=493)	Cowley et al., 2015
Hepatopancreatitis (Pir-AB)	No published percentage of positive detections in detection. Surveillance in 2016: 0% detection (n=980) from 13 farms.	Condon and Bochow, 2016
Whitespot syndrome Virus (WSSV)	Mass mortality and pre-emptive destruction of stock from seven *P. monodon* farms in SE Queensland.	Glanville et al., 2017

3.4.2 Consequence of the presence of Endemic Pathogens in Australian prawn aquaculture

Whilst, the impacts of an introduction of YHV-1, WSSV or other exotic pathogens into Australian prawn farms would be substantial, there is uncertainty in identifying which endemic pathogens pose a risk for Australian prawn farming operations. Historically, the endemic pathogens have been associated with disease outbreaks rather than demonstrated to cause mortality. The detection of a pathogen does not demonstrate the pathogen is the cause of the disease outbreak, but rather is present during the disease outbreak. All of the pathogens reported to impact on production in Australian prawn farms have been associated with disease incidents, yet, have also conversely been detected as chronic infections in healthy prawns (Elliot and Owens, 2015; Sellars *et al*., 2019, Moody *et al.,* 2017).

In some cases, mortality has been induced in experimental challenges using crude viral extracts that are not characterised to identify the specific genetic strain of the target pathogen and that could also contain multiple non-target, yet potentially virulent, pathogens. The absence of specifically characterised pathogens with demonstrated virulence has contributed to a degree of ambiguity of the impacts of pathogens in Australian prawn farming. Based on reports in the public domain, the list of pathogens historically associated with disease in Australian prawn farms includes HDV, MBV, GAV, IHHNV, YHV-7, Vibriosis and WSSV (Table 2).

Table 2 Summary of pathogens or disease reported from Australian farmed Penaeid species

Year	Pathogen/Disease	Description of Report	Reference
1987	MBV	*Penaeus esculentus* mortality in post larval stages	Doubrovsky et al., 1988
1991	IHHNV	Mass mortality approaching 100% in *P. monodon* x P. *esculentu*s (Australian Institute of Marine Science) domestication research program.	Owens et al., 1992
1991	Vibriosis	Mass Mortality in hatcheries	Muir, 1991
1994	Midcrop mortality syndrome (MCMS)	80% mortality in affected ponds. Demonstrated to be an infectious agent. Gill Associated Virus and Spawner mortality virus were both proposed as causes. Separate research groups (CSIRO & JCU) each report experimental challenges conducted with crude viral extracts that are positive for the detection of GAV or SMV respectively lead to mortality.	Owens et al., 1992; Spann et al., 1992
1997	HPV	In *Penaeus japonicus*	Spann et al., 1997
2008	IHHNV	Detected in Australian farmed prawns, no disease outbreaks described.	OIE, 2008
2011	IHHNV	Detected in pond collected samples 1.39-5.33% a percentage of positive detections no disease	Moody et al., 2011
2012	Yellowhead Virus-7 (YHV-7)	Detected in broodstock associated with significant but undefined mortality.	Cowley et al., 2019
2015	Hepatopancreatitis	Pir-AB toxins in Vibrio. Up to 90% mortality from 3 farms: Bundaberg and Cardwell QLD. Experimental challenge with Bundaberg strains induced 100% mortality. Cardwell strain variable mortality.	OIE, 2016; Moody, 2018
2016	WSSV	Mass mortality and pre-emptive destruction of stock from seven *P. monodon* farms in SE Queensland.	Glanville et al., 2017
2017	Yellowhead Virus-7 (YHV-7)	Farms displaying characteristics typical of MCMS. Experimental challenge: mortality in *P. monodon* with crude viral extract. High loads of YHV-7 detected. GAV loads not significantly different from control animals that did not display mortality.	Cowley et al., 2019; Moody et al., 2019

Under Aquaculture Licensing regulations, farms are required to notify Biosecurity Queensland of any disease outbreaks. However, pond mortality incidents can also be reasonably attributed to harmful algal blooms rather than infectious pathogens. When incidents are attributed to a pathogen, unless the disease is caused by a notifiable (typically exotic) pathogen, incidents that occur on farms are not publicly disclosed. Furthermore, pathogens can impact on farm production by causing chronic effects such as low-grade mortality, reduced growth rate, delayed stocking, or decreasing the visual appeal and farm-gate price of the crop. Sub-optimal water temperature and salinity can also lead to low grade mortality and reduced growth rate. Sellars *et al.* (2019) presented the only study investigating the impact of IHHNV on *P. monodon* production in Australian ponds. Sellars *et al.* (2019) noted an association between higher mean IHHNV detection (copies/ug TNA) and reduced growth after 120, 140 and 155 days of culture in *P. monodon* reared in experimental ponds. However, similar to other studies reporting an impact of IHHNV on *P. monodon,* there was not an experimental challenge with IHHNV; the study did not determine the strain/s of IHHNV detected, and the study did not exclude the involvement of the range of other pathogens that naturally infect *P. monodon* in Australia or the range of pathogens that have been associated with slow growth in *P. monodon*.

The ambiguity in recognising and reporting disease incidents and defining chronic impacts of pathogens in prawn farms contributes to the absence of industry-wide information available to estimate the consequence of an endemic pathogen infection, or frequency of disease outbreaks across industry. Annual prawn aquaculture production statistics (by volume) for Queensland published within the Lobegeiger report and Australian Bureau of Agricultural and Resource Economics (ABARES) indicate the Queensland prawn aquaculture industry has averaged annual increased volume in production of 9% ± 19% (mean ± SD) since the 1991-92 season to the most recent 2018-19 season (Figure 3). However, since the peak in production of 5,115 t in 2009-2010, Queensland prawn aquaculture production volumes have varied. Seven of the last 9 years since 2009-10 have had reported decreased volume compared to the previous season (Figure 3). Although a range of factors could impact on total industry production, reported disease events coincide with four of the seven less productive seasons (Figure 3).
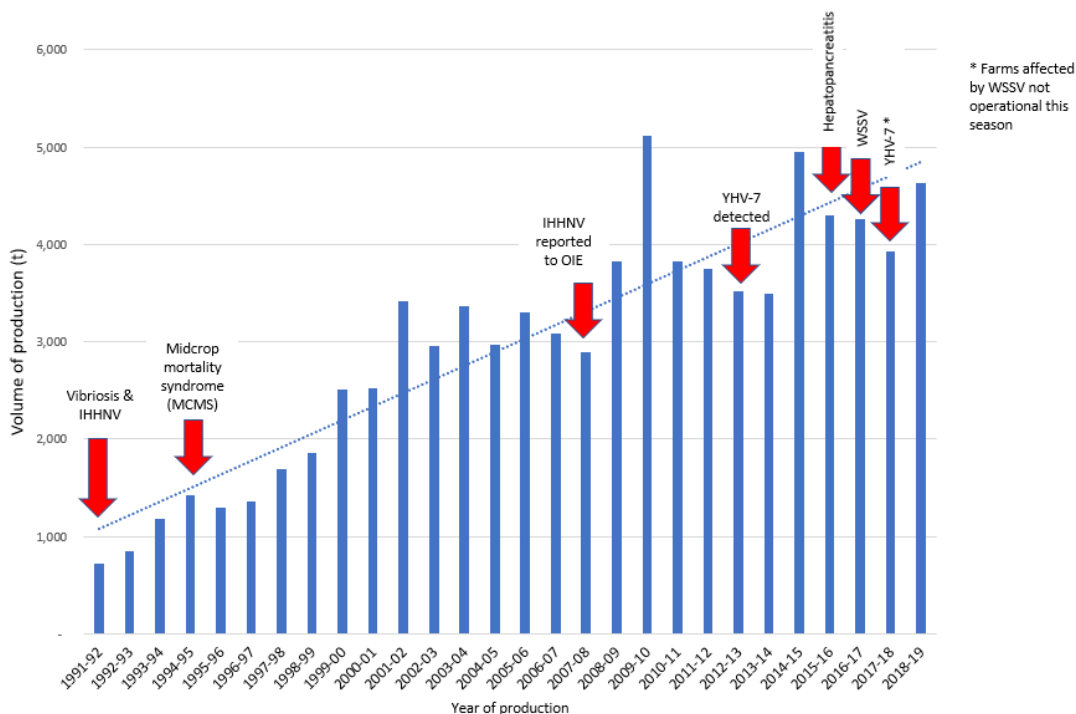
Figure 3 Annual volume (t) of Queensland prawn aquaculture production including indication of reports of prawn disease from QLD farms. (Production data sourced: ABARES and QDAFF Lobegeiger reports; Pathogen data sourced Table 2)

3.5 Principles of Biosecurity Management

There are four broad approaches to biosecurity management, namely, exclusion, eradication, containment and asset-based protection (SCAAH, 2016). Different approaches are applied based on the prevalence and severity of the pathogen, the availability of treatments, the geographic distribution of the pathogen and economic consequences of the pathogen becoming established. Figure 4 outlines the characteristics and the prawn pathogens that align with each biosecurity approach in Australia. The approaches can be applied at national, state, regional and farm level.
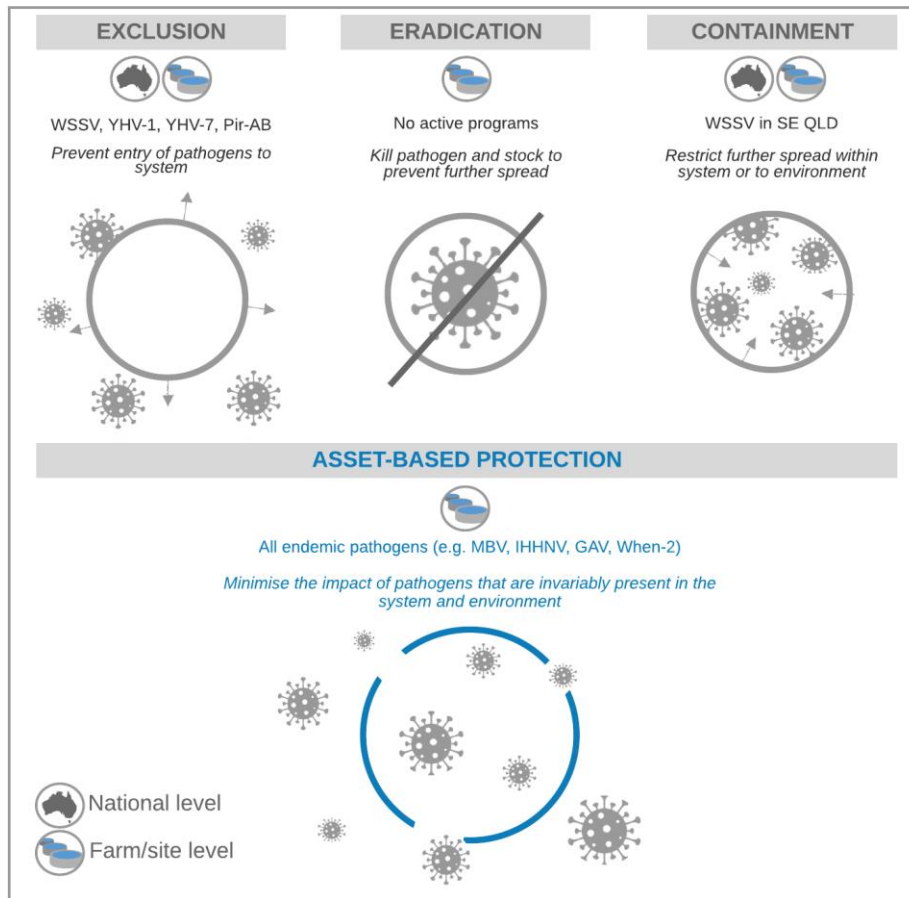
Figure 4 Different approaches of Biosecurity management of a pathogen indicating level of exclusion, eradication and containment varies with the prevalence and impact of the pathogen. Current approaches in Australia include exclusion of WSSV and YHV-1 at National and Industry level, exclusion of YHV-7 at NSW State level, exclusion of Pir-AB at industry level and containment of WSSV in SE Queensland.

3.5.1 Exclusion

Exclusion involves actions to actively prevent the entry of a pathogen into an area known to be free of the pathogen. Exclusion is applied to the pathogens that cause the most severe mortalities and economic losses. Exclusion is more achievable if a pathogen is not highly prevalent, or the culture system is contained within biosecure facilities. Exclusion is applied at National, State and individual enterprise level to prevent the entry of WSSV and YHV-1 into the Australian environment and prawn farming enterprises.

Government approaches to exclusion of prawn pathogens

Australia operates on a national "exclusion" approach for WSSV and exotic pathogens that are not currently present in Australia. Historically the global transfer of prawn pathogens, WSSV, IHHNV, TSV and YHV has been linked with the transfer of live crustaceans (Jones *et al.,* 2012). The detection and experimental transmission of viruses from imported uncooked frozen commodity prawns has also been demonstrated (Nunan *et al.*, 1998; Durand *et al.*, 2000; McColl *et al.,* 2004 and Scott-Orr *et al.,* 2017). The Australian government bans the import of live crustaceans and defines the import conditions for uncooked commodity prawns. The import conditions are devised based on the Import Risk Assessment

(IRA) which accepts an "appropriate level of protection" (ALOP) to prevent the entry of viable pathogens in imported crustaceans. The current ALOP is "very-low, but not zero". Testing of imported prawn consignments to confirm the ALOP adopts a surveillance regime that will detect a 5% prevalence with 95% confidence (https://www.agriculture.gov.au/biosecurity/risk-analysis/memos/ba2017-12#5-review). Following the WSSV incidents of 2016/17, which have been proposed to occur through the import of WSSV infected uncooked prawns (Glanville, 2017), the IRA for frozen uncooked commodity prawns was submitted to review (Biosecurity Advice 2017/07). While the IRA review is in progress, testing conditions for the detection of WSSV and YHV-1 are applied to 100% of imported uncooked frozen prawn consignments. Public submissions to the IRA review noted the emergence of a number of pathogens that were not currently included in the IRA, despite causing significant economic impact to current overseas prawn production (Diggles, 2018). An Animal Biosecurity Advice (2020-A03) issued 14th May 2020 recognised the risk of import of *Enterocytozoan hepatopenaei* (EHP) was greater than the ALOP and detailed an upgraded protocol stating, in addition to testing to confirm freedom of WSSV and YHV-1 at country of origin and on arrival in Australia:

"uncooked prawns arriving in Australia on or after 1 July 2020 must be certified by the competent authority to have undergone a deveining step during processing." (Cookson, 2020).

Within Australia, surveillance for the detection of WSSV is conducted by government and industry. The Australian Government Department of Agriculture has undertaken a two-year national surveillance program of crustaceans to determine the distribution of WSSV. The details of the National surveillance program are not publicly available, however, to date, all samples that have been collected and tested within States and Territories excluding QLD have returned negative results (https://www.outbreak.gov.au/current-responses-to-outbreaks/white-spot-disease). The Queensland component of the surveillance program reported the detection of WSSV from wild crustaceans from a region in northern Moreton Bay in 2018 and 2020 and from two prawn farms on the Logan River in April, 2020. Refer to the interactive map:

https://www.daf.qld.gov.au/business-priorities/biosecurity/animal-biosecurity-welfare/animal-health-pests-diseases/a-z-list-of-significant-animal-pests-and-diseases/white-spot-disease/white-spot-disease-surveillance

Farm level approaches to exclusion

WSSV, YHV-1 and AHPND are the only pathogens that are actively managed industry-wide by exclusion at farm level in Northern Australia. Since the WSSV incidents of 2016, the Northern Australian prawn farming industry has operated under increased biosecurity to prevent the entry of WSSV, YHV-1 and AHPND through the screening of all wild caught broodstock translocated into QLD and NSW prawn hatcheries. Analysis of faecal samples to detect the Pir-AB toxin associated with AHPND occurs for each batch of broodstock. The detection of the Pir-AB toxin gene does not constitute the definition of AHPND, hence detection of Pir-AB via translocation testing is not publicly reported. If the Pir-AB toxin genes are detected, due to the disease being associated with a *Vibrio* bacterial host, farms can exclude the pathogen by culling or eradicate with anti-microbial treatments. There are no treatment options for viral pathogens of prawns. Protocols to translocate wild caught broodstock to hatcheries (FAMPR001: Queensland Government), also require analysis by qPCR on a pleopod tissue sample from every broodstock to ensure freedom of detection of WSSV and YHV-1. There have been no notifications of detection of WSSV or YHV-1 in wild- captured broodstock. Arbon *et al.* (in review) reported zero detection of WSSV and YHV-1 from 3,882 wild captured broodstock collected from Australian hatcheries from August 2018-November 2019.

Some hatcheries actively screen and exclude pathogens beyond those required under FAMPR001. Exclusion of YHV-7, Pir-AB, and reduction of IHHNV, are the most frequently adopted biosecurity management approaches. However, there is no uniform approach across industry and estimating the costs and likelihood of exclusion of endemic pathogens is hindered by the limited data available about their relative presence, distribution and impact on productivity. The National

Biosecurity Guidelines for Australian Prawn Farms advise screening for the presence of WSSV, YHV-1, AHPND and Infectious myonecrosis virus (IMNV), and provide no recommendations for managing any of the pathogens that are endemic to Australia. While the aforementioned instances of reduced annual industry output by volume could result from a range of scenarios, impacts of endemic pathogens would be reflected in annual production. Endemic pathogens that are found to be present in a low percentage of positive detections could reasonably and cost effectively, be managed by exclusion; however, there is little data available to industry to indicate which pathogens might be present in low prevalence.

3.5.2 Eradication

Government and Industry-wide approach

After a pathogen enters a system, there is a short time frame for destruction of the pathogen and infected carrier organisms (cultured and non-cultured species). Eradication was attempted by the Queensland Government when WSSV was detected in the prawn farms on the Logan River in QLD in 2016. However, considering the wide host range of WSSV, eradication was unlikely to be successful (Glanville *et al.,* 2017). The subsequent detection of WSSV in wild crustaceans from an area in northern Moreton Bay indicates eradication is no longer an option to manage WSSV at a national level.

Risks to the success of eradication of a pathogen include:

- Time to diagnosis,

- Engineering capability to achieve containment,

- Time to enact eradication process,

- Capacity to implement eradication: access to sufficient knowledge, workforce and chemicals to complete eradication.

Time to diagnosis and enact containment is delayed when investigating mortalities in prawn farming operations. Delays in diagnosis occur because some mortality is expected in prawn farming operations, so disease investigations are not typically enacted until more significant mortalities are observed. When disease investigations are instigated, the delays in time to diagnosis can be inhibitory to eradication or other biosecurity approaches. During the WSSV incidents in SE QLD, samples for genetic testing were collected on Infected Property 1 (IP1) on Thursday 22nd and 25th November 2016 and disease testing occurred on the Wednesday 30th November (OIE, 2016 Ref 21737). The delay between mortality and diagnosis of WSSV prevented a rapid containment and eradication of WSSV within the index property.

Diagnosis of a notifiable disease such as WSSV, YHV-1, IMNV, TSV, EHP, DIV-1, or any other exotic notifiable pathogen is restricted in Queensland to the activities of the Biosecurity Sciences Laboratory (BSL) by the QLD Biosecurity Act. Although prawn farms in SE QLD are located within 50 km (45 min drive) to the Biosecurity Sciences Laboratory (BSL), farms outside SE QLD are considerably distanced from BSL and samples may be in transit for 48[+] hours from regional locations. Under extreme weather events such as cyclones or floods, which are forecast to increase in frequency and intensity with global climate change, and the current COVID-19 environment, freight delivery to/from regional areas to metropolitan Brisbane or interstate is often delayed. The reliance of industry on an insecure freight network for access to the sole approved provider of testing of notifiable exotic pathogens in response to on-farm mortality presents a significant impediment to rapid diagnosis, response and eradication of an exotic disease agent from a farm located in regional Northern Australia. Other laboratories in close proximity to industry, are not authorised to conduct analysis on notifiable agents to investigate disease outbreak, despite having the required capability and quality control

systems of NATA accreditation. The establishment of a regionally located laboratory for the detection of aquatic pathogens in real time was recommended in the "Scaling up, Joint Select Parliamentary Committee on northern Australia: Inquiry into opportunities for expanding aquaculture in northern Australia (2016)", yet government policy prevents the application of the laboratory capability to rapidly detect an exotic pathogen from a disease incident on farm.

Individual farm approach

The design and operational protocols of prawn hatcheries are regulated and allow for detection, containment, destruction of stock and disinfection (eradication) of facilities if a notifiable pathogen is detected within any enterprise system. Although difficult, eradication is a possible management response to disease at the farm level. The semi-open design of prawn grow-out ponds make containment, destruction of stock and disinfection of facilities more challenging. Significant volumes of chemicals are required that are not readily available in regional locations and are also reliant on the aforementioned freight network. Despite the difficulties, the absence of recurrent disease incidents such as Hepatopancreatitis/*Penaeus monodon* mortality syndrome (PMMS) (OIE, 2016) or YHV-7 (Cowley *et al.*, 2016) from historically affected properties indicates farm-level eradication is possible, dependant on the pathogen, level of preparedness, and speed of enacted response by affected farms.

3.5.3 Containment

Containment involves the zoning and restricted movement of materials to prevent the spread/transport of a pathogen to an area beyond its current distribution. While containment can be effectively implemented in terrestrial agriculture, or potentially in a farm-based scenario, limiting the spread of an aquatic pathogen is very difficult once a pathogen has entered natural water bodies or infected wild host animals. The Queensland government imposed a White spot disease movement restriction area to prevent the movement of uncooked high risk-animals such as prawns, yabbies and marine worms outside of the restriction zone.

Refer to the attached link detailing the WSSV restricted movement zone:

https://www.daf.qld.gov.au/business-priorities/biosecurity/animal-biosecurity-welfare/animal-health-pests-diseases/a-z-list-of-significant-animal-pests-and-diseases/white-spot-disease

Whilst restrictions on movement will slow the spread of WSSV by human economic activity, the natural dispersion of WSSV via wild animal movement and sea currents cannot be prevented. Crabs and birds were particularly problematic in the management and destruction of WSSV infected stock in SE QLD. The possible spread of WSSV by birds was indicated when WSSV was detected on the last farm (Infected Property-7) to become infected in the Logan River catchment on 11th February 2017 despite the farm ceasing any water intake after December 2016 (OIE, 2017: ref 22856 and farmer's personal communication).

At hatchery and farm level, containment is actively practised by separation of facilities into different rooms, segregation of equipment and/or workers, compartmentalised management of groups of ponds and a flow of movement from an area of "lowest risk" to highest risk. However, the semi-closed nature of prawn pond culture limits the ability to contain pathogens that are able to infect multiple species that may be in the environment within the vicinity of a disease-affected property. Detection of WSSV from wild mudcrab *Scylla serrata*, and *P. monodon*, *P. esculentus* and *Metapenaeus bennettae* in close proximity to infected properties was reported during the period of attempted eradication of WSSV in SE QLD (OIE, 2017: reports 22770, 22953 and 22856).

3.5.4 Asset-based protection

Asset-based protection refers to management processes that aim to limit losses when a pathogen has become established in an area. The majority of prawn farms have varying degrees of biosecurity practises to achieve asset-based protection. Different farms have different acceptable levels of protection (ALOP) to manage/prevent disease outbreaks caused by the prawn pathogens known to be endemic in Australia. Farms that have historically experienced significant disease outbreaks tend to accept a lower level of risk in farm biosecurity plans. Effective asset-based protection requires investment in an array of operational processes such as settlement and water treatment ponds, reduced stocking density and implementation of nursery ponds. Implementation of the full range of biosecurity processes requires a significant capital investment. Prioritisation of investment to biosecurity is often downgraded because:

- Farmers consider their facility to be at low risk of a disease outbreak,

- Farmers estimate the cost of sporadic disease outbreaks is less than the cost to implement thorough preventative actions,

- In the absence of evidence and data, there is a degree of uncertainty about the efficacy of the preventative actions,

- The costs of the most effective biosecurity measures are beyond the farm operational budget over the short term.

Improving biosecurity can lead to unintended consequences which impact on investment. Whilst improved biosecurity options should reduce risk and increase confidence in investment, this does not always occur. The Seafarms Group Ltd ASX share price dropped from 0.09 to 0.08 AUD during 17-21st October after the company improved biosecurity through the construction of two intake settlement ponds (circa AUD $1 million) from 9 production ponds on 21st October 2019 (Seafarms, 2019). Nonetheless, industry considers improving biosecurity and animal health a research priority and many farms have progressively implemented improvements to farm infrastructure and operations to strengthen biosecurity. Seafarms Group was awarded the Department of Agriculture: 2020 Farm Biosecurity Producer of the Year Award.

(https://www.agriculture.gov.au/biosecurity/australia/public-awareness/aba/aba-booklet)

3.6 Systems approach to preventing disease outbreaks

Although the exclusion of a pathogen can prevent a disease outbreak, infection with a pathogen does not inevitably lead to a disease outbreak. With the exception of highly virulent pathogens, disease outbreaks tend to occur when there is an alignment of factors that reduce the health of cultured animals with factors that favour the proliferation of a pathogen. In the absence of exclusion, eradication and containment, farm management practises to prevent disease outbreaks must understand and manage the multi-component interaction between the pathogen, the host (prawn) and the environment. (Figure 5). Approaches to prevent disease outbreaks require a strong understanding of the three component contributors to disease or other impacts on production.

BIOTIC ENVIRONMENT
Other crustaceans
Non-beneficial microbes
Harmful algal species
Beneficial microbes
Algal bloom

ABIOTIC ENVIRONMENT
Salinity
Temperature
pH
Organic load
Dissolved oxygen
Toxic Nitrogen
Phosphorous
Drought/Rain
El Nino/ El Nina
Carbon sources
Season

HOST
Age
Sex
Genetic line
Moult stage
Growth rate
Immune status
Microbiome
Density stocked
Nutritional status
Development stage

DISEASE OUTBREAK

PATHOGEN
Strain/Type
Numbers/Density
Other pathogens
History of association with host

Figure 5 Diagram illustrating the interaction of pathogen-host and environmental conditions that contribute to disease outbreaks in prawn farms

3.6.1 Pathogen component

Although disease outbreaks can be induced if a particularly destructive or virulent/strain of a pathogen/s are present, there are very few pathogens that will induce a disease outbreak irrespective of host or environmental factors. With the exception of WSSV, none of the pathogens identified from Australian *P. monodon* are ubiquitously associated with disease outbreaks in farmed Australian prawns (Elliot and Owens, 2015; Cowley, 2015; Moody, 2008; Sellars *et al.*, 2018). Hepatopancreatic Necrosis Virus, *Monodon* Baculovirus, IHHNV, GAV, YHV-7, WSSV and more recently Wenzhou shrimp Virus-2 have been detected in Australian penaeid prawns in the absence of disease incidents. Multiple strains exist for the majority of pathogens and although strain variation is known to impact on viral pathogenicity, few researchers investigating prawn viruses identify the specific strain of virus involved.

Different levels of pathogenicity occur within the Yellowhead complex of viruses, which includes YHV-1, YHV-7 and GAV, the latter two being endemic in Australian prawns. There have been no studies investigating variable virulence between GAV strains. There are only three complete genome sequences (genome length = 26253 nt) of GAV in the NCBI database. Other GAV sequences range from 1203 nucleotide (nt), 662 nt, or 231 nt, which represent between 4.5-0.8% of the complete genome. Australia is the only country to have reported disease in association with GAV, despite records of GAV detection from Thailand, Philippines, Indonesia, India, Mozambique, Vietnam and Taiwan (NCBI

accessed 21/6/20). Some recognition of strain variation has been indicated for IHHNV (Dhar, 2010). A significant variation in the genome sequence of IHHNV in prawn populations in the Gulf of California has been detected and the monitoring of genetic variance in IHHNV was recommended because slight changes in genome sequence can lead to increased virulence of viral pathogens (Dhar, 2020). The specific identification and monitoring of the more virulent strains of viruses is required to support targeted management against the most problematic pathogens. Whilst there are assays that detect different strains of virus within the Yellowhead complex (e.g. YHV-1, YHV-7, GAV, YHV-6), there are not targeted assays that can differentiate between strains of GAV or IHHNV. As these pathogens are specifically removed in overseas SPF programs, there has been no attempts to develop differential tools to identify potentially more virulent strains for any of the prawn viruses.

Purification of viruses is required to robustly demonstrate a causal relationship between pathogen and disease. Few studies have completed experimental challenges using purified viruses. Notably when purification has not been performed, few studies acknowledge the possibility of other viruses. Owens and Anderson (2001) are one of few researchers to note the use of a crude viral extract does not provide definitive evidence of the pathogenicity of an agent. Although Owens and Anderson (2001) demonstrated infectivity of an experimental challenge inoculum, they retained the terms Mid-Crop Mortality syndrome and acknowledged the presence of multiple viruses, including Spawner mortality virus and Gill Associated Virus, which were present in the experimental challenge extract. Few other studies have presented this potential ambiguity, with many subsequent studies building upon the ambiguity of initial studies.

3.6.2 Prawn host component

There is variance in susceptibility to pathogens between species and between individuals within the same species. Although domestication was a major factor contributing to the majority of global prawn production converting from *P. stylirostris, P. indicus, P. japonicus* and *P. monodon*, the reduced susceptibility of *L. vannamei* to viral infections was also a contributing factor in the shift towards production of this later species. Similar difference in interspecies susceptibility to disease occurs in Australian prawn species. Moody (2017) reported 90% mortality in *P. monodon* during an experimental challenge with YHV-7. Banana prawns, *F. merguiensis*, exposed to the same viral challenge experienced limited mortalities despite having a similar viral copy number detected by qPCR (Moody, 2019).

Within species, host-related factors including variance in genes such as heat shock protein 70 or 40 (HSp70/ Hsp40), nutritional status, moult stage, developmental stage (larval, juvenile and broodstock) spawning Component (pre or post spawn) and ablation status (pre or post ablation) all contribute to the outcome following exposure to a pathogen (Figure 3). Although genetic and biological prawn factors such as age, moult cycle, genetic variants and nutritional status are known to contribute to disease outbreaks, there is an absence of the genetic resources to investigate the critical biological factors in *P. monodon.* In the absence of genetic tools, selection relies on selective experimental challenge (selective breeding) and culture experiments which take many years to produce a second and third generation of offspring (Huang, 2019). Some researchers report selectively bred individual genetic lines of *P. monodon* that are resistant to WSSV (Huang, 2019). In an Australian specific study, heritability of survival to GAV infection (0.11 ± 0.03) was reported in *P. monodon* following experimental challenge (Noble *et al.*, 2019). The same authors noted heritability of natural GAV infection (0.06 ± 0.03) on-farm were not correlated with laboratory-based GAV resistance traits determined from experimental challenge studies and suggested GAV infection and GAV mortality may involve different immune defence mechanisms such as resistance to viral infection and tolerance of environmental stressors (Noble *et al.,* 2020). However, the experimental GAV inoculum was not purified and the naturally infected prawns were not assessed for a broad range of pathogens potentially confounding relatively sensitive genetic parameter analysis. Genetic resistance in *P. monodon* has not been investigated for any of the other pathogens considered endemic to Australian.

3.6.3 Environment component

Pathogens and prawns are each affected by environmental conditions and many conditions that are sub-optimal for prawns are conversely associated with increased mortality in the presence of particular pathogens (Table 3). Environmental stress, caused by osmotic and temperature stresses, can result in viral epizootics (Elliot and Owens, 2015). If pathogens are present in the culture system, there is a need to manage the biotic (living) and abiotic (non-living) environmental factors to maximise host health and reduce the incidence of disease (Elliot and Owens, 2015) (Figure 3). Although the multicomponent nature of disease expression is well established, there are few published studies that adopt a multicomponent approach (Elliot and Owens, 2015). The vast majority of disease studies have focussed on experimental challenges testing a range of concentrations of a single pathogen. Consequently, farm environmental factors that contribute to disease outbreaks are not well documented. Lower water temperature has been demonstrated to increase mortality following exposure to WSSV and YHV-7 (Rahman *et al.,* 2007 Moody *et al.,* 2019). Similarly, average copy of IHHNV per 50 ng of *L. vannamei* DNA increased in prawns held in cool water (24.4 ± 0.5 °C) compared to warm water (32.8 ± 1.0°C) (Montgomery-Brock *et al.,* 2007). Millard *et al.,* (2020) specifically identified knowledge gaps relating to the influence of temperature, salinity, DO, pH and pCO_2 and Nitrogen as critical to improving the understanding of penaeid prawn physiology and susceptibility to WSSV. A machine learning (ML) approach has recently been applied to identify the combination of factors that contribute to AHPND (Khiem *et al.*, 2020). Indeed, this later study highlights the considerable scope for the application of ML when applied to environmental data to improve the understanding of disease outbreaks.

Table 3 Culture conditions that are optimal for the production of *P. monodon* and factors that are documented to increase mortality in *P. monodon* in the presence of pathogens.

Parameter	Species investigated	Detrimental condition	Minimum ideal condition	Pathogen
P.monodon studies of optimised growth				
Dissolved oxygen	*P.monodon*	< 4 ppm	4 ppm	no pathogen
Nitrite Levels	*P.monodon*	>1.28 mg/L	<1.28 mg/L	no pathogen
Un-ionised ammonia	*P. monodon*	> 0.01 mg/L	< 0.01 mg/L NH3-N	no pathogen
pH	*P.monodon*		7.8	no pathogen
Salinity	*P.monodon*		15-25 ‰	no pathogen
Temperature	*P.monodon*		24-34°C	no pathogen
Acute Hepatopancreatic Necrosis Disease				
pH	*prawns*	8.7-9.0		AHPND
Temperature	*prawns*	> 30.5°C	25-27 °C	AHPND
Salinity	*prawns*	25 ‰	5 ‰	AHPND
Gill Associated Virus/ Yellow head virus complex				
Days in pond	*P.monodon*		<113 days	GAV
Dissolved oxygen	*P.monodon*	<4.5 ppm	> 4.5 ppm	GAV
pH	*P.monodon*	<7.5	Overnight 7.6–7.8	GAV
Salinity	*P.monodon*		33–38 ‰	GAV
Temperature	*P.monodon*	> 29 °C	<29 °C	GAV
Temperature	*P.monodon*	25°C (~60% mortality)	30°C (10-30% mortality)	YHV-7
Whitespot syndrome Virus				
Alkalinity	prawns	9.7		WSSV
Dissolved oxygen	*P.monodon*	as low as 0.8 ppm		WSSV
pH	prawns	6.5		WSSV
Salinity	*P.monodon*	< 15 ppm		WSSV
Temperature	*P.monodon*	25°C	32-33°C	WSSV
Other agents				
Days in pond	*P.monodon*	110-120 days		MCMS
Secchi	*P.monodon*		<20.5 cm	MCMS
Temperature	*P.monodon*	Shift from 20-21°C to 27°C	20-21°C no mortality	MCMS
Temperature	*L.vannamei*	24°C	32-33°C	IHHNV
Bacterial disease (excluding AHPND)				
Salinity	*P.monodon*	5‰, 15‰ and 35‰	25 ‰	*P. damselae subsp. damselae*
Salinity	*P.monodon*	35‰		*V. harveyi*
Temperature	*P.monodon*	28-32 °C		*V. harveyi*
Dissolved oxygen	*P.stylirostris*	1 mg/mL		*V. alginolyticus*

Although hatchery systems are highly biosecure and containable facilities, pond systems are less insulated from surrounding environmental conditions. Managing environmental conditions of water temperature and salinity is particularly difficult in pond grow out systems. The majority of prawn farmers located in Northern Australia face the annual challenge of managing the crop under highest density stocking during Summer, which is the period of most active growth, favourable market demand and market price, yet is also the time of year with the most volatile weather conditions. Conversely during the cooler Easter season when the market is also favourable, prawns grown through summer are large and at high density, yet have reduced feeding and frequency of moulting which can be accompanied by increased fouling and diseases (farmer's personal communications). Management of DO, pH and Secchi is achievable, however, involves increased production costs and thus requires demonstration of economic benefit.

4 Aims of the Project

The aims of this project were to collect data to enable the Australian prawn farming industry to make evidence-based decisions to improve Biosecurity. Specifically, the project sought to:

Project Goal	Justification of activity
1. Identify the presence and distribution of endemic pathogens in Australian prawn farming operations.	Knowledge of pathogen distribution is required to complete a biosecurity risk assessment and determine most appropriate biosecurity management activity
2. Determine the exposure of industry to the introduction of endemic pathogens under present biosecurity management practises	Knowledge of pathogen distribution at multiple stages of production is required to assess the efficacy of current biosecurity practises and determine future strategies to strengthen biosecurity.
3. Determine if endemic or presently unknown pathogens are involved in disease outbreaks or incidents of reduced productivity within farming operations	Knowledge of impact of pathogen is required to complete a biosecurity risk assessment and prioritise biosecurity management practises to most damaging pathogens
4. Provide training and improve the knowledge of Australian prawn farmers to meet their GBOs.	Under the Biosecurity Act (2014) farmers are expected to responsibly manage disease incidents.

5 Methods

5.1 Project sample collection

Figure 6 provides an outline of sample collection for the project activities. Briefly, the project commenced seeking expression of interest to participants from industry. Five farms were included in two long term studies involving scheduled sample collection. All farms were invited to provide ad hoc samples for analysis. Scheduled and ad hoc-collected samples were analysed by quantitative PCR (qPCR) for the detection, estimation of the percentage of positive detections and quantification of endemic pathogens. Ad hoc-collected samples were analysed by High Throughput sequencing (HTS). Following HTS, a suite of targeted qPCR assays were developed to detect bacterial toxin genes. The bacterial toxin gene assays were applied to the schedule and ad hoc-collected samples.

Scheduled				
Within aquaculture system			External	
Aquacultured: *P. monodon*			Wild: *Acetes sp. & Palaemon sp.*	
Broodstock	Post-larvae	Pond prawns	Within-pond	Farm intake
n = 967	n = 411	n = 666	n = 59	n = 191

Ad-Hoc			
Within aquaculture system			External
Aquacultured: *P. monodon*			Imported: *P. vannamei*
Broodstock mortalities	Hatchery mortalities	Slow-growth ponds	Imported retail
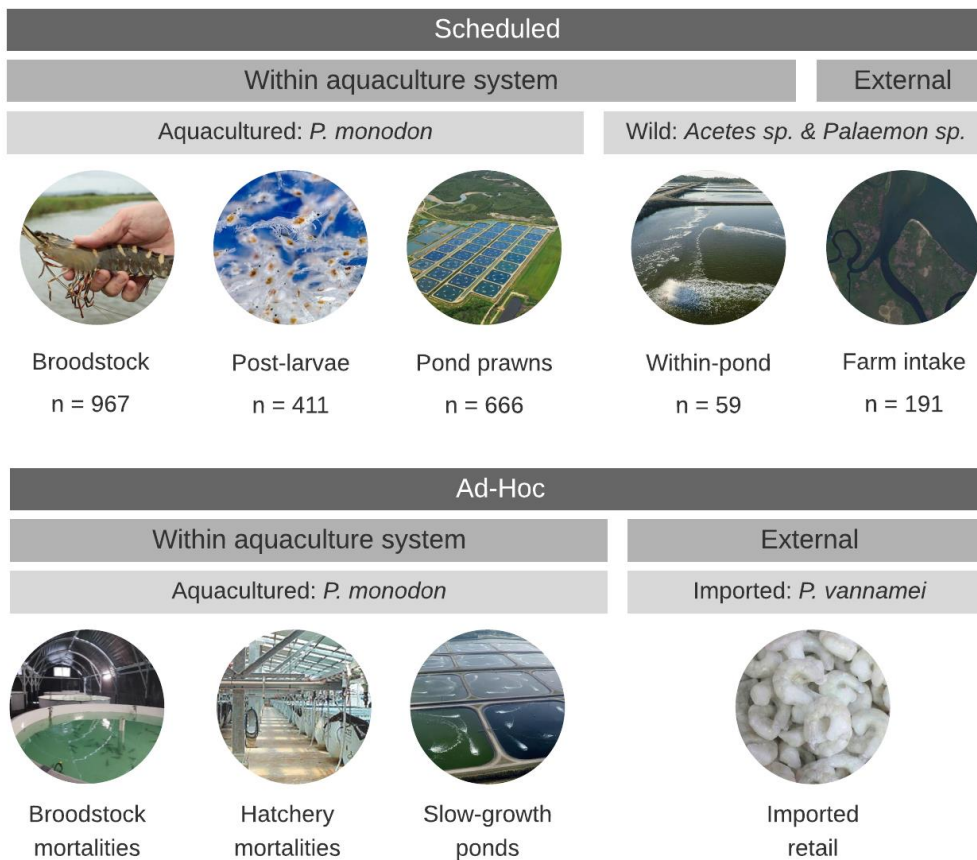
Figure 6 Summary of Scheduled and Ad-Hoc collected samples for the entire project

5.1.1 Scheduled sample collection

On-site visits were conducted to brief industry partners and complete an overview of farm biosecurity processes (Appendix A). Scheduled sample collection was conducted for genetic analysis to detect endemic pathogens from:

- Hatchery production: Broodstock and post-larval samples,

- Pond production: Prawns and glass prawn,

- Environmental locations: glass prawn and jelly prawn from farm intakes.

Complete details of the results of qPCR assays are provided in Appendix B. Detailed description of the sample numbers and collection are provided in each of the progress reports (Sections 12 and 23 in this report)

5.1.2 Ad hoc sample collection

Samples were provided from industry to investigate sub-optimal productivity. Ad hoc investigations were directed towards:

1. Unexplained, sustained hatchery mortalities,

2. Pond collected samples experiencing slow growth or lower than expected survival at harvest,

3. Broodstock mortality,

4. Retail Frozen imported prawns: 1kg of frozen prawns. Tested as 42 pools of 5 animals.

5.2 Genetic analysis by qPCR

Briefly, qPCR is the assay recommended by the World Health Organisation of Animal Health. qPCR is a specifically targeted approach that amplifies a pre-determined genome sequence of a pathogen. The qPCR result is expressed as the cycle threshold value (Ct) and provides an indication of both the presence of the pathogen genome and of the number of copies (quantity) of the genetic sequence. The lower the Ct value (range from 10 to 45), the higher the quantity of genome detected. Though qPCR is the most sensitive specific quantitative assay recommended by the OIE for the detection of prawn pathogens, it is not able to determine if the sequence that has been detected is from a viable ("alive") pathogen. For this reason, the detection of a pathogen genome by qPCR will refer to detection of the "target" rather than detection of the virus or bacteria. qPCR can only be applied when the gene sequence of the pathogen target is known or can be accurately estimated.

5.3 Genetic analysis of scheduled collection samples

Samples were collected from hatcheries consisting of broodstock and post-larval stage prawns and the pond culture consisting of ~45 day and 100 day collected samples. The methods and results of the Hatchery and Pond collected analysis have been presented in:

Report 1: Hatchery component.

Report 2: Grow-out component.

Samples submitted for qPCR analysis consisted of:

- Broodstock samples: a single pleopod sample from individual broodstock having undergone 1 to 4 spawns (average 2, ± SD 1) after 1 to 45 days (average 24, ± SD 9) within the hatchery.

- Post larvae samples: 150 Post larvae, tested as 5 separate extractions.

Pond prawn samples: 33 prawns were sampled from each pond. A single pleopod was excised from each prawn. Samples were tested as pools of three prawns. Sample numbers were selected to detect a 20% percentage of positive detections with 95% confidence. Presumably at least 20% of the pond population would be expected to be infected by a

pathogen for disease or other impacts on production to be observed. Briefly, prawns stocked from four farms at a density ranging from 30 to 80 prawns per m^2 were sampled at ~45 days post stocking, or at harvest (100 days).

Acetes sp. and *Paleamons* spp.: 191 pools of 10 samples were collected by farms from points on the farm prior to prawn ponds. With consideration of safety to farm staff, with particular awareness of crocodiles, the points of sample collection were not standardised across farms. Glass shrimp (*Acetes* sp.) and jelly shrimp (*Paleamons* spp.) samples were collected from intake canals, intake reservoirs and points of intake near the farm intake pump station. Dr Ben Diggles kindly collected samples from SE QLD locations.

5.3.1 Frozen imported retail prawns

A 1kg bag of frozen imported uncooked prawns were purchased from a local Townsville supermarket. A tail muscle and telson portion were removed and submitted for Total Nucleic Acid (TNA) extraction applying the MagCORE Max extraction kit on a Kingfisher Magnetic particle extraction robot. Samples were extracted and tested as pools of 5 individuals. Total nucleic extracts were analysed for the presence of WSSV, YHV-1, Enterocytozoan, YHV-7, IHHNV, HDV, When-2, GAV, Pir-A, Zon occludens toxin gene, Repeats in toxin gene and Hemolysin D toxin gene.

5.4 Distribution of positive target detections in 55 ponds from four farms

The percentage of positive target detections and average and minimum Ct value of all pond samples from the four participant farms was visually linked for presentation using Lucidchart (Lucid software Inc. Ent3_V1). The farm and pond numbers were de-identified and arbitrarily presented as Farm A to D and ponds 1 to 55. The pond numbers indicated are not accurate representations of the source pond number from any individual farm i.e. Pond 1 indicated in Farm A was not collected from Pond 1 of the de-identified farm.

5.5 Correlation of pathogen detection with prawn pond production

Pond productivity data was kindly provided by the participant farms. To protect commercially sensitive information, high level indexes including stocking density, food conversion ratio (FCR) and % survival were selected to represent productivity. The respective descriptors include:

1. Stocking density= number of prawns per m^2 of pond bottom area

2. Food conversion ratio = volume of feed/volume of crop harvested

3. % Survival = Number of PLs stocked/Number of prawns harvested

The pond productivity data was mapped across farms and ponds in Lucidchart (Lucid software Inc. Ent3_V1).

5.6 Tracked mapping of pathogen target detection through the prawn production cycle

The project conducted tracked sampling of broodstock at pre- and post-spawn and continued the collection of samples from progeny through the production cycle. Linked samples consisted of the post-larval batches and juvenile prawn samples that were collected from pond cultures at ~45 days or 100 days of culture. Results of qPCR analysis were recorded by cycle threshold value (Ct) and graphically illustrated in Lucidchart (Lucid software Inc. Ent3_V1). Data was linked from Broodstock to Post larvae to Pond according to records provided by the hatchery and managers of each farm. The broodstock value represents the results from the collective of individuals that contributed to the spawn. Post

larvae data represents the PL tank results from the contributing cohort of broodstock. Linked lines from post larvae to pond indicate the dispersal of larval rearing tanks into ponds. The percentage of positive target detections (%), average Ct and the minimum Ct (indicating highest copy of target genome detected) of the multiple contributors to any grouped cohort were indicated in the Lucidchart (Lucid software Inc. Ent3_V1).

5.7 Statistical analysis

5.7.1 Pearson's correlation

Descriptive parameters of pathogen detection, including the percentage of positive detections and average and minimum Ct of positive detections (as an indicator of quantity of target detected) were calculated for each individual pond based on replicate sample data. Productivity parameters, including FCR, stocking density and survival (inversed to mortality) were kindly provided by each respective farm for the ponds relevant to this study. Parameters of pathogen detection were statistically compared to the productivity parameters for each pond. Pearson's correlation coefficient was calculated for each comparison using pearsoncorr within the Python scipy.stats package. Heatmaps of the correlation coefficients were subsequently generated using heatmap within the seaborn package to visually assess the potential relationships between the parameters. All analysis was conducted using Python V.3.6.7. Pearsons' correlation matrix is indicative of association/correlation and not indicative of a causative relationship.

5.7.2 Multiple linear regression analysis

Multiple linear regression analysis was conducted using linear_model from the sklearn package (http://scikit-learn.org) in Python (Py 3.6. 7. http://python.org). Twenty-nine independent input variables were used to predict the outcome variable (survival %) for the 55 ponds sampled in this study. Input variables included pathogen target prevalence (n = 8), average (n = 9) and minimum (n = 9) Ct values of positive target detections, in addition to three feature engineered variables relating to the relative cumulative pathogen loading of each pond. The model was generated using the pathogen detection variables and the actual pond survival, as provided by each farm. The multiple linear model (eg. $AX_1 + BX_2 + … + ZX_n = Y$, where $X_{1, …,} X_n$ are the input values (e.g. prevalence of IHHNV), A, …, Z are scalar coefficients which are used to moderate the relative 'effect' of each of the input variables (e.g. if GAV prevalence has a small impact on survival, its coefficient will be closer to zero) and, Y is the outcome variable (predicted survival %)) was used to generate 'predicted' survival values for each pond. The predicted survival values were compared to the actual survival values, where the coefficient of determination (proportion of variability in the actual survival values that was described by the linear model) were calculated. The significance of the correlation between the predicted and actual survival values was assessed using Pearson's correlation.

5.8 Genetic analysis by High Throughput sequencing of ad hoc collected samples

The high throughput sequencing approach also amplifies genomic sequence. However, unlike qPCR, HTS is not targeted and will amplify the vast magnitude of genomic sequences within a sample. HTS approaches are expensive and require significant computer processing capability to process the data output, but importantly, enable the identification of unknown targets within a sample. In this study the HTS approach was applied to explore the presence of unknown viruses and bacterial genes in samples provided by industry that were experiencing productivity issues.

5.8.1 HTS application to Hatchery mortality

A commercial tiger prawn *Penaeus monodon* hatchery suffered significant mortality in larval rearing tanks for a sustained period. Mortality was characterised by the rapid overnight mortality of mysis-2 to PL-15 stages within a 12 to 24-hour period. Salinity was between 35-37 ppt and temperature ranged between 29-31 °C.

Post larval prawn sample collection and DNA extraction

Post-larval (PL) prawns were collected from six tanks displaying signs of disease and were placed into approximately 50 ml of 90% ethanol. Sample were transported at room temperature for analysis. Extractions of ~ 20-40 PLs were completed using the MagCORE Max (Applied Biosystems) kits on a Kingfisher Magnetic Particle bead extraction robot (Life Technologies) following the manufacturer's instructions. TNA extracts were eluted into 100µl elution buffer (Applied Biosystems). TNA extracts were analysed by qPCR for the presence of Gill Associated Virus (GAV), Yellowhead virus-1 (YHV-1), Whitespot syndrome Virus (WSSV), Yellowhead virus-7 (YHV-1), Infectious hypodermal hematopoietic necrosis virus (IHHNV), Hepatopancreatic Necrosis Virus (HPV), Infectious myonecrosis virus (IMNV), Taura syndrome virus (TSV), Wenzhou shrimp virus-2 (When-2) and the Pir-A toxin gene (Pir-A). The TNA extracts from PLs were submitted for library preparation and sequencing.

Bacterial sample collection and DNA extraction

Bacterial culture was conducted at the prawn hatchery. Two samples of 100 µl of culture water was collected from affected hatchery tanks and spread onto Thiosulphate-citrate-bile salts-sucrose (TCBS 1.5% NaCl) agar plates. Prior to plate inoculation, one plate had no antibiotic infusion (Sample A) and the second plate was surface infused with 100µl of 5ppm erythromycin (Sample B). Agar plates were incubated overnight at a temperature of ~35 °C. Following incubation, a confluent culture of small yellow colonies was collected from a surface scrape of each of the agar plates and placed into a 1.5ml microfuge tube containing 90% ethanol. Samples were transported at room temperature to the JCU AquaPATH laboratory for analysis. Total nucleic acid (TNA) extracts were prepared from the ethanol preserved colonies. Extractions were completed using the MagCORE Max (Applied Biosystems) kits on a Kingfisher™ Magnetic Particle bead extraction robot (Life Technologies). TNA was eluted into 100 µl of elution buffer (Applied biosystems).

Library preparation and sequencing of hatchery collected samples

Library preparation and sequencing was carried out at the Australian Genome Research Facility (AGRF). All libraries were prepared using the Illumina Nextera XT protocol. The two bacterial samples were sequenced on a single MiSeq V2 flow cell with 150 bp paired-ends reads. The post-larval sample was sequenced on a single, shared HiSeq lane producing 125 bp paired-end reads. Raw sequences are available on Genbank under short read accessions SRR10074419 to SRR10074421, within biosample accessions SAMN12697362 to SAMN12697364 in project ID PRJNA563984.

Bioinformatical analysis of bacterial and post-larval isolates from hatchery samples

FastQC (0.11.8, Andrews, 2010) was used to assess the quality of the sequences. Centrifuge (1.0.3-beta, (Kim, 2016) was used together with the compressed Bacteria and Archaea database (www.ccb.jhu.edu/software/centrifuge, last updated 15/04/2018) to assess the taxonomy of the two samples. From this a Kraken-style report was generated for further analysis. The analysis was carried out with Spades (3.12.0, (Bankevich, 2012), wrapped within the Shovill

pipeline (1.0.1, github.com/tseemann/shovill). Prokka (1.13.3, (Seemann, 2014) was used to find coding regions (CDS) and a basic annotation. Blastp (2.6.0, (Altschul, 1990;Camacho, 2009) against the NCBI non-redundant amino acid database (nr, retrieved 11-July-2018) was used for a more in-depth annotation of the CDS. The quality of the assemblies was assessed using QUAST (5.0.2, (Gurevich, 2013). To determine the presence of the toxins in post-larval prawns, the generated raw reads and the putative toxin contigs identified in qPCR assay design were loaded reads into Geneious 10.2.6 (https://www.geneious.com; (Kearse, 2012). The raw reads were quality trimmed and aligned reads against toxin genes to check presence and calculate mean coverage. Additionally, Centrifuge (1.0.3-beta, (Kim, 2016) was used together with the complete NCBI nucleotide non-redundant sequence database (www.ccb.jhu.edu/software/centrifuge, last updated 15/04/2018) to assess the taxonomy of the post-larval sample.

Software available in conda were installed and used through Miniconda (4.6.14). Contigs of length < 200 bp were removed using bioawk (https://github.com/lh3/bioawk) for submission to Genbank as per NCBI regulations. Assembled contigs are available on Genbank under project ID PRJNA563984. Selected toxin sequences added to GenBank can be found under accession numbers MN895997 to MN896003.

5.8.2 HTS application to Pond collected samples

Two hundred and sixteen samples were collected from 20 ponds that displayed slow growth or reduced survival at harvest. Thirteen ponds were sampled in Summer 2019, four ponds were sampled in Autumn 2019 and 3 ponds were sampled in Autumn 2020. Seasonal sampling of ponds may be skewed by pond production cycles rather than any influence of season on sub-optimal performance. Samples that were negative or very low copy number for the detection of the endemic pathogens were submitted for HTS sequencing.

NGS library preparation and sequencing of pond collected samples
Total Nucleic Acid samples were sent to the Australian Genome Research Facility (AGRF), Melbourne, for library preparation and sequencing. The DNA samples were prepared using the Illumina Nextera FLEX library preparation kit (Illumina, San Diego, USA), while the RNA samples were prepared using the Zymo-Seq RiboFree Total RNA Library Kit (Zymo Research, Orange, CA, USA). DNA and RNA libraries where then separately sequenced on two MiSeq sequencing runs using a MiSeq V3 cartridge 600 cycle cartridge (Illumina, San Diego, USA).

Bioinformatical methods of pond collected samples
Quality trimming and adapter removal for both DNA and RNA samples was carried out using Cutadapt (Martin, 2011) within TrimGalore (Kruger, 2012), and using FastQC as quality control (Andrews, 2012).

To process the DNA samples, the assemblies were carried out in Megahit (Li *et al.,* 2015; Li *et al.,* 2016), quality control was done using Quast (Gurevich *et al.,* 2013), and TransDecoder (Haas & Papanicolaou, 2016) was used to find expressed gene sequences. To process the RNA samples, the assemblies were carried out in Trinity (Grabherr *et al.,* 2011), quality control was done using TransRate (Smith-Unna *et al.,* 2016), and TransDecoder (Haas & Papanicolaou, 2016) was used to find expressed gene sequences.

For annotation, amino acid sequences predicted by TransDecoder were split into blocks of 5,000 sequences using awk, and then annotated with blastp (Altschul *et al.,* 1997) against a subsection of the NCBI database containing Penaeid and

viral sequences only (Downloaded 19 July 2019). Lastly, makeblastdb was used to create a database of each DNA and RNA, and reciprocal blastp searches were carried out to assess the overlap between the different samples.

6 Results
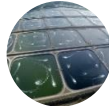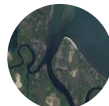
6.1 Scheduled sample collection: Compilation of pathogen presence and intensity of detection in all production stages.

A total of 2, 294 samples from scheduled collection times were analysed by qPCR for the detection of WSSV, YHV-1, YHV-7, GAV, IHHNV, HDV, When-2 and Pir-A. Detailed results of hatchery and grow-out components are provided in:

Report 1: Hatchery component (Section 12) and Report 2: Grow-out component (Section 23)

A summary of the percentage of positive pathogen target detections for the project is presented in Table 1. GAV and IHHNV were the most frequently detected targets and were detected at the highest calculated copy numbers in all stages of production. When-2 was also detected in high load in broodstock and pond collected samples, but was not detected in post larval stage samples. WSSV, IHHNV, GAV, When-2, HDV and Pir-A were detected in *Acetes* spp. collected from the farm environment. WSSV and YHV-1 were not detected from any prawn samples. Frozen imported commodity prawns were positive for the detection of When-2 (100% Average Ct=25.94 ±1.24) and IHHNV (57% Average Ct=35.93 ±0.60)

Table 4 Summary of the most frequently detected pathogen targets from scheduled sample collection from the hatchery and grow-out components. Total number of samples analysed, total percentage of samples with positive detections (%) and the most commonly detected pathogens of each component including highest calculated copy number of the respective targets (copies per µl Total Nucleic Acid extract). All broodstock and PL samples were *P. monodon.*

Sample source		No. of Samples analysed	Proportion positive	Most frequently detected pathogens	Highest quantity detected (copies per µl of nucleic acid extract)
Hatchery					
Broodstock		967	93%	GAV (89%)	4.2×10^9
				IHHNV (47%)	1.2×10^8
				When-2 (32%)	1.3×10^9
Post larvae		411	88%	GAV (88%)	6.4×10^8
				IHHNV (61%)	1.3×10^8
				HDV (19%)	44
Grow-out Ponds					
P.monodon		666	99%	GAV (98%)	6.5×10^6
				IHHNV (62%)	1.5×10^7
				When-2 (22%)	1.8×10^8
Acetes sp. (glass shrimp)		59	73%	When-2 (49%)	5.3×10^5
				GAV (24%)	3.3×10^4
				IHHNV (20%)	1.9×10^2
				HDV (20%)	66
Farm intake (prior to prawn ponds)					
Acetes sp. & Palaemon sp. (jelly shrimp)		191	39%	IHHNV (23%)	1.8×10^3
				HDV (20%)	6.8×10^3
				WSSV (9%)	8.2×10^3
				When-2 (1%)	4.2×10^3
				Pir-A (1%)	18

6.2 Detection of endemic pathogens in farmed pond cultured prawns.

6.2.1 Gill Associated Virus (GAV).

GAV was detected in all ponds at all farms tested in this project (Figure 7). Except for two ponds (1 & 2), GAV was detected from >83% of samples within each pond (Figure 5.1). GAV was detected in ponds at an average Ct value of 32.72 and minimum Ct value of 23.83. No farms or hatchery indicated any active management strategy for GAV.
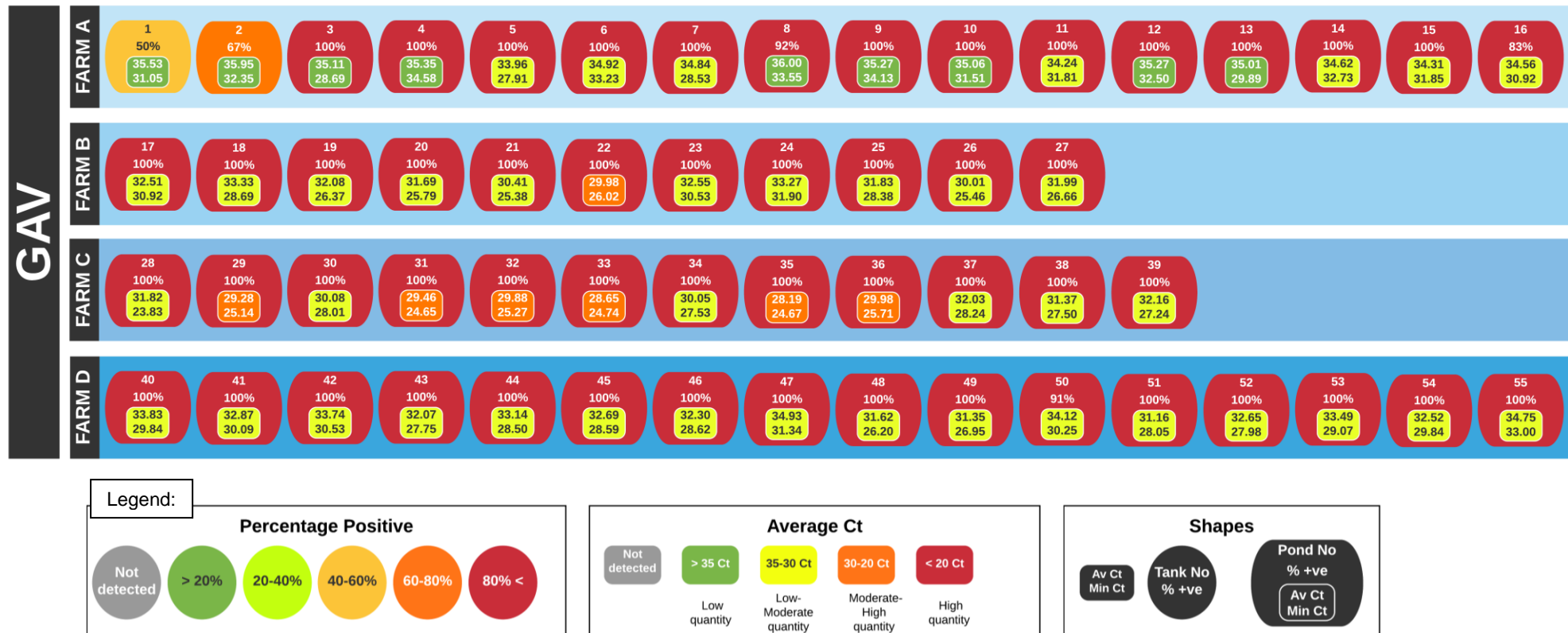
Figure 7 Detection of GAV from 55 ponds sampled from 4 farms. Detection is indicated as a percentage of positive detections (%) and the average and minimum Cycle threshold (Ct) of the positive detections. Refer to Legend for indication of colour coding of percentage positive, average and minimum Ct value

6.2.2 Infectious Hypodermal Haematopoietic Necrosis Virus syn Penaeus stylirostris Densovirus (IHHNV/ PstDV)

The percentage of positive detections and detected quantity of IHHNV was highly variable across all ponds from each farm. Only one pond, namely 46, had an average Ct value below 25 (22.32) (Figure 8). Three farms reported targeted hatchery-level management to reduce the introduction of IHHNV. In this limited analysis an absence of management at hatchery level coincides with the highest overall percentage of positive detections, but not highest calculated copy number of IHHNV in pond samples.
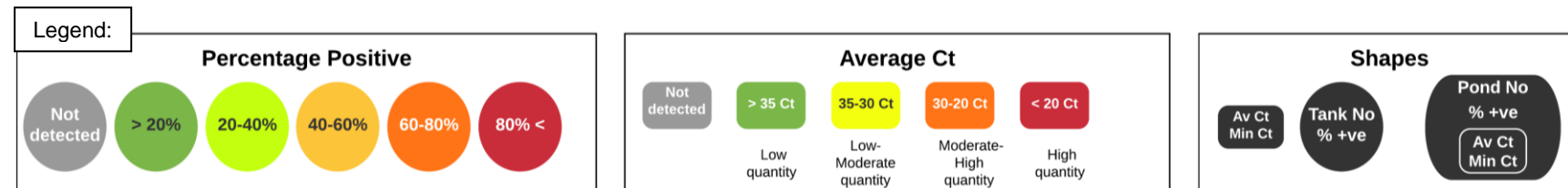
Figure 8 Detection of IHHNV from 55 ponds sampled from 4 farms. Detection is indicated as a percentage of positive detections (%) and the average and minimum Cycle threshold (Ct) of the positive detections. Refer to Legend for indication of colour coding of percentage positive, average and minimum Ct value

6.2.3 Wenzhou Shrimp Virus-2 (When-2)

Wenzhou shrimp virus-2 was detected from 24 of 55 ponds (Figure 9). When-2 detection displayed a strong association to two specific farms with high variability in the percentage of positive detections and Ct value within the farms. No farm reported targeted management of When-2, however, in some cases When-2 exclusion or reduction in When-2 occurred at hatchery level though coincidental management of IHHNV. Notably, samples collected from the farm with no active management against IHHNV also had the highest percentage of positive detections of When-2. Only four ponds (1, 2, 6 and 23) were positive for the detection of When-2 with an average Ct below 25. Unlike GAV and IHHNV, there was not widespread high rates of low quantity detection of When-2 from pond collected samples. High percentages of positive When-2 detections tended to be accompanied by higher quantity of detection (lower Ct value). Conversely, low percentages of positive detections in any single pond tended to also be a low quantity of target detection. The results of When-2 detection suggest When-2 is not introduced to farms via PLs or testing of early stage PLs is not suitable for reliable detection of When-2.
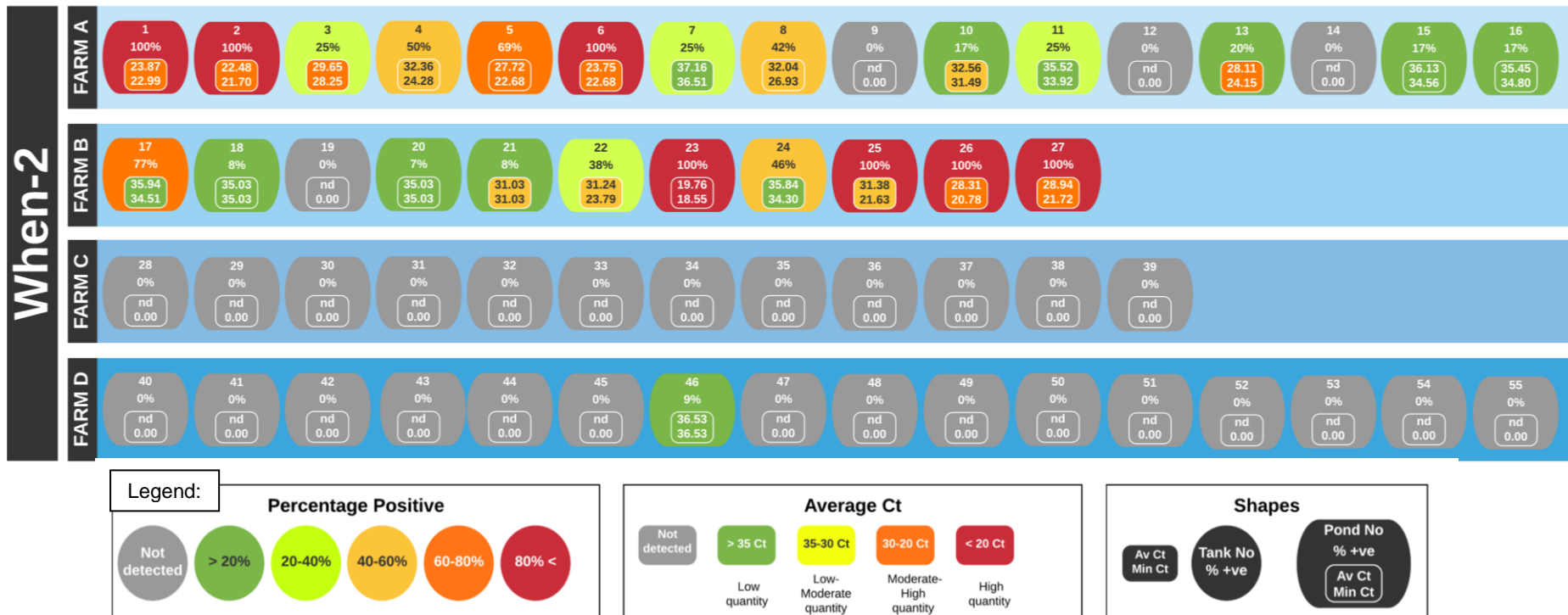
Figure 9 Detection of When-2 virus from 55 ponds sampled from 4 farms. Detection is indicated as the percentage of positive detections (%) and the average and minimum Cycle threshold (Ct) of the positive detections. Refer to Legend for indication of colour coding of percentage positive, average and minimum Ct value

6.2.4 Hepatopancreatic Necrosis Virus *syn* Hepandensovirus (HPV/HDV)

Hepatopancreatic Necrosis Virus was positively detected in a low percentage of pond samples and at high Ct values. No farms reported targeted management of HDV.

The percentage of positive detections (19%) of HDV in pond samples was similar to that detected in PL samples (~ 20%).
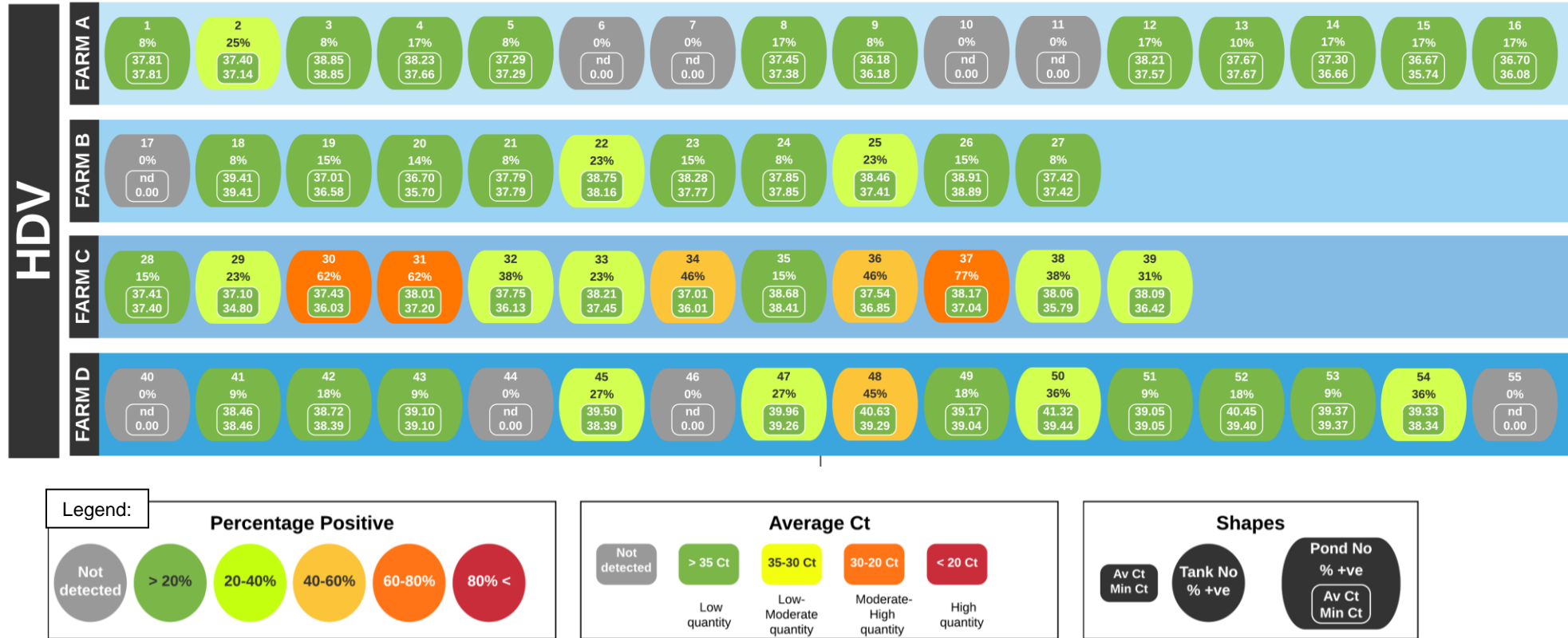
Figure 10 Percentage positive and Average and minimum Ct of HDV from pond collected samples. Detection of HDV from 55 ponds from four prawn farms. Pleopods from 38 prawns were collected from each pond and analysed as pools of 3 pleopods. Refer to Legend for indication of colour coding of percentage positive, average and minimum Ct value

6.3 Tracking of endemic Pathogen targets from Broodstock to Pond

6.3.1 Tracking GAV

GAV was detected in a high percentage of broodstock samples at pre-spawn with high Ct (low quantity of target) (Figure 12). The copy number of GAV increased by a factor of x10-100 in post-spawn samples (data not shown, estimated from the Ct value change). All PL batches and pond collected samples were also positive for the detection of GAV. GAV quantity increased ~10 to 1000-fold in PL tanks compared to post-spawn broodstock. GAV Ct value increased by ~ 6, indicating a decrease in quantity by a factor of ~100 in pond samples compared to PLs. Only one pond maintained a similar Ct value in detection from PL to pond (Pond 22). Notably, Pond 22 also had the highest survival (70%) and FCR (1.89) for the associated farm.

6.3.2 Tracking IHHNV

The percentage of positive detections and the quantity of IHHNV detected increased from pre- to post-spawn (Figure 11). The percentages of positive detection of IHHNV in individual post-larval tanks was more similar to the broodstock pre-spawn than post-spawn samples, noting the time and number of spawns between the pre and post-spawn sample collection was not standardised and ranged from 1- 45 days and 1-4 spawns, respectively. Four PL batches, negative for IHHNV, were reared from broodstock that were negative for the detection of IHHNV pre-spawn. Additional PL batches negative for the detection of IHHNV were reared from broodstock groups with a percentage of positive detections less than 100% and average Ct values higher than 34 (ie. low quantity of target). The mixing of PL tanks into ponds combined tanks that were negative for IHHNV with tanks that were positive for IHHNV. Although only seven of the 20 PL tanks were positive for the detection of IHHNV, the mixing of PL tanks for pond stocking resulted in only 2 ponds being stocked with PLs that were negative for the detection of IHHNV (Pond 21 & 26). However, all ponds were positive for the detection of IHHNV albeit at a low quantity of target.

6.3.3 Tracking of When-2

When-2 was detected in a high percentage of broodstock at pre-spawn with high Ct (low quantity of target), but in a low percentage of broodstock at post-spawn (Figure 13). There was no detection of When-2 in any of the PL samples, yet only one pond was negative for the detection of When-2. There was considerable variation in the percentage of positive detections and Ct value associated with detection of When-2 from pond samples. One pond recorded a low Ct of 18 which was the highest calculated copy number detected from all pathogen targets across all ponds analysed in this project (Pond 23). Pond 23 was one of the two ponds with a percentage of positive detections less than 100% of IHHNV (Figure 13).

6.3.4 Tracking of HDV

HDV was positively detected in a low percentage of samples, at high Ct values of detection (low quantity of target). HDV was not detected in a high percentage of samples or at high calculated copy numbers at any stage of production (Figure 14Figure 17). The rate of HDV target detection increased from pre to post-spawn. HDV was most frequently detected in PL stages.

6.3.5 Tracking of Pir-A

The percentage of samples positive for the detection of Pir-A and the quantity of the target detections increased from pre to post-spawn (Figure 15). The number of broodstock tanks positive for Pir-A pre-spawn increased from 12 to 19 post

spawn. Despite the high percentage of positive detections of Pir-A from broodstock tanks, only 2 post larval tanks and 0 ponds were positive for the detection of Pir-A.
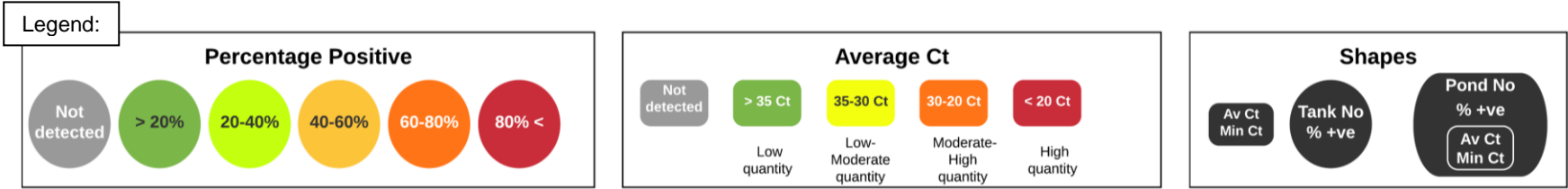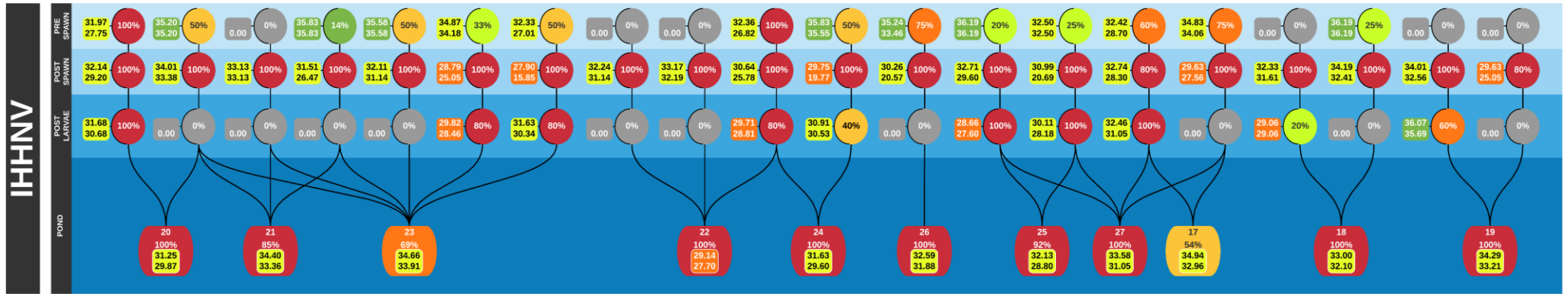
Figure 11 Tracking of IHHNV detection from Broodstock (pre and post spawn) to Post larvae and Pond collected sample. Percentage positive (%) and average Ct and minimum Ct of detection are indicated. Refer to Legend for indication of colour coding of percentage positive, average and minimum Ct value
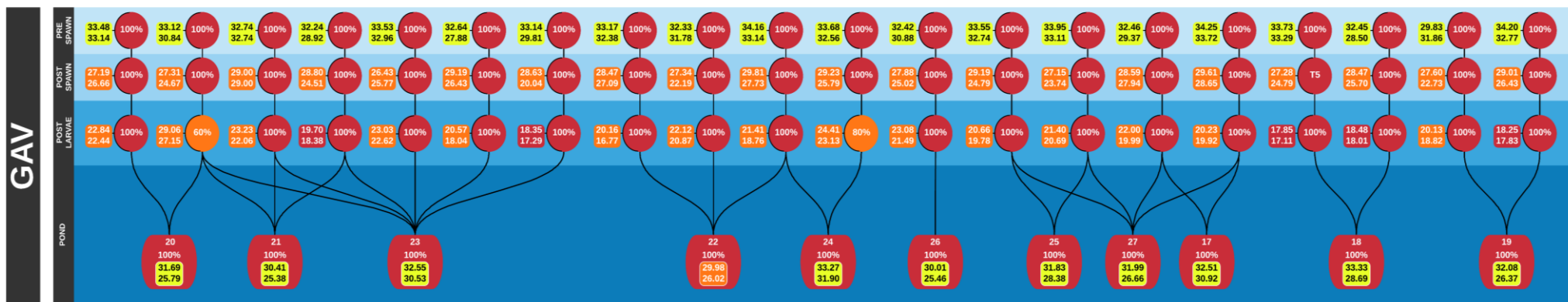
Figure 12 Tracking of GAV detection from Broodstock (pre and post spawn) to Post larvae and Pond collected sample. Percentage positive (%) and average Ct and minimum Ct of detection are indicated. Refer to Legend for indication of colour coding of percentage positive, average and minimum Ct value
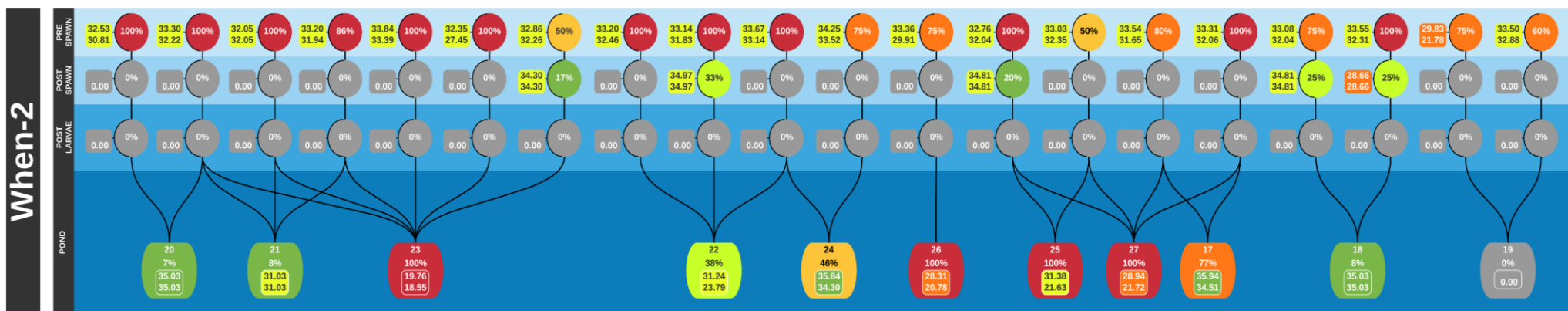
Figure 13 Tracking of When-2 detection from Broodstock (pre and post spawn) to Post larvae and Pond collected sample. Percentage positive (%) and average Ct and minimum Ct of detection are indicated. Refer to Legend for indication of colour coding of percentage positive, average and minimum Ct value
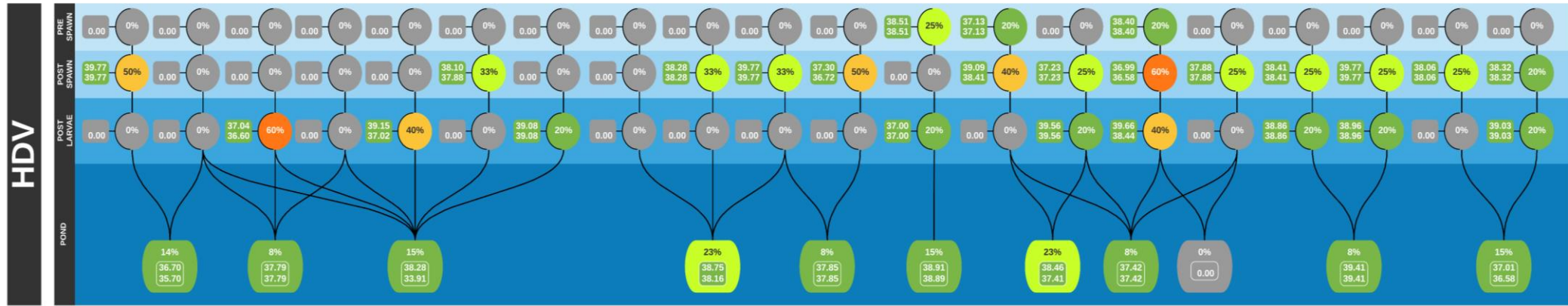
Figure 14: Tracking of HDV detection from Broodstock (pre and post spawn) to Post larvae and Pond collected sample. Percentage positive (%) and average Ct and minimum Ct of detection are indicated. Refer to Legend for indication of colour coding of percentage positive, average and minimum Ct value
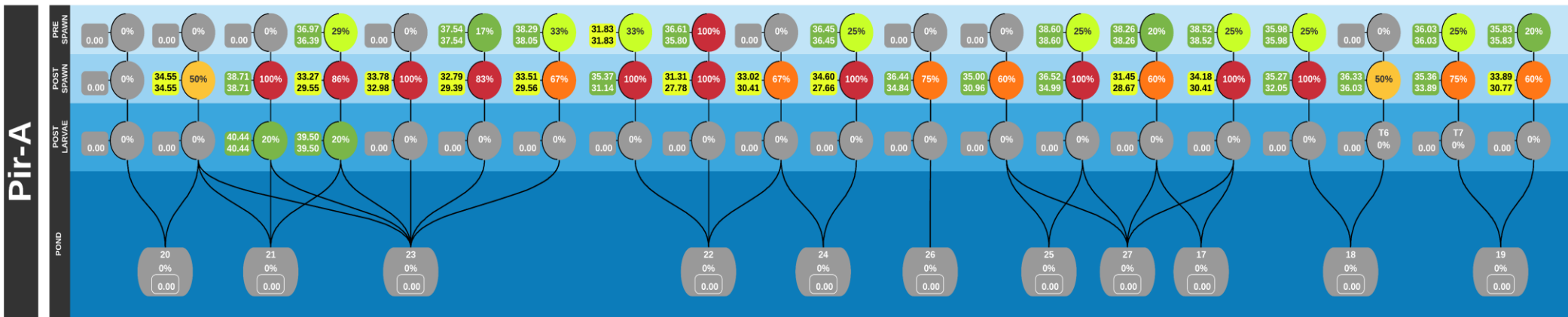
Figure 15: Tracking of Pir-A detection from Broodstock (pre and post spawn) to Post larvae and Pond collected sample. Percentage positive (%) and average Ct and minimum Ct of detection are indicated. Refer to Legend for indication of colour coding of percentage positive, average and minimum Ct value
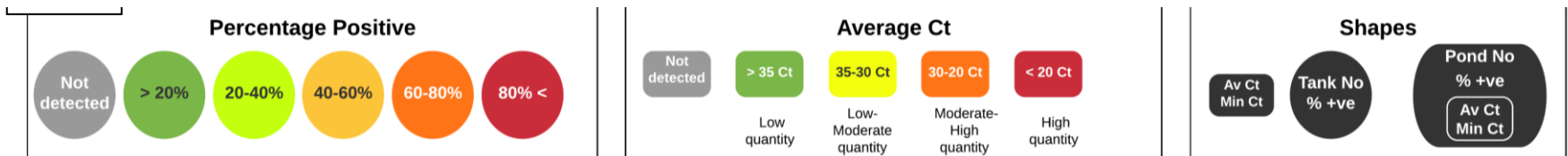
6.4 Results of High Throughput Sequencing (HTS) Analysis

6.4.1 Hatchery related mortality

Results of initial pathogen screening of Post larvae

Analysis by qPCR to detect viral pathogens and the Pir-A toxin gene did not support a differential diagnosis relating to any of the pathogens targeted by the initial analysis of qPCR (data not shown). Attempted treatment of tanks with erythromycin did not reduce mortalities, but a bacterial aetiology was indicated when mortality was effectively reduced following treatment with oxytetracycline.

Sequencing results and Bioinformatical analysis of the bacterial cultures

The MiSeq sequencing run resulted in 6.13 M and 6.42 M reads for the two bacterial culture samples, respectively. FastQC showed the sequences were of high quality with minimal adapter contamination. The taxonomic classification with Centrifuge indicated both samples contained >99.99% bacterial reads, with the remainder being either unclassified or archaeal. Both samples were dominated by the Genus *Vibrio*, with 91.2% (sample A) and 98.1% (sample B), respectively. Within the genus *Vibrio*, sample A (no erythromycin infusion) contained the *V. harveyi* group (38.6%), dominated by *V. alginolyticus,* and the *V. orientalis* group (48.2%), dominated by *V. tubiashii*. In contrast, sample B (plus erythromcyin infusion) was dominated by the *V. harveyi* group (78.9%), dominated by *V. alginolyticus*, and a lower proportion of the *V. orientalis* group (16.7%), which was also dominated by *V. tubiashii*.

Selection of bacterial toxin genes, qPCR assay design and application

The HTS approach lead to the identification of seven toxin genes of putative significance from a bacterial lawn cultured from water samples collected from a tank containing affected post larvae (Table 5). Four Taqman qPCR assays were designed to target genes with putative links to virulence (Sequences: MN895998, MN895999, MN896001, MN896003) namely those with high homology to genes encoding the Zona occludens toxin (ZON) (NCBI WP_063494373), Repeats-in-toxin (RtX) (NCBI EEZ80939), YaFO toxin (NCBI WP_025441780) and Hemolysin D (hemo) (NCBI WP_005464076) (Table 6). The Taqman assays were applied to the TNA extracts that were submitted to HTS analysis. Analysis by qPCR confirmed the presence of the genes in the bacterial culture TNA extracts and the six batches of PLs collected from tanks displaying mortality.

Table 5 Seven putative toxin genes identified in Sample B (plus erythromycin) showing NCBI accession number, length in base pairs and amino acids, blast hit information from NCBI and if the sequence was found in Sample A (no erythromycin). Genes targeted for qPCR analysis are indicated **in bold.**

NCBI accession number	Length nt	Length aa	NCBI hit gene name	NCBI hit Vibrio species	NCBI hit accession number	Query coverage [%]	Pairwise Identity [%]	e-value	Sample A equivalent based on blastn search
MN895997	654	217	hemolysin III family protein	*V.europaeus*	WP_069668035	100	99.5	1.30E-153	Two hits (100% PI, 100% QC / 92.2% PI, 100% QC)
MN895998	4701	1566	**putative RTX toxin**	*V.alginolyticus 40B*	EEZ80939	82.8	99.1	0	One hit (85.5% PI, 39.5% QC)
MN895999	1425	474	**toxin**	*V.parahaemolyticus*	WP_063494373	100	98.5	0	One hit (100% PI, 100% QC)
MN896000	2541	846	hemolysin	*V.tubiashii*	WP_004748518	100	95.5	0	One hit (100% PI, 100% QC)
MN896001	1038	345	**hemolysin D**	*V. parahaemolyticus*	WP_005464076	100	99.7	0	none found
MN896002	327	108	thermolabile hemolysin	*V.tubiashii*	WP_004743772	26.2	96.3	3.68E-70	One hit (99.1% PI, 100% QC)
MN896003	402	133	**toxin YafO**	*Vibrio parahaemolyticus*	WP_025441780	100	100	4.48E-94	none found

6.4.2 **Pond collected samples**

The metagenomic assembly of the DNA samples resulted in 660 k contigs with an N50 of 500 bp for NGS08 and 903 k contigs with an N50 of 502 for NGS09 (Table 6). After the processing with TransDecoder, a total of 126,415 and 143,929 amino acid sequences were identified for NGS08 and NGS09, respectively (Table 6).

The transcriptomic assembly of the RNA samples resulted in 1.6 M contigs with an N50 of 311 for NGS02 and 1.3 M contigs with an N50 of 302 for NGS08 (Table 7). After the processing with TransDecoder, a total of 91,485 and 181,229 amino acid sequences were identified for NGS08 and NGS09, respectively (Table 6). The volume of data collected from tissue samples from sub-optimally performing ponds is significantly more complex to configure and analyze. The results of HTS analysis did not identify any sequence with homology to any of the pathogen targets which were not detected by the targeted qPCR analysis. Results of blast analysis on the data set revealed a high number of putative prawn genes, bacterial genomes, bacterial phage genome and endogenous and potentially infectious viral genomes (Table 7). The absence of a mapped prawn genome and characterized viruses of crustaceans, makes the separation of genetic sequence of prawn from that of potentially uncharacterized viruses difficult, yet also provides insight into the plethora of organisms that are present and unconsidered in the prawn health studies. DNA and RNA transcripts with high homology

to viral genetic sequence were identified (Table 7). Because crustaceans share genetic homology with insects, an initial research approach to search for novel crustacean viruses can seek to align genome sequence with homology to characterized insect viruses. Each of the aforementioned crustacean viruses including IHHNV, HDV, TSV, MBV, DIV-1, display homology to insect viruses which have tended to be more intensively studied due to the presence of insect cell lines and the long historical interest in removing destructive insects from horticultural production. Most notable results of HTS analysis in this project include the acquisition of DNA sequence and RNA transcripts of *Penaeus monodon* endogenous nimarvirus and a range of insect infecting viruses including sequences with homology to Densovirus, Insect iridescent virus, granulovirus, Iflavivirus and Nucleopolyhedrovirus (Table 7). Each of the homologous sequences align with viral sequences that have been reported as endogenous, host-genome integrated sequences and infectious viral particles. Further investigation is required before robust conclusions on the significance of the sequences can be presented. The existence of endogenous integrated sequences is more frequently being reported in crustacean health research (Bao et al., 2020; Kawato et al., 2019). The significance of the presence of the endogenous sequence in the crustacean genomes and their role in crustacean health are not understood. Proposed mechanisms include facilitating immune response to the specific virus or conversely contributing to the viral replication process when infection by homologous virus occurs. Regardless, the presence of such sequences must be considered in the design of molecular assays that are targeted to support farm biosecurity, or epidemiological investigations. For example, the qPCR recommended in the OIE assay to detect IHHNV detects a sequence that is reported as endogenous sequence in Australian *P. monodon* and hence alternate assays must be applied to the detection of IHHNV in Australian sourced prawns. In addition, epidemiological studies based on the molecular detection of strain variation in WSSV must ensure interpretations are based on the variation in viral derived-sequence and not a reflection of variation in endogenous nimarvirus sequences across multiple species, or multiple geographic sources. Ideally, to avoid erroneous conclusions, epidemiological studies, particularly those that will influence national biosecurity policy, should be based on comparison with entire viral genome sequences, or at a minimum demonstrate the presence of complete viral genome in the analysis.

Table 6 Summary of HTS output from pond collected samples including DNA and RNA sequence of each sample

	DNA NGS08	DNA NGS09	RNA NGS02	RNA NGS08
Number of sequences	660,482	903,253	1,570,638	1,335,347
Larges sequence [bp]	24,849	373,828	20,426	14,564
Number of bases	323,933,380	443,212,584	499,799,744	409,208,243
Mean fragment length [bp]	490.4	490.7	318.2	306.4
Number over 1 kbp	19,886	21,094	9,330	3,757
Number over 10 kbp	13	74	63	14
Number with ORF	39,757	65,718	41,554	15,550
n50	500	502	311	302
gc	40.2 %	36.9 %	44.6 %	43.6 %
TransDecoder amino acid sequences	126,415	143,929	91,485	181,229
Number of sequences with amino acid Homology in the NCBI database	202,309	250,608	143,832	73,368
Number of sequences with amino acid homology to viruses the NCBI database	18,567	17,456	50,317	13,671
Length of longest sequence with aa homology to viral sequence	6,118	6,144	5,955	3,037

Table 7 Summary of viral sources of HTS output alignments targeted at viruses linked with disease in insects or known crustacean viruses. Including viral group, DNA or RNA genome of viral group, project sample number and alignment source (DNA or RNA), number of sequences aligned to the homologous virus, length of amino acid alignment, average length of alignments and % identity by amino acid homology.

Homology to	Type of viral genome	Project sample ref	Number of blast matches with viral genome records	Range of Length of blast alignments (amino acid)	Average length of alignment (amino acids)	% identity (by amino acid)
Endogenous nimavirus	DNA	#8 DNA	100	12 to 6118	123	26-100%
		#9 DNA	488	19 to 6114	266	24-100%
		#8 RNA	832	16-3032	365	22-100%
		#2 RNA	1205	24-5995	355	19-100%
Densovirus	DNA	#9 DNA	6	25-143	63	30-62%
		#8 RNA	10	46-96	70	27-96%
		#2 RNA	19	31-111	66	23-45%
		#8 DNA	3	53-78	62	28-24%
Nuclopolyhedrovirus	DNA	#9 DNA	44	17-593	76	23-59%
		#8 RNA	134	27-122	58	21-55%
		#2 RNA	361	18-209	63	21-66%
		#8 DNA	87	25-153	65	22-50%
Iridescent virus	DNA	#9 DNA	465	17-209	58	14-62%
		#8 RNA	215	25-142	63	20-64%
		#2 RNA	383	24-459	71	19-63%
		#8 DNA	1096	20-177	78	21-70%
Iflavivirus	RNA	#9 DNA	24	26-124	63	25-42%
		#8 RNA	21	50-89	55	38-43%
		#8 DNA	10	37-162	64	22-58%
		#2 RNA	76	26-139	65	22-56%
Picornavirus	RNA	#9 DNA	256	21-168	64	20-61%
		#8 RNA	243	13-144	60	21-69%
		#2 RNA	858	17-177	67	20-65%
Vibrio phages	Both	#9 DNA	61	28-694	210	23-100%
		#8 DNA	13	44-104	70	28-45%
		#8 RNA	13	38-80	54	29-42%
		#2 RNA	90	27-203	77	25-64%
Phage	Both	#9 DNA	428	21-776	100	22-75%
		#2 RNA	530	22-326	88	20-63%
		#8 DNA	65	27-141	62	22-55%
Granulovirus	DNA	#2 RNA	168	13-262	66	20-100%
		#8 DNA	29	12-360	68	18-100%

6.5 Results of qPCR analysis developed from HTS analysis: scheduled sample collection

6.5.1 HTS from hatchery collected samples

HTS analysis conducted on samples collected from a mass mortality event in a hatchery detected the presence of multiple bacterial toxin genes. Three of the genes, namely, Zonula occludens toxin (Zon), Repeats-in toxin (Rtx) and the hemolysin D (Hemo) gene were selected for further study. The Zon, Repeats-in-Toxin (RTX), YaFO and hemolysin D (hyld) genes were detected with high prevalence and with average Ct values ranging from 22.82 to 24.47 in the PL samples collected from the six larval rearing tanks suffering mass mortality. The four targeted genes were also detected in high copy number in the bacterial cultures prepared from hatchery water samples with average Ct values ranging from 15.3 to 18.77. The significance of detecting the genes specifically targeted by qPCR cannot be determined without experimental challenge, however, a review of the literature indicates tangible associations with virulence to warrant further study.

Repeat-in-toxin (RTX) proteins are produced by a variety of Gram-negative bacteria and perform diverse roles (Linhartova et al., 2010). Analysis applying a 454 GS-FLX pyrosequencing approach to *Vibrio harveyi* (strain CAIM72), the aetiological agent of "bright red syndrome" in *Litopenaeus vannamei*, identified >100 genes that encode for putative virulence factors including, amongst others, type 1-4 and type 6 secretion systems and RTX toxins (Espinosa-Valles et al., 2012). In the "insect-equivalent" of AHPND, experimental challenge of insects with *Photorhabdus luminescens*, the host of the Pir-AB toxin gene, demonstrated the expression of multiple virulence factors. A RTX-like metalloprotease was reportedly expressed as a virulence factor by *Photorhabdus luminescens* during insect infection (Daborn et al., 2001). Of relevance to virulence in *Vibrio* spp., RTX proteins have been shown to cause rounding of epithelial cells, act as bacteriocins, contribute to defence against environmental aggression and contribute to the activity of the Type I secretory pathway (Linhartova et al., 2010). Combined with hlyd, RTX proteins within the type I secretory pathway form components of the multidrug efflux pump that contributes to antibiotic resistance in *Vibrio* HylD and RTX proteins were identified by whole genome sequencing as virulence factors in *Shewenella algae*, *V.parahaemolyticus* and *V.anguillarum* isolated from Italian aquaculture centres (Zago et al., 2020).

Both the Zon and hylD genes have been identified within the genome of AHPND strains, but are rarely discussed as contributors to the disease. The Zon gene has been proposed as a potential gene of interest that could encode a virulent toxin in its own right, or at least one that modifies AHPND virulence (Prachumwat et al., 2018). The gene is homologous to the ZON gene of *Vibrio cholerae* and was also present in the VP$_{AHPND}$ isolate from China (Yang et al 2014) and many other AHPND associated Vibrio isolates within the National Centre for Biotechnology Information (NCBI) database (Table 7). Beyond AHPND-related studies, the ZON and Cholerae toxin genes (Ctx) were detected in a *Vibrio cholerae* strain that was highly pathogenic to larval *P.monodon* (Joseph et al., 2015). Parvathi (2011) reported the prevalence of the ZON gene (15%) as one of five virulence-related genes targeted by PCR when investigating *Vibrio harveyi* isolates collected from shrimp hatchery systems. Although no toxin gene was identified, a pathogenic *V.proteolyticus* strain was reported to affect the zonulae adherents of the gut of *Artemia*, whereby the damage of the cell junctions between gut epithelial cells allowed bacterial cells to penetrate the gut epithelial layer (Verschuere et al., 2000).

The Zon, hemo and RTX genes detected in this study require further investigation to ascertain their significance in hatchery mortality and prawn health. The molecular detection of genes does not demonstrate the expression of the

various factors. However, preliminary HTS,followed by qPCR represents a fit for purpose tool to monitor the presence of multiple genes, and combined with other analysis, presents a specific, sensitive, rapid turnaround, high throughput, quantitative and cost effective option to monitor the presence of genes to determine an association with virulence and toxin genes in bacterial diseases in shrimp production systems. Although Vibrio bacteria are ubiquitous in the marine environment, there is limited knowledge of the presence of specific toxin genes. Analysis of the pond collected samples was conducted to gain a snap-shot of the frequency of detection of the Zon, hemo and RtX genes.(Refer to Appendix 3 for Primer and Probe sequences)

Table 8 List of Vibrio strains in the NCBI database collected from shrimp samples that contain the Zon occludens toxin gene. Table includes bacterial species, host species, country of origin, bacterial strain if provided, NCBI database number and any disease that was associated with the accession.

Species	Host species	Country of origin	Strain	NCBI ref	Disease
V.campbelli	shrimp-undefined	Thailand	VIB391	MNLF01000022.1	ND
V.campbelli	shrimp-undefined	Thailand	VIB392	MNLF01000043	ND
V.campbelli	*P.monodon*	Thailand	1114GL	CP019634.1	ND
V.cholerae	shrimp pond sediment	Bangladesh	CRA_S9	QJSG01000030	ND
*V.harveyi**	L.vannamei	Mexico	CAIM 1792	CP033144.1	BRS
V.owensii	L.vannamei	China	V180403	CP033144.1	AHPND
V.parahaemolyticus	*P.monodon*	Malaysia	MSR17	Ahmed et al., 2019	ND
V.parahaemolyticus	shrimp-undefined			LFYM01000076.1	ND
V.parahaemolyticus	shrimp-undefined	Vietnam	13-028/A2	NZ_JOKT01000001.1	AHPND
V.parahaemolyticus	*L.vannamei*	South Korea	SM4	NZ_QPQE01000002.1	AHPND
V.parahaemolyticus	shrimp pond water	Malaysia	NA1	POBW01000024.1	AHPND
V.parahaemolyticus	*L.vannamei*	Malaysia	ND13	POBH01000024.1	AHPND
V.parahaemolyticus	*L.vannamei*	Malaysia	ND12	POBI01000025.1	AHPND
V.parahaemolyticus	*L.vannamei*	Malaysia	ND11	POBJ01000025.1	AHPND
V.parahaemolyticus	*L.vannamei*	Malaysia	ND16	POBE01000022.1	AHPND
V.parahaemolyticus	*L.vannamei*	Malaysia	ND15	POBB01000005.1	AHPND
V.parahaemolyticus	*L.vannamei*	Malaysia	ND22	POAYO01000025.1	AHPND
V.parahaemolyticus	*L.vannamei*	Malaysia	ND24	POAWO1000025.1	AHPND
V.parahaemolyticus	shrimp-undefined	Vietnam	M1-1	PDDQ01000038.1	AHPND
V.parahaemolyticus	*L.vannamei*	Thailand	NCKU_TV_3HP	JPKS00000000	AHPND
V.parahaemolyticus	*L.vannamei*	Thailand	NCKU_TV_5HP	JPKT00000000.1	AHPND
V.parahaemolyticus	*L.vannamei*	China	NCKU_CV_CHN	JPKU00000000	AHPND
V.parahaemolyticus	*P.japonicus*	China	20140722001-1	CP034292.1	AHPND
V.parahaemolyticus	shrimp-undefined	China	CHN25	CP010884.1	ND
V.parahaemolyticus	shrimp-undefined	China	PB1937	CPO22243	AHPND
V.vulnificus	shrimp-undefined	China	Vv004	QCYB01000004.1	ND
V.xuii	shrimp culture water	China	DSM 17185	LHPK01000016	ND

6.5.2 Percentage of positive detections and distribution of Zona occludens toxin gene (ZON) from farm collected prawns

The Zon toxin detection was variable across farms and ponds (Figure 16). The Zon toxin was not detected in a large proportion of pond collected samples. Only 9 ponds were positive for the detection of Zon toxin gene with greater that 50% detection. The majority of ponds had less than 38% of samples positive and 24 of 55 ponds were negative for the detection of Zon. Only 6 ponds had 100% of samples positive for the detection of Zon. Only one pond, namely 29, had an average Ct value detection below 30.
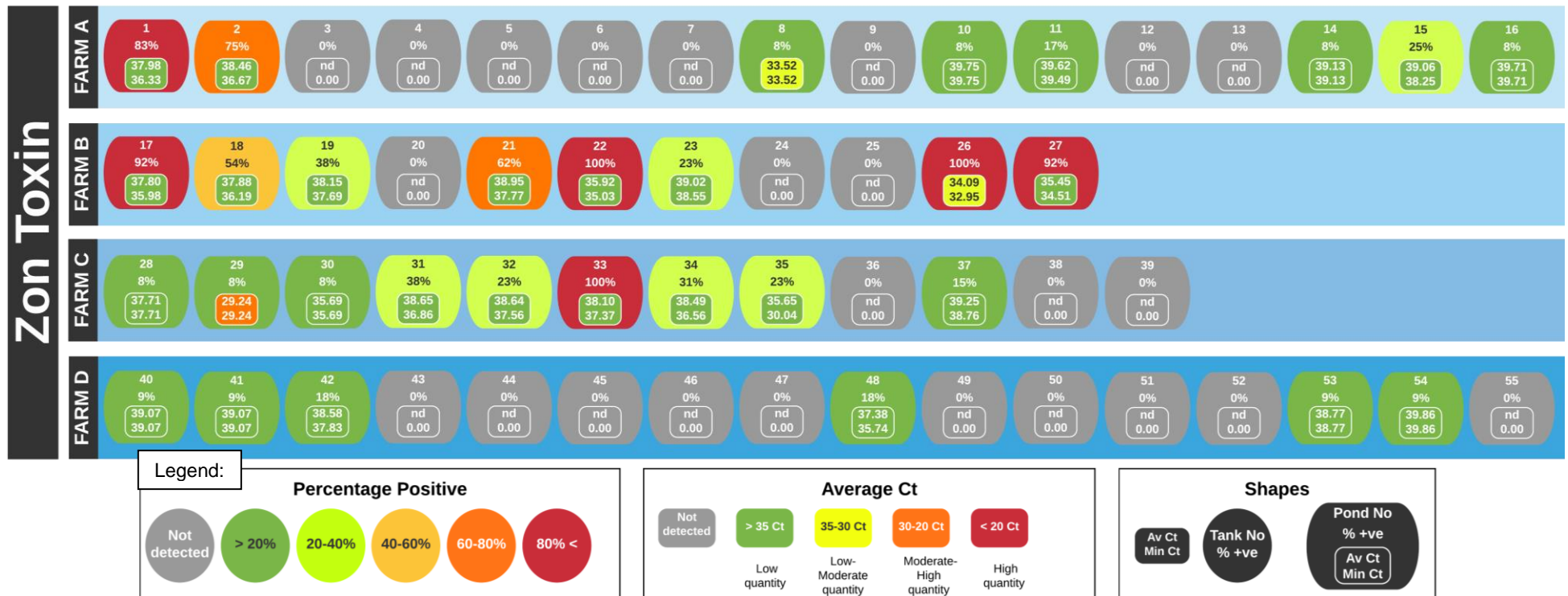
Figure 16 Detection of the Zona occludens toxin gene (Zon) from 55 ponds sampled from 4 farms. Detection is indicated as the percentage of positive detections (%) and the average and minimum Cycle threshold (Ct) of the positive detections. Refer to Legend for indication of colour coding of percentage positive, average and minimum Ct value. Refer to Legend for indication of colour coding of percentage positive, average and minimum Ct value

### 6.5.3 Percentage of positive detections and distribution of Repeats in toxin- toxin gene (RtX) from farm collected prawns

Repeats-in-toxin was the most frequently detected bacterial toxin gene (Figure 17). RTX was detected in a very high proportion of samples in one farm (Farm C) and variably from low to high percentage of positive in two farms. The gene was generally only detected at a very low quantity of target (Ct over 30) with only one singe pond (Pond 35) having an average Ct below 30.
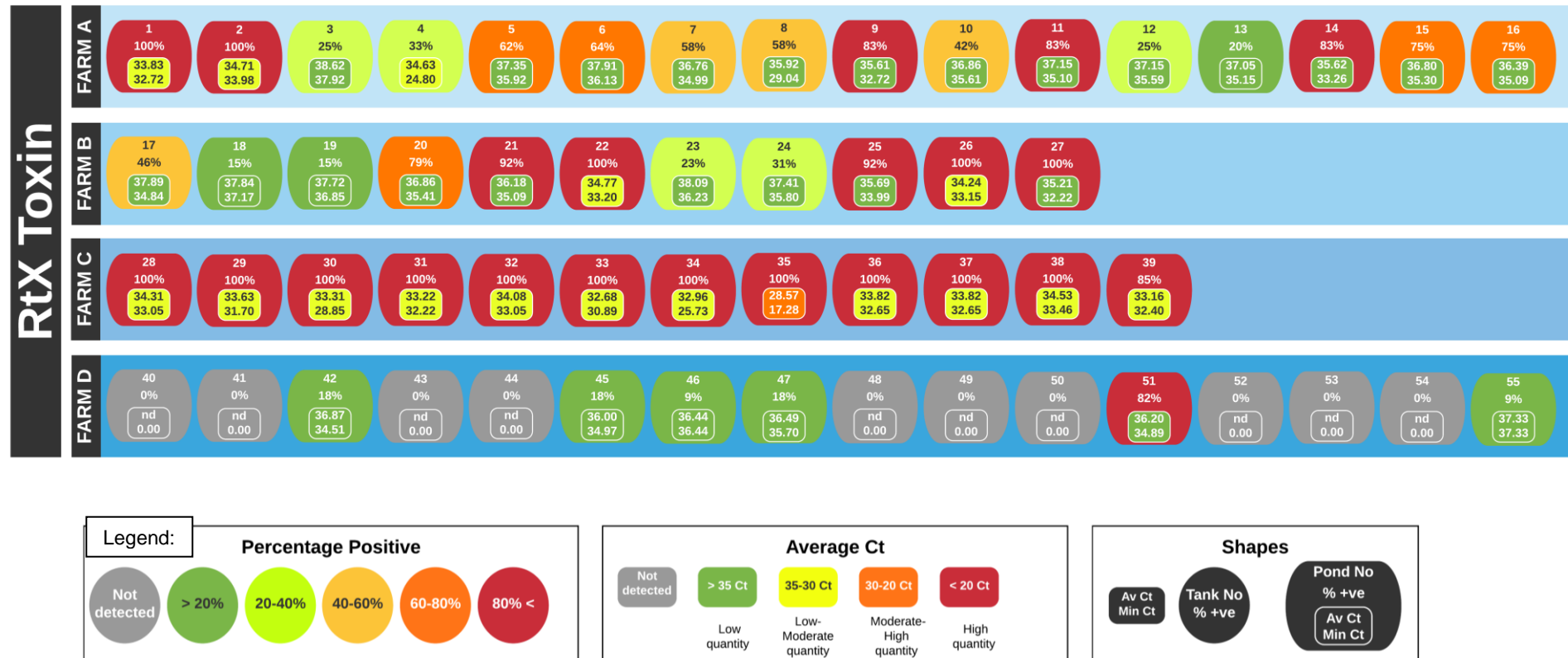
Figure 17 Detection of Repeats-in-Toxin gene (Rtx) from 55 ponds sampled from 4 farms. Detection is indicated as the percentage of positive detections (%) and the average and minimum Cycle threshold (Ct) of the positive detections. Refer to Legend for indication of colour coding of percentage positive, average and minimum Ct value

6.5.4 Percentage of positive detections and distribution of Hemolysin D toxin gene (hemo) from farm collected prawns

Aside from Pir-A, which was not detected in any pond collected sample, Hemolysin D was the least frequently detected bacterial toxin gene (Figure 18). Hemolysin D was detected in a high percentage of samples from a single farm. There were no high copy number detections of Hemolysin D from any pond collected sample. Although Farm C had the highest percentage of positive detections of Hemolysin D, it had very low frequency of positive detections of the Zon occludens toxin.

Figure 18 Detection of Hemolysin D toxin (Hemo) gene from 55 ponds sampled from 4 farms. Detection is indicated as the percentage of positive detections (%) and the average and minimum Cycle threshold (Ct) of the positive detections. Refer to Legend for indication of colour coding of percentage positive, average and minimum Ct value

6.6 Detection of pathogen targets from ad hoc, non-scheduled pond collection (sub-optimal pond production)

Samples were submitted from 20 ponds that exhibited sub-optimal production. Only nine of the 20 ponds that exhibited sub-optimal productivity had high percentage of positive detections and high viral calculated copy number for any of the endemic pathogens analysed in this study (Figure 19 and Figure 20). IHHNV, GAV and When-2 were the only pathogens detected in a high percentage of samples, with a high calculated copy number (low Ct) (Figure 19). Twelve sub-optimal production ponds had lower average and minimum Ct for the detection of IHHNV than that detected in scheduled sampling for IHHNV (32.37-18.55). Seven of the sub-optimal production ponds, also had lower average and minimum Ct for the detection of When-2 than the scheduled sampling ponds (28.66-18.55) and six of the ponds had lower average and minimum Ct for the detection of GAV (32.59-23.83). All of the ponds with high GAV and high When-2 detection were also positive for high IHHNV detections. Because DOC was not standardised, no statistical comparison between sub-optimal and scheduled sampling pond data has been conducted. There was an increased number of ponds that had 100% detection of IHHNV, When-2, HDV, Zon, hemo and Rtx in the sub-optimal production ponds compared to the scheduled sampling collection ponds (Table 9). The majority (n=11) of ponds that experienced sub-optimal productivity did not display any association with any particular pathogen within the targeted qPCR analysis. In some cases, the pond samples were not temporally aligned with the point of reduced productivity i.e. were collected at the time of harvest after the pond had displayed reduced productivity over a number of months. Sampling at the time of incident would have provided more robust investigation, however, in many cases the time of incident did not obviously present to the farm, possibly indicating an event early in the stocking process. The analysis on sub-optimally performing ponds indicates no single pathogen can be directly consistently linked with poor production. It is possible the cumulative loading of pathogens impacts on prawn health. The role of multi-pathogen infection on prawn health has not been described in Australian prawn production and rarely considered in experimental research analysis directed at the effect of particular pathogens.

Table 9. Percentage of ponds with 100% positive detection of targets from scheduled sampling and sampling of sub-optimal production ponds.

Pathogen Target	Percentage of Ponds with 100% positive detection (%)	
	Scheduled sampling	Sub-optimal production
GAV	100	100
IHHNV	30.9	75
When-2	12.7	40
HDV	0	15
Rtx toxin gene	29	50
Zon toxin gene	5.4	40
Hemo toxin gene	1.8	10
*No detection of WSSV, YHV-1, YHV-7 or Pir-A from any of the pond collected samples		
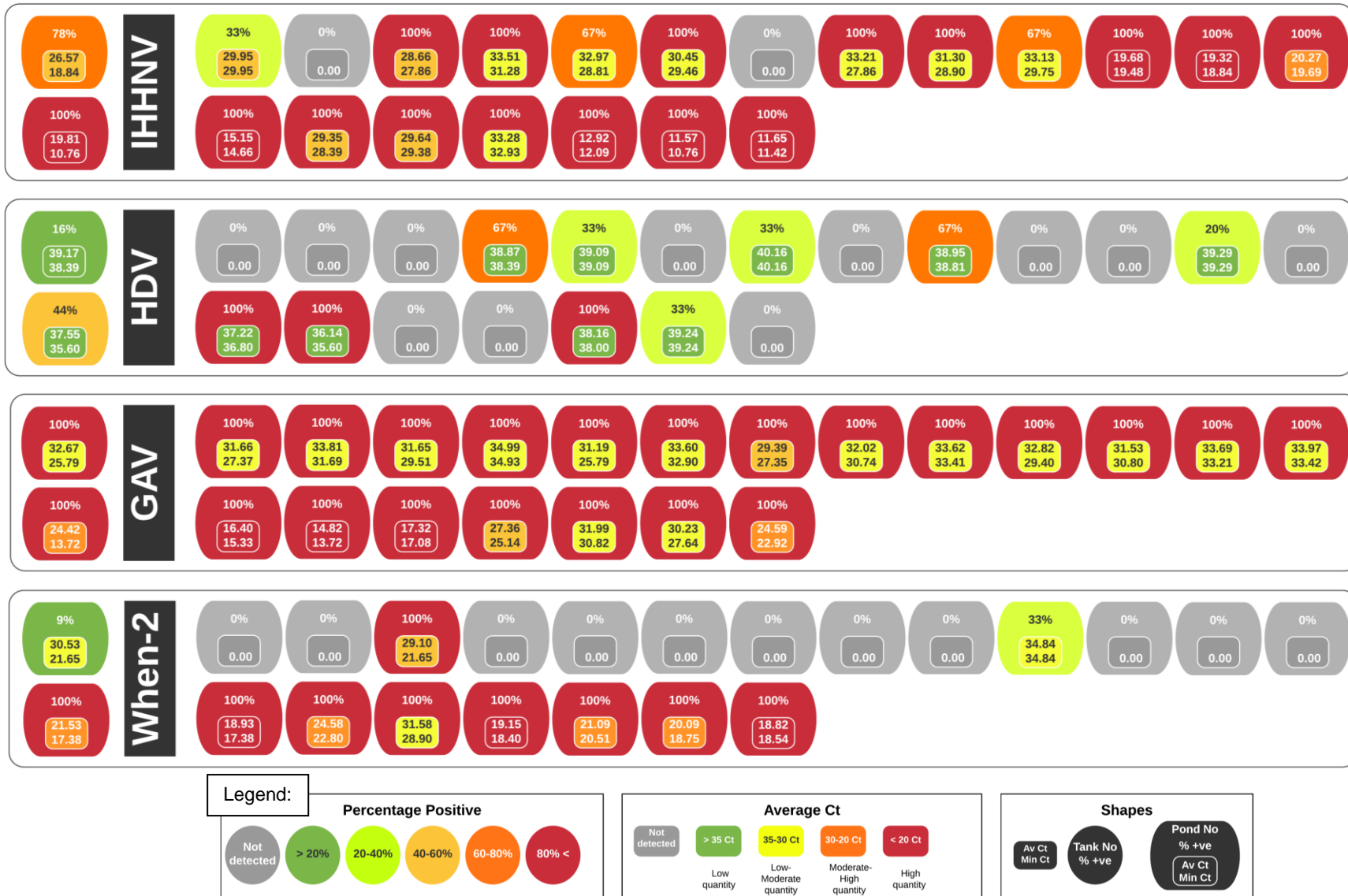
Figure 19 Summary of Viral target detection from ponds exhibiting sub-optimal production. Refer to Legend for indication of colour coding of percentage positive, average and minimum Ct value
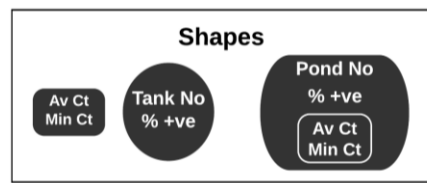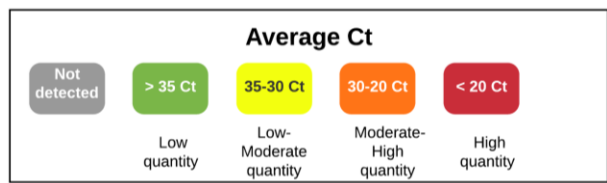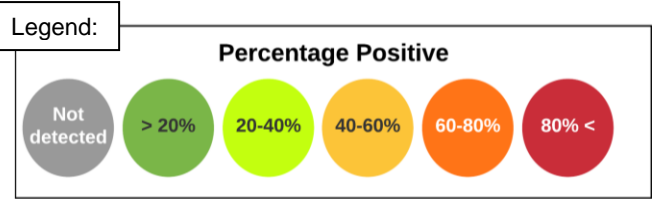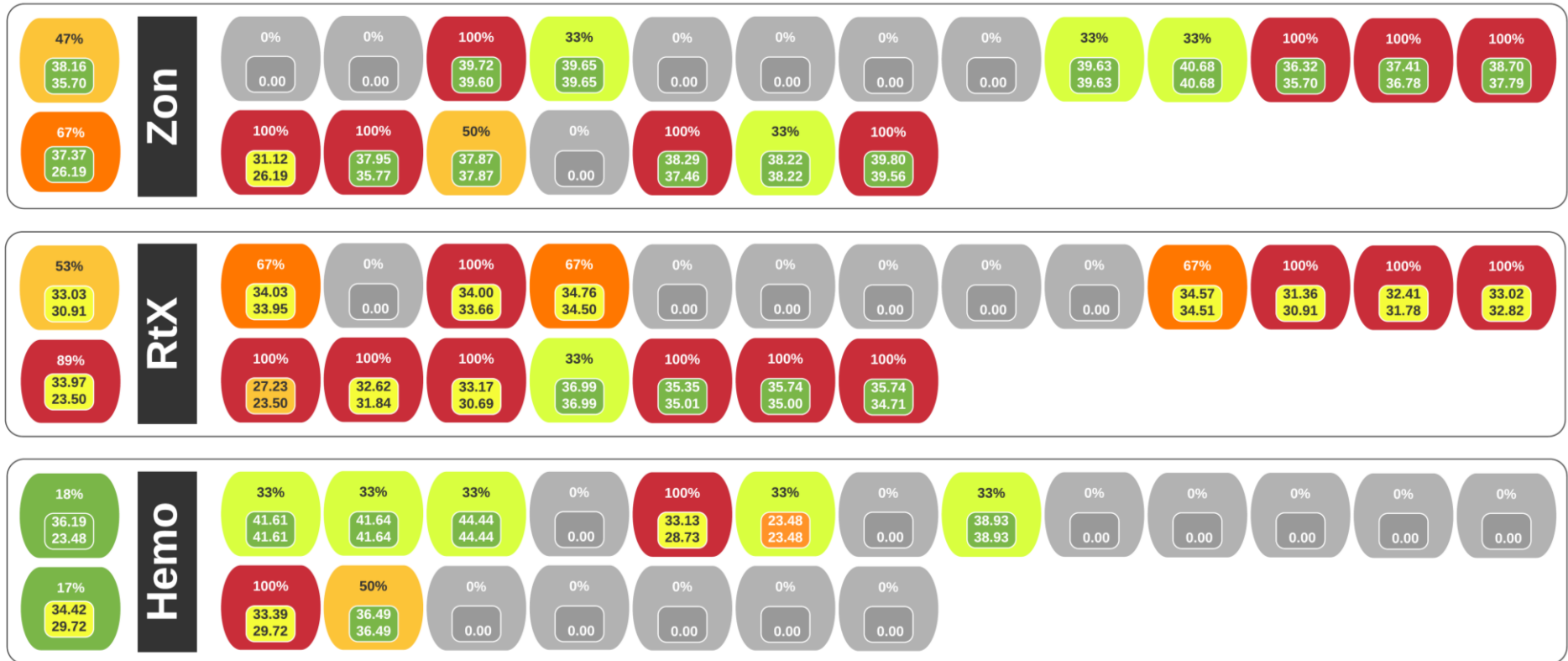
Figure 20 Summary of Bacterial toxin gene detection from samples collected from ponds exhibiting sub-optimal production. . Refer to Legend for indication of colour coding of percentage positive, average and minimum Ct value

6.7 Statistical analysis between pathogen presence and intensity with productivity in grow-out prawn production

Overall survival (%), food conversion ratio (mass of feed (g) required to produce 1 (g) of prawn) and stocking density for each of the subject 55 ponds was provided by participant farms. Each parameter varied across farm and pond. Survival ranged from 26-100%, FCR ranged from 1.7 to 2.38 and stocking density ranged from 30 to 80 prawns per m². Overall pathogen and production matrices are indicated in Figure 21. The variation in survival, stocking density and FCR create considerable difference in profit per farm. A broad indication of variation in gross farm gate sale ($), total food purchased (kg), total feed costs and differential costs of gross farm gate, less feed costs, are estimated in Table 10. Additional costs including labour and electricity, which are a considerable proportion of farm operating costs are not indicated. Statistical analysis of Pearson's correlation and multivariable linear regression modelling was conducted between farm production data and pathogen data to investigate any correlation or trends. The data set to conduct this analysis is not ideal and no strong conclusions of the correlations are proposed, but rather trends for future consideration indicated.

Table 10 Gross estimates of harvest weight, farm gate price and cost of feed and differential (farm gate – feed costs) at best (100%) and worst (26%) case survival (%) and best (1.7) and worst (2.38) case food conversion ratio on pond production assuming a harvest weight of 30g and feed cost of $3/kg.

Production		Sale Price		Total feed required (kg)				Total Feed costs ($) assuming $3/kg			
Stocking density (prawns/ m²)	Harvest weight (kg)	Farm gate ($)		FCR 1.7		FCR 2.38		FCR 1.7		FCR 2.38	
		Survival		Survival		Survival		Survival		Survival	
	Survival										
26%	100%	26%	100%	26%	100%	26%	100%	26%	100%	26%	100%
70	5460 / 21000	$109,200.00	$420,000.00	9282	35700	12994.8	49980	$ 27,846.00	$107,100.00	$ 38,984.40	$149,940.00
50	3900 / 15000	$ 78,000.00	$300,000.00	6630	25500	9282	35700	$ 19,890.00	$ 76,500.00	$ 27,846.00	$107,100.00
30	2340 / 9000	$ 46,800.00	$180,000.00	3978	15300	5569.2	21420	$ 11,934.00	$ 45,900.00	$ 16,707.60	$ 64,260.00

Stock. Den.	Differential Cost comparing FCR and Survival			
	23%	100%	23%	100%
70	$ 81,354.00	$312,900.00	$ 70,215.60	$270,060.00
50	$ 58,110.00	$223,500.00	$ 50,154.00	$192,900.00
30	$ 34,866.00	$134,100.00	$ 30,092.40	$115,740.00

Figure 21 Mapped Pond productivity factors including stocking density, Survival (%), Food Conversion Ratio (FCR) and Percentage of Positive detections and Average Ct value of detection of Viral and Bacterial Toxin gene targets.

6.7.1 Correlation between productivity factors: Stocking density, FCR and Survival/Mortality

Pearson's correlation coefficient (ρ) measures linear correlation between two variables. The coefficient ranges between 1 to -1, with each extreme representing perfect positive (y increases as x increases) or negative (y decreases as x increases e.g. survival vs. mortality) correlation between the two variables, respectively. The application of this analysis is limited as the robustness of the correlation estimates can be degraded when heavy noise is present (eg. in complex systems where many factors may contribute to the variation present in each variable). The results should not be interpreted as supportive of causative outcomes as there are many variables that contribute to outcomes that were not incorporated in this analysis. Here, Pearson's correlation analysis is applied to provide preliminary and interpretable statistics to identify potential variables and associations of interest for further investigation.

The summarised output of the Pearson's correlation matrix between the production statistics and the percentage of positive detections of each pathogen target are provided (Table 11). Analysis indicated significant correlation between the productivity variables (FCR, stocking density and survival). There was a strong negative correlation between FCR and Survival (ρ = -0.54), whereby higher survival rates were correlated with lower FCR values (Table 11). This relationship is expected and is indicative of a more efficient conversion of feed input to prawn production. The complementary inverse relationship of increased mortality and increased FCR was also consequently observed (ρ = 0.54), indicative of an inefficient production system (Table 11). A highly significant positive correlation was observed between stocking density and mortality (ρ = 0.69) and the complementary inverse relationship between stocking density and survival (ρ = -0.69) (Table 11).

Of the pathogen parameters, the percentages of positive detections for RtX and Hemo were observed to have the highest absolute correlation with density and survival (and inversely, mortality) (Table 11). Counter-intuitively these pathogens were strongly negatively correlated with density (ρ ≈ -0.6; i.e. RtX and Hemo were more prevalent when ponds were stocked at lower density) and were strongly positively correlated with survival (ρ ≈ 0.5; i.e. survival was higher in ponds where RtX and Hemo were more prevalent). It is highly likely that these correlations are a product of confounded factors relating to the specific farm management activities rather than causative correlations. The restriction to protect commercial information prevents further expansion on this topic. The percentage of positive detections of IHHNV was also positively correlated with both density and mortality (ρ ≈ 0.5). That is, with increasing density, the percentage of positive detections of IHHNV increased and with increasing percentage of positive IHHNV detections, mortality rate increased. Again, these results should not be interpreted as causation relationships as there are many variables that can contribute to outcomes that were not incorporated in this analysis. Instead, these values are aimed to provide preliminary and interpretable statistics to identify potential variables and associations of interest for further investigation.

Table 11 Pearson's correlation matrix between pond productivity factors of Mortality (%), Survival (%), Density (prawns/sq.m) and Food Conversion Ratio (FRC) and the a percentage of positive detections (%) of each pathogen target (IHHNV, HDV, When-2, GAV, Zon, Rtx and Hemolysin). Highly significant correlations (p<0.001) are indicated with *.

	% IHHNV	% HDV	% When-2	% GAV	% Zon	% RtX	% Hemo	FCR	Density	Survival	Mortality
FCR	0.27	0.00	0.32	0.00	0.26	0.08	-0.09	1.00*	0.24	-0.54*	0.54*
Density	0.50*	-0.19	-0.05	0.17	0.06	-0.58*	-0.59*	0.24	1.00*	-0.69*	0.69*
Survival %	-0.46*	0.29	-0.13	-0.18	-0.10	0.47*	0.51*	-0.54*	-0.69*	1.00*	-1.00*
Mortality %	0.46*	-0.29	0.13	0.18	0.10	-0.47*	-0.51*	0.54*	0.69*	-1.00*	1.00*

Although the analysis presented between pathogen detection and pond production practises and outcomes are not robust, there is an opportunity to develop models to identify risks to farm productivity. This study identified correlations with a less than ideal dataset. The Pearson's correlation identifies trends between two variables and does not incorporate the contribution of multiple variables to influence a dependant variable. Considering the outcomes of production are most likely multifactorial, it is expected Pearson's correlation is not a robust analysis. The absence of a dominant correlation between any 2 variables is evident in the mapped summary of all parameters indicated by Figure 21.

6.7.2 Multiple linear regression model

Multiple linear regression analysis was performed to investigate potential relationships between the pathogen detection results and pond survival (%). Multiple linear regression analysis differs from Pearson's correlation analysis as it includes multiple input variables (29 pathogen detection variables) within the analysis to estimate an outcome variable, in this case pond survival (%). The analysis did not identify any single variable to be highly significant as a predictor of survival; however, cumulatively the model was able to estimate survival, with a coefficient of determination to the actual survival values of $r^2 = 0.73$ ($p < 0.05$) (Figure 22). Comparison of the predicted and actual survival values indicated 6 ponds had an actual survival 12-22% greater than the predicted survival. Conversely, 10 ponds had actual survival 12-30% lower than predicted survival. These results highlight the limitations of the model, likely derived from the nature of the small dataset and potential farm-based confounding factors (e.g. different management strategies) which were not incorporated in the model. More detailed investigation into the pond records may identify risk factors or management interventions for further inquiry. Investigation into the application of pathogen and other pond data is on-going. The application of machine learning and neural networks to identify relationships and trends across complex systems represents a large and relatively under-utilised opportunity for the prawn farming industry. Models incorporating a range of water quality parameters, productivity data which is currently collected by farms, and quantitative pathogen screening data, may enable the early prediction of disease outbreaks or forecasting of crop success (Kheim *et al.*, 2020). Such progression would facilitate highly informed management practices and may improve understanding of the prawn-pathogen-environment model.
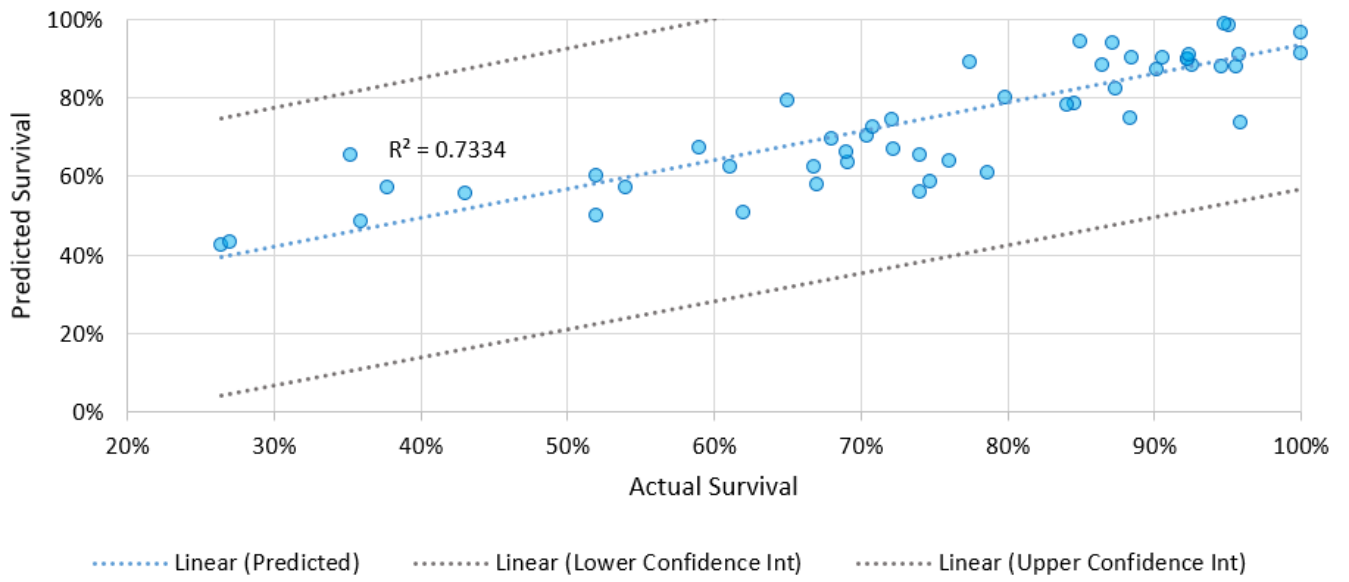
Figure 22 Predicted versus Actual Survival (%) in ponds based on multiple linear regression model incorporating pathogen detection data to predict pond survival

7 Findings and Recommendations

Effective biosecurity is an essential component to support profitable and sustainable prawn aquaculture. This project has significantly increased the volume of data describing prawn pathogen distribution in the Australian prawn farming industry. The project completed analysis of ~ 2,500 samples for the detection of eight pathogen targets. Whilst this is the most significant dataset available to support industry to guide biosecurity management, the project has highlighted significant gaps in the current understanding of prawn pathogens in the Australian prawn farm ecosystem. Although the current planned expansion of the industry has potential to deliver significant gains to Northern Australian economies, such potential is threatened by the risk of disease outbreaks which are likely to increase as a consequence of industry expansion, impacts of global climate change and as the geographic range of WSSV expands. The need to improve biosecurity is not restricted to the prawn farming industry and was recognised broadly by the aquaculture sector in the Northern Australian Aquaculture Situational analysis (NAASA) (Cobcroft *et al.*, 2020). The number one key priority of the actions identified for the development of the Northern Australia Aquaculture sector were to bolster biosecurity (Cobcroft *et al.*, 2020). Specifically, the sector analysis indicated:

- Review of policy and meeting the requirements for improved risk assessment and R&D programs to better understand biosecurity risk and management at the border.

- Increased pathogen understanding, documented risks, transmission pathways, and practical surveillance implemented for the aquaculture industry in northern Australia.

- Establishment of the most effective structures to develop high health lines for key production species (Cobcroft *et al.,* 2020).

This project supports the broad recommendations proposed by the NAASA with more specific details relating to the Australian Prawn Farming industry. The key finding and recommendations of this project are presented in the following sections.

8 Key Findings

8.1.1 No detection of WSSV from wild captured broodstock

The absence of detection of WSSV and YHV-1 in any broodstock sample suggests the current broodstock fisheries are a source of WSSV-free and YHV-1 free broodstock.

Although WSSV has been detected in wild crustaceans in SE Queensland, there was no detection of WSSV or YHV-1 from any broodstock sampled from the northern sites of broodstock collection. Based on overseas occurrences where WSSV has expanded in geographical range following initial disease incidents, the timeline for continued access to WSSV free stock may be closing for the industry and with this predicament domestication of *P. monodon* should be pursued as a high priority.

8.1.2 Detection of Wenzhou Shrimp Virus-2

Wenzhou Shrimp Virus-2 was detected in retail imported frozen prawns and East Coast Queensland sourced broodstock.

Wenzhou Shrimp Virus-2 was originally detected by HTS in wild *P. monodon* and *Exopalaemon carinicauda* purchased from fisherman in Wenzhou China between 2011 and 2013 (Li *et al.,* 2015). The only other published record regarding When-2 is the detection from Australian *P. monodon* by HTS (Huerlimann *et al.*, 2018). The present study detected When-2 from only EC QLD sourced broodstock, pond collected samples, glass shrimp and 100% of the imported retail prawns. The When-2 target was not detected in any NT sourced wild broodstock. The virus is not characterised; however, in this study, When-2 was associated with a number of the ponds that exhibited sub-optimal production. The historical prevalence of When-2 and origins of the virus is unknown. Further research is required to characterise the virus and determine the significance of the Wenzhou Shrimp Virus-2 on farmed prawn production.

8.1.3 High percentage of positive detections of pathogens in farmed prawn production line

There is a high percentage of positive detections of the pathogens targeted in this study, in particular GAV (~90%) and IHHNV (>43%). The pathogens are present in a high percentage of samples in all stages of production from broodstock to pond collected samples. The findings of this report demonstrate the ubiquity of viral and bacterial pathogens throughout all stages of prawn production in Australia.

The presence of pathogens in broodstock, post-larvae and across both pond prawns and cohabitating organisms (in ponds and the external environment), are indicative of limited attempts to exclude these pathogens and gaps in the effectiveness of current biosecurity measures. Results of the current project indicate there is a high likelihood of introduction of endemic pathogens into the prawn culture system with wild captured broodstock. The pathogens that were most prevalent in broodstock continued to be detected in a high percentage of samples and at varying levels of infection throughout the other stages of prawn production, indicating that while industry relies on the intake of wild captured broodstock to produce the annual crop, there will be limited opportunity to adopt an exclusion or containment approach to manage many of the endemic pathogens. The high percentage of positive detections of GAV and IHHNV in wild broodstock in this project suggest industry will not have access to sufficient quantities of Australian wild captured broodstock to establish SPF lines that are free of GAV and IHHNV and retain sufficient genetic diversity for long term sustainability.

8.1.4 Not all pathogens present in broodstock transfer to pond production

> Not all of the pathogens present in broodstock were transferred to post-larval batches and pond production.

The results of this study suggest that improved biosecurity management at the hatchery level will lead to reduction in pathogens in the PL and subsequent pond production stages. This applies specifically to the pathogens that were present at lower frequency in the samples, namely, Pir-A and Wenzhou Shrimp Virus-2. Although both targets were detected in broodstock, Pir-A was not detected in any pond samples and Wenzhou shrimp virus-2 was present in 24/55 ponds, 23 of which were from two farms, one of which had no active management of any of the targeted pathogens. When-2 was not detected in any pond samples from one farm and only detected in two samples from another farm. Under current conditions, for industry to ensure access to sufficient quantities of broodstock to stock ponds, infected broodstock will need to be accepted in their production lines. Noting, acceptance of infected broodstock will require on-going monitoring and management of those pathogens rather than previous programs whereby endemic pathogens, except GAV, have been largely ignored.

8.1.5 Multiple infections were frequent at all stages of production

> In total, 72.4% of broodstock, 50% of post larvae samples and, 76% of pond prawn samples were simultaneously positive for the detection of multiple (2 – 5) pathogens in the absence of disease incidents. The percentage of positive detections of multiple pathogen detections increased in ponds with reduced productivity.

Many previous studies investigating productivity associations with pathogen presence have focused only on the detection of a specific single pathogen target (Sellars *et al.,* 2019; Noble *et al.*, 2017; Spann *et al.,* 1997). The results of the present study demonstrate that the majority of Australian prawns are infected with multiple pathogens, in the absence of disease, and an increase in the occurrence of 100% detection of IHHNV, When-2, HDV, Zon and Hemolysin D toxin was noted in ponds with reduced productivity. With the exception of a single pond, all instances of high quantity of GAV detection in ponds was accompanied by high quantity detections of IHHNV or When-2, yet if analysis was conducted for GAV only, an association with disease and GAV detection would be concluded. These results highlight the level of ambiguity that exists in determining the degree of risk presented by the presence of a pathogen based on a single pathogen result. A review of the historic literature indicates the impacts of most endemic viral pathogens have not been comprehensively demonstrated (and perhaps overstated). Research is required to enable evidence based and prioritized management of endemic pathogens on-farm, and to better understand the potential effects of multiple pathogen infections on the health and productivity of *P. monodon*. Experimental challenge with purified characterized viral pathogens should be completed with urgency, as this will allow industry to prioritize management of pathogens with robustly demonstrated pathogenicity.

8.1.6 The percentage of positive pathogen target detections and intensity of detection varies with geographic source of broodstock

> Comparatively, the percentage of positive pathogen detections and intensity of positive detection varied with geographic origin of broodstock samples. Samples collected from East Coast Queensland sources were positive more frequently and in higher average quantity for the detection of GAV and When-2, compared to NT-sourced stock. Conversely NT-sourced broodstock were more frequently positive for Pir-A and were detected with higher average quantity of IHHNV and Pir-A.

The findings of this study suggest that preferential selection of NTWM broodstock will reduce the likelihood of pathogen introduction into the hatchery. In total, 14% of NTWM broodstock analysed were negative for the detection of all pathogens targeted, compared to only 0.6% of the ECWM broodstock. However, the intensity of pathogen detection was variable both within and between genetic sources, suggesting that data-driven broodstock selection, from individual broodstock pathogen screening results will allow for greater management of pathogen presence and quantity in stocked animals. Analysis on pond collected samples indicate both NTWM and ECWM PL stocked ponds are susceptible to infection with all pathogens. The differential detection of pathogens from wild broodstock between geographic regions should not be interpreted as a genetic superiority of NT sourced broodstock, but could be due to a range of factors, one of which may be the anthropological activities of East Coast Queensland. Although this project has completed the largest multi-pathogen, multi-life stage analysis ever completed in Australian prawns, analysis of a larger set may reveal different trends. There was limited temporal variation in the broodstock analysed in this study so seasonal and annual fluctuations in pathogen detection were not identified. Seasonal variation in prawn pathogen detection from wild prawns is reported from overseas studies and the repeated WSSV detections from wild crustaceans in northern Moreton bay have each occurred during the March sample collections.

8.1.7 Glass prawn, *Acetes spp,* are confirmed by qPCR as carriers of prawn pathogens

Glass prawn, *Acetes* spp., were confirmed through qPCR analysis as substantiative carriers of prawn pathogens. When-2, IHHNV, GAV and HDV were detected from glass prawn co-habituating in prawn ponds. Glass prawn and Jelly prawn, *Palaemon* spp., collected from farm intakes or reservoirs, were positive for the detection of IHHNV, HDV, WSSV, When-2 and Pir-A. Glass prawn and jelly prawn may act as reservoirs and sources of infection in prawn farms and the surrounding environment.

Knowledge and consideration of potential pathogen reservoirs within the culture environment is of critical importance for biosecurity management. Although other crustacean species also pose a risk as reservoirs of prawn pathogens, this project focussed on glass and jelly prawns species due to their frequent presence and high number across tropical aquaculture farms. Management or exclusion of these organisms will likely be challenging due to their ubiquitous nature and small size and the semi-open characteristics of Australian prawn production systems. The presence of reservoir species should be considered within the context of development of domesticated lines, especially those targeting freedom from pathogens. Regardless of status at stocking, even considering the low numbers of samples tested in this project, it is evident carrier species such as glass prawn are very likely to enter the pond environment. Industry will need to consider the benefits of SPF, compared to SPT/SPR approaches, noting that stocking with SPF will not confer protection from infection if/when the stock is exposed to pathogens during grow-out production. Some overseas production systems report successful super-intensive production of prawn in highly biosecure facilities. In the absence of high levels of exclusion, the risk of transmission of pathogens via glass prawn should be considered within farm biosecurity management plans. The introduction of WSSV via this path poses a significant threat to prawn production in regions where WSSV exists.

8.1.8 The presence of a pathogen does not invariably lead to a disease incident

The high percentage of positive pathogen target detections was ubiquitous throughout all production stages in the absence of disease outbreaks.

The capacity to structure and apply asset-based protection is reliant upon comprehensive understanding of the pathogens present, or potentially threatening a system and the environmental factors which may underwrite disease

outbreaks. This study has highlighted the extensive presence of endemic pathogens within each stage of prawn production in Northern Australia. Only 7% of incoming broodstock were negative for the detection of all pathogens targeted in this study. Similarly, only 12% of the post larvae from these broodstock and 1 % of the samples collected from ponds were negative for the detection of the targeted pathogens. Despite the near absolute positive detections of some pathogens, such as GAV (>96%), throughout larval rearing and grow out production stages, no disease outbreaks were reported across any of the ponds. The absence of disease outbreaks does not consider the impact of an endemic pathogen on productivity indexes such as survival (%), growth rate, feed conversion ratio or, length of days till harvest, all of which contribute significantly to the cost and profitability of a business. Survival in the scheduled sample ponds ranged from 26% to 100% and FCR ranged from 2.34 to 1.7.

Increased research focus on the interaction of host-pathogen-environment factors is required to better understand the dynamics and occurrence of disease in commercial prawn production systems and how this information might be integrated into biosecurity management strategies. Options within asset-based management to avoid disease incidents when pathogens are invariably present include: to reduce the length of the pond culture duration though utilising an intermediate enclosed nursery stage, genetic selection, improved nutrition and approaches that improve the manageability of pond conditions such as reduced stocking density, increased water exchange, increased dissolved oxygen levels and, recognising a high likelihood of introduction of pathogens to prawn pond cultures, approaches to reduce risk of disease could consider the application of machine learning to identify risks to productivity (Kheim *et al.,* 2020).

8.1.9 Pathogen presence and intensity of detection is variable between farms and ponds within farms.

There was highly variable intensity and percentage of positive detections for the pathogens targeted in this study. Between individual ponds from the same farm, the differences in percentage of positive detections varied substantially. Such variability was also present between farms, with some farms positive for low or no detections of pathogens (e.g. When-2, Zon, RtX and Hemo), and other farms consistently high percentages of positive detections across multiple ponds. These patterns were also observed for the intensity of pathogen detections.

Here, results varied considerably in pathogen detection and productivity across ponds within farms and between farm sites. The causes of such marked variation are not defined or well understood. These findings indicate that pathogen management strategies using a systems-based approach, integrating site-specific environmental condition and pathogen screening data will be valuable at a within-farm and broader industry level.

8.1.10 Pir-A was detected from non-destructive pleopod samples

The Pir-A toxin gene was detected from pleopod samples using qPCR in 17% (81/475) of the broodstock sourced from the NT (none of the ECWM were positive for the detection of Pir-A). Positive detections ranged from 8.6 copies μl^{-1} to 3.8×10^4 copies μl^{-1}. The Pir-A status determined by qPCR from pleopod tissues was confirmed through testing of cultures from faecal samples conducted at the Biosecurity Sciences Laboratory (hatchery manager, pers communications).

This study reports detection of Pir A directly from broodstock pleopod samples. Commonly, the detection of the Pir-A toxin gene is facilitated via an enrichment step (Dangtip et al., 2015; Dong et al., 2019; Restrepo et al., 2018; Sirikharin et al., 2015), or is analysed from gut-associated tissues and organs, as stated within the OIE Manual of Diagnostic Tests for Aquatic Animals (2019).

Detection of Pir-A using pleopod samples does not indicate a systemic infection, and hence may not be suitable as a diagnostic sample; however, the ability to use a sample that is non-destructive, traceable to individuals (unlike faecal samples) and already collected in current broodstock biosecurity screening programs provides an opportunity to evaluate broodstock as a vector of Pir-A into aquaculture systems. Incorporating Pir-A screening with other analysis conducted on pleopod samples will improve understanding of the distribution of the Pir-A toxin gene in the absence of disease outbreaks and enable further studies investigating the risk factors and hence management priorities leading to disease outbreaks when Pir-A is present in the prawn culture system.

8.1.11 Viral and bacterial toxin gene quantity and percentage of positive detections increased from pre to post spawn broodstock

The quantity of detection of IHHNV, GAV and Pir-A markedly increased from pre-spawn to post-spawned broodstock samples. IHHNV and GAV quantity of detection increased circa 1.5×10^6 viral copies μl^{-1} and Pir-A quantity increased by 2.4×10^3 copies μl^{-1}. The percentage of positive detections of both IHHNV and Pir-A increased by ~60%. GAV was positively detected in 100% of broodstock samples at both pre-and post-spawned stages.

Increase in the percentage of positives and quantity of viral and bacterial target detection in the hatchery environment was substantial. This stage represents a key point of focus for pathogen management, with the potential for pathogen introduction via incoming broodstock and subsequent amplification due to increased stress (de la Vega *et al.,* 2004). These findings suggest initial preferential selection of broodstock based on viral and bacterial screening results coupled with pre-spawn broodstock quarantine and may minimize pathogen transmission and amplification, reducing the potential for disease outbreaks or productivity loss. In the absence of intervention, this study indicates significant transfer of pathogen targets within the broodstock maturation area. Unless improvements to quarantine are improved during broodstock capture until the broodstock screening is complete, hatcheries will face the prospect of repeated widespread culling of broodstock if WSSV is detected in a broodstock cohort. The culling of broodstock will increase pressure on an already restricted supply. Effective quarantine practices will take time to develop and Industry is advised to investigate more stringent isolation and quarantining protocols to avoid the risk of increased culling if the geographic distribution of WSSV extends.

8.1.12 Gaps in knowledge of endemic pathogens

There are significant gaps in the viral genome sequence data of pathogens collected from Australian prawns. Knowledge of genetic sequence is particularly important for guiding the design of specifically targeted assays to support the diagnosis and management of impact of particular pathogen strains on prawn production.

The GAV genome is ~ 26 000 nt. There are three complete sequences of GAV in the NCBI database, of which only one originates from Australia. All other sequences in the database are partial sequences of ~662 or 231 nt which equates to 0.8 to 2.5% of the entire GAV genome. Three sequences in the NCBI database refer to sequence derived from Midcrop mortality syndrome samples. Two of the sequences align closely with the GAV reference sequence and a third sequence is further removed from the reference sequence aligning more closely to YHV-7. The most recent GAV addition to the NCBI database collected from an Australian sample is 20 years old and there has been no recent addition of substantial length of genome sequence for GAV in the database from Australian samples since 1997. The most recent additions to the Yellow head virus complex from Australia are the YHV-7 sequences obtained from samples collected in 2012 (Refer to Figure 24). It is possible the GAV genome being detected through current activities has diverged into a non-pathogenic cluster and monitoring and management of GAV is not being targeted to a specific pathogenic strain/s.

Coupled with a degree of ambiguity that arises from the historical experimental challenges involving filtered tissue homogenates to demonstrate GAV pathogenicity, a confirmation of GAV pathogenicity involving purified sequenced viral extract is recommended. Outputs of such challenges should present the full GAV sequence to the NCBI database and be accompanied with complete genome sequencing analysis of multiple recent GAV detections to ensure biosecurity management is being directly targeted to pathogenic strains.
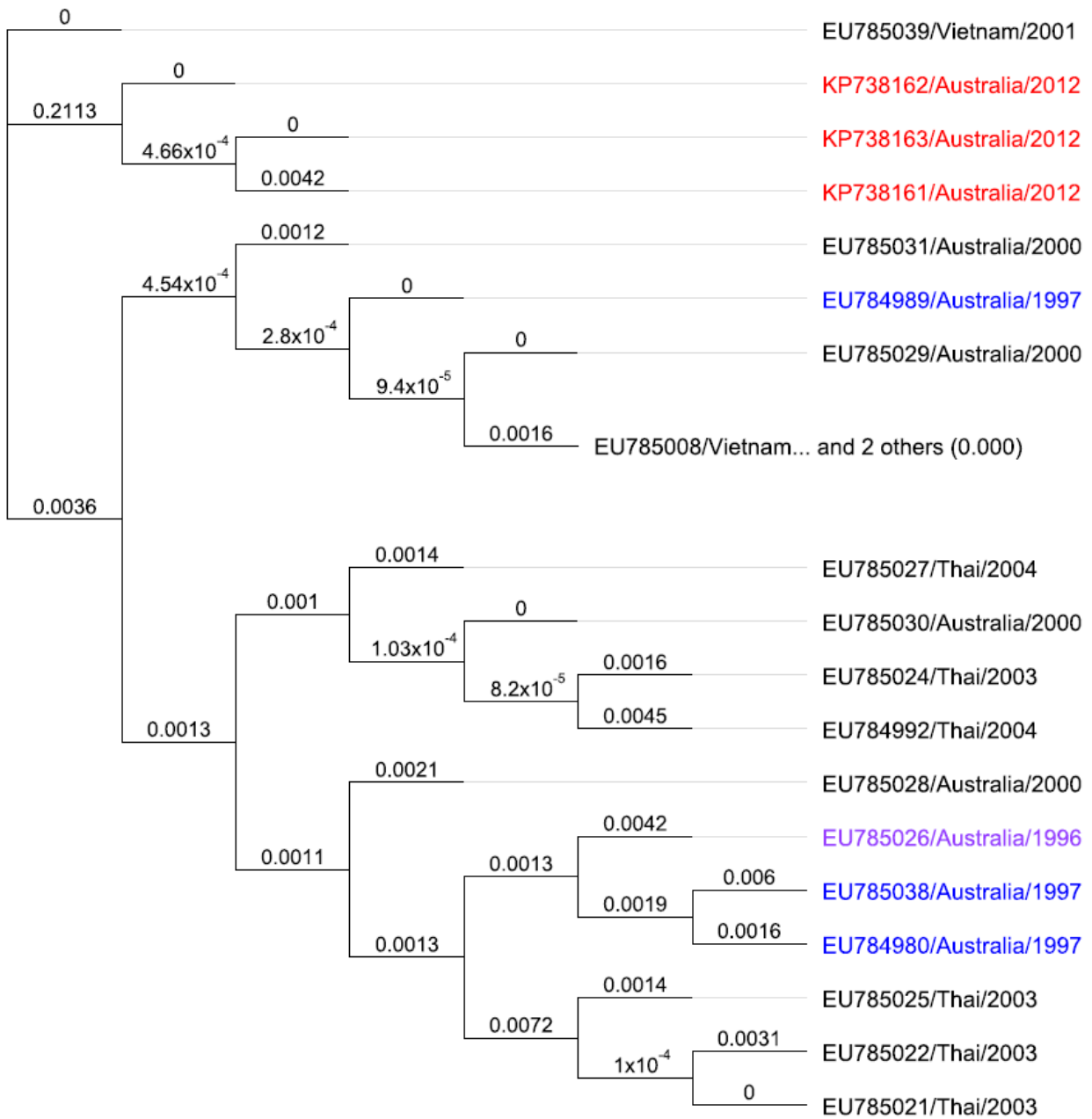
Figure 23 Neighbor-joining tree of the GAV and YHV-7 sequences from the NCBI database. Sequence is indicated by NCBI reference number/country of origin/year of sample collection. The GAV strains from Midcrop Mortality Syndrome (MCMS) are indicated in BLUE , The GAV reference sequence from 1996 is indicated in PURPLE . The YHV-7 strains are indicated in RED. GAV sequences are each 662 nt and YHV-7 sequences are 305nt.

The IHHNV genome is ~4000 nt. Eight near complete genomes of IHHNV collected from Australian prawns (collected 1991 to 2008) are in the NCBI database. Comparison of the near complete IHHNV genomes collected from Australian strains indicate homology to reference sequences ranging from 85 to 100%. One of three IHHNV sequences obtained from HTS in this project shared 99% identity with the sequence obtained from the 1991 IHHNV associated mortality of *P. monodon* x *P. esculentus*. Variation in genome sequence can lead to the generation of false negative results in qPCR analysis and efforts to remove IHHNV may be inhibited if qPCR assays are not optimized to detect all economically relevant strains of IHHNV. The impact of IHHNV has never been conclusively demonstrated in *P. monodon* and the impact of strain variation in IHHNV on prawn health has not been considered in Australian industry. A significant variation in the genome sequence of IHHNV in prawn populations in the Gulf of California has been detected and the monitoring of genetic variance in IHHNV was subsequently recommended because slight changes in genome sequence can lead to increased virulence of viral pathogens (Dhar, 2020).

8.1.13 Application of High through-put sequencing to improve understanding in prawn disease

Analysis of the data output generated from the HTS application in the Project's hatchery component will extend beyond this project. In the hatchery analysis, the application of HTS to gather the genetic sequence of a repertoire of putative virulence factors from bacterial cultures in the prawn hatchery. Diseases caused by pathogenic Vibrio spp. have long impacted on shrimp production systems (Table 2). While a number of species including *V.harveyi, V.alginolyticus, V.proteolyticus, V.parahaemolyticus* have been associated with disease in shrimp (Kadriah & Nurbaya, 2019), the same species are also reported as ubiquitous in the marine environment (Zhang et al., 2018). The molecular mechanisms that contribute to disease outbreaks involving marine Vibrio spp. in shrimp aquaculture are not well understood and hinder science-based management approaches to prevent disease. The realization that AHPND is associated with the expression of a toxin gene has led to a paradigm shift in the management of bacterial disease in prawn production from a reliance on culture-based tools to the application of qPCR. The HTS approach in this project identified additional putative toxin genes in association with hatchery mortalities. This project selectively focused on a number of bacterial toxin genes to consider for further analysis. On an encouraging note, the targeted qPCR analysis on pond collected samples indicated a variable, but generally low percentage and low calculated quantity of positive detections of Zon and hemolysin D toxin genes, indicating the genes are not highly prevalent in the pond environment under typical culture conditions. The low baseline detection of the genes may assist in future studies if the genes are detected in high prevalence and high calculated copy number in association with disease outbreaks and alternately, the longer term monitoring of the genes over multiple seasons may provide insight into correlations between toxin gene prevalence and environmental or pond conditions.

Although the presence of bacterial toxins has long been associated with Vibrio infections, relatively few specific toxin genes are actively monitored in marine aquaculture, other than the Pir-AB toxin gene, or toxins associated with human health. The role of the microbiome is an emerging topic in the study of animal health and the HTS approach provides a powerful tool to study the complex interaction of bacterial contributions to health. Present applications of HTS have been limited by the lack of characterized bacterial genomes and the outcomes of many studies have identified changes in bacterial community structure at high taxonomic levels. Rather than focusing on the taxonomic characterization of bacterial communities, an alternate approach of assessing the genomic capacity for pathogenicity, such as identifying toxin genes, antibiotics resistance genes, and other genetic drivers of virulence, may yield more industry-translatable outcomes. The high cost and requirement for high performance computing power to analyse HTS data reduces routine implementation of HTS. However, costs will likely decrease and in the interim the combination of HTS and qPCR are

effective tools to improve the understanding of the role of the microbiome and bacterial toxin genes in prawn disease. The forecast impacts of climate change and emergence of antibiotic resistant bacteria perpetuate an increased likelihood of bacterial disease and understanding the multitude of factors that contribute to bacterial and viral-induced disease may become more critical to disease management in aquaculture more broadly as disease in tropical systems increase and the geographic range of tropical marine bacteria extend into temperate zones.

9 Strategic Recommendations

9.1 Recommendation 1: Strengthen National Border control: keep exotic pathogens exotic.

- Short-term: Strengthen the prawn IRA conditions (policy driven requirement)

- Short-medium term: Establish cost sharing arrangements with industry to enable surveillance for exotic pathogens with the prawn translocation testing to strengthen claims of freedom of exotic pathogens (policy driven requirement with some government contribution to current industry investment)

Exclusion of pathogens poses the most cost-effective biosecurity action with indicative economic returns of $100 for every $1 invested (SCAAH, 2016). Australian agriculture has benefited from the presence of relatively few serious animal diseases. Exclusion of pathogens through National level quarantine has traditionally provided a high level of protection to Australian agriculture to prevent the importation of exotic pathogens and pest species. The WSSV disease incidents severely impacted the infected properties and the possibility of continued WSSV incidents remain a serious concern for farms situated on the Logan River. Although the immediate costs of WSSV have been calculated, the permanent on-going costs to production and potential lost profitability due to WSSV cannot be accurately estimated and will be borne by future prawn and crustacean farming and fisheries operations. The sustainable development of prawn farming, and other emerging crustacean species, such as tropical rock lobster and slipper lobster, in northern Australia relies heavily on strong national level border quarantine to prevent the entry of other exotic pathogens. Although WSSV is the most economically devastating of the viral prawn pathogens, other viral diseases have, and will likely continue, to emerge in overseas prawn farming systems.

The importation of frozen, uncooked commodity prawns presents a clear path allowing the introduction and uncontrollable release of material containing viable exotic viruses into the Australian consumer market. The IRA needs to be strengthened to ensure it robustly protects industry to exclude the entry of viable exotic prawn pathogens. Specifically, the IRA would be strengthened if it:

- Accepted a lower level of risk. The risk of import of viable prawn pathogens would be most effectively mitigated if only cooked product was imported.

- Considered the <u>volume</u> of non-compliant product that enters Australia rather than a "very-low but not zero" rating;

- Was supported by a sampling regime that aligns with the risk posed by <u>volume</u> of product being imported rather than targeted to detect a prevalence of 5% of non-compliant material;

- Were proactively and frequently reviewed rather than following an exotic disease outbreak;

- Recognised new and emerging pathogens.

The ever-present risk of transfer of viable pathogens in uncooked prawns could be effectively mitigated though cooking pre-import. The import of cooked product would effectively mitigate the import of emerging or unknown pathogens that are not specifically listed as notifiable diseases Cooking post-import would also reduce the risk but would also require significantly more resource to ensure post-border biocontainment and compliance.

An ALOP that considers the <u>volume</u> of non-compliant product rather than a level "very-low but not zero" would provide more robust protection to industry. Since this project commenced in July 2018, 145 of 10770 (1.3%) consignments of imported frozen commodity prawns have tested positive for WSSV while held under biosecurity control. The WSSV

positive consignments were detected during a heightened import testing regime that necessitates sampling of 100% of consignments. The current levels of detection of WSSV (1.3 %) are below the level that would be reliably detected within a surveillance program designed to detect 5% prevalence of positive detections of WSSV. Whilst the percentage of detections is very low, it must be noted that a shipping container (assumed 10T in this context) constitutes the maximum size of a single consignment and hence, the 145 shipments could be in excess of 1450 T of WSSV positive material, or slightly more than ~30% of total Australian aquaculture prawn production in 2018-2019, which arguably, is not a "very low but not zero" volume of potentially viable WSSV positive product. Refer to the link for the summary of testing on uncooked imported prawn consignments (accessed 23/7/2020).

https://www.agriculture.gov.au/import/goods/uncooked-prawns/uncooked-prawn-consignments

The IRA review process should be more proactively scheduled to maintain a level of protection that is aligned with current knowledge of prawn disease rather than reactive review after an exotic disease outbreak. An increased frequency of the review will reduce the onerousness of the review process and allow national biosecurity processes to nimbly respond to the changing risks in the global transfer of prawn pathogens. A review every 3 years and whenever a new Chapter or revision of the OIE Aquatic Manual occurs would ensure the IRA process aligns with most recent scientific knowledge in global prawn pathogens. Current conditions of import do not require testing for the presence of EHP, TSV, IMNV or Covert Mortality Nodavirus (CMNv) despite the pathogens being recognised as endemic to source countries and as significant pathogens in Australia's national list of reportable diseases of crustaceans (Animal Health Committee, 2018). A recently emerging virus, Decapod Iridescent Virus-1 (DIV-1) has been associated with significant losses in China and Vietnam causing estimated 80% mortality in pond prawns (Kearns, 2020). The virus contributed to a 0.3 million tonne decline (more than 64x the Australian total prawn aquaculture production in 2019) in production of *L. vannamei* in the 2018 China fishery statistical yearbook (Huifeng, 2020). DIV-1 has not been included in the OIE Aquatic Manual or Australia's list of reportable diseases of crustaceans. Moody et al., (2015) reported the detection of YHV8 and YHV10 in imported commodity prawns from China. The detection of When-2 (100%) and IHHNV (57%) in samples tested from a single 1kg package of imported retail prawns, conducted during this project, also raises concern. There is no information about the prevalence or impact of When-2 on shrimp production in China and many disease incidents could be mistakenly attributed to other pathogens based on non-specific clinical signs such as pale hepatopancreas being historically linked to a particular pathogen. A cohort study of 196 Thai shrimp ponds found over 50% of the ponds that were reported as Early Mortality Syndrome were negative for diagnosis of AHPND which highlighted that assigning cause of mortality without confirmatory diagnosis could lead to a failure to recognise other important causes of mortality (Sanguanrut *et al.*, 2018). This project highlights the potential for accidental import of presently unlisted, unknown and uncharacterised viral pathogens in uncooked prawns. Professor Lightner (2005), a world renowned expert in prawn disease, stated that the lag time between the recognition of a significant disease and the development of diagnostic methods to detect them contributed to the international movement of significant diseases including IHHNV, TSV and WSSV. Considering the propensity of EC QLD stock to test positive for When-2 compared to NT sourced broodstock, and the proximity of EC QLD broodstock fisheries to areas where recreational fishing is particularly popular, it is possible the recent detection of When-2 in Queensland sourced prawns is the consequence of an undetected introduction of When-2 into the Queensland environment via the use of frozen imported prawns as bait. Although Australia has reported the presence of IHHNV, and members of the Yellow head virus complex, multiple strains of the viruses exist and their presence should also be considered within the IRA. The presence of different strains of virus within the same organism provide a mechanism for genetic recombination between strains which can lead to the emergence of more pathogenic strains. The importance of strain variation in viral pathogenicity is recognised in studies of terrestrial animals and fish diseases but largely neglected in prawn disease studies.

An impediment to Australia adopting a more robust IRA is the absence of surveillance to support claims of freedom from disease to justify exclusion of infected stock through import. The high cost of traditional specific structured surveillance prohibits implementation by government. A model for cost efficient surveillance of pathogens in Australian prawns could involve a cost share arrangement between prawn hatcheries, NATA accredited laboratories completing translocation testing, and government. Industry currently funds the cost of annual sample collection and analysis for excess of 5,000 broodstock for the detection of WSSV and YHV-1 and AHPND. A National survey to demonstrate freedom from WSSV and YHV-1 completed analysis on 3,081 samples (East *et al.*, 2015). Cost-effective analysis for the detection of additional targets such as EHP, DIV-1 and IMNV could be achieved through collaborative contributions from industry, government and NATA accredited service providers to align analysis with current translocation testing. Such an arrangement would provide industry with strong evidence to claim historical freedom of a pathogen if another exotic disease outbreak were to occur. The extension of surveillance within current industry practises would position farmers at the forefront of disease surveillance, generate data to support Australia's claims of freedom, provide early detection of exotic pathogens and, strengthen the capability of the network of laboratories that provide service to industry. The embedding of increased exotic pathogen testing within active laboratory operations would also enable a rapid National surge capacity or continuation of service to farms in unaffected areas if an exotic disease emergency were to occur. The proposed changes require a shift in current government policy rather than a large investment of funds to extend biosecurity capability.

9.2   Recommendation 2: Strengthen regional capability and capacity in prawn health and aquatic biosecurity response

- Short-Medium-term: Establish a Northern Australia aquaculture node of aquatic animal emergency response capability (policy driven requirement and some investment in training (FRDC AAHS Training program).

- Short-term: Extend permission for NATA accredited laboratories to test for exotic pathogens in all applications rather than only translocation testing (Policy driven requirement).

- Short-term: Include providers of prawn translocation testing in the network of laboratories available for surge capacity in the case of an emergency aquatic disease response in Northern Australia (policy driven requirement and collaboration of laboratories and, some investment to extend the Aquatic Animal disease proficiency program).

- Short-term: Train prawn farm staff in emergency disease response procedures (policy driven requirement and investment in training/disease simulation exercise (FRDC AAHS Training Program or Australian Government Department of Agriculture).

If national level biosecurity does not prevent the entry of viable exotic prawn pathogens further exotic disease incidents are possible. If an emergency prawn disease event were to occur in Northern Australia, it would be difficult to mount an effective eradication and containment operation with current resources. Farms located in regional northern Australia have:

- Sub-optimal access to laboratory diagnostic services with permission to rapidly detect exotic pathogens.

- Poor access to a trained and sufficiently resourced workforce with strong aquatic animal emergency disease response knowledge.

- Unreliable and delayed access to large quantities of chemicals and other resources required for eradication and containment of a viral pathogen.

These characteristics are not isolated to the prawn industry and apply generally to all marine aquaculture production systems in Northern Australia. Freshwater systems face similar levels of isolation, but are less exposed to the biosecurity risks posed by human activities and the high interconnectivity of the marine based industries. Although this project specifically targets means to improve biosecurity in Australian prawn farming, addressing policy and investment to develop a northern Australian capability in aquatic animal disease response would benefit all aquaculture systems. The recommendation is not isolated to this report. The Joint Select committee on Northern Australia (2015) recognised the need for regional capability to diagnose disease outbreaks in real time and the need to strengthen regional biosecurity was one of the key recommendations of the Northern Australia Aquaculture Situational Analysis (Cobcroft *et al.*, 2020).

At present, government policy, rather than a lack of infrastructure or technical capability, hinders the NATA accredited facilities that provide service to regionally located industry from being fully applied to support industry to rapidly identify a disease outbreak associated with an exotic pathogen. A number of laboratories provide rapid, high throughput, pathogen detection services to regionally located prawn farmers to support prawn translocation protocols; however, the laboratories that are external to government are not approved to conduct testing for exotic pathogens in response to a disease incident despite holding the quality assurance of NATA accreditation. Extending current permissions for NATA accredited laboratories to perform detection of a range of exotic aquatic pathogens across a range of applications, in addition to the sending of samples to government laboratories such as Biosecurity Queensland, would enable industry to launch a rapid disease response.

COVID-19 disruption has highlighted critical points of weakness in the current framework of Australian diagnostic veterinary service provision whereby all network laboratories approved to test for exotic pathogens to diagnose mortalities are located in areas at higher risk of COVID-19 disruption e.g. Melbourne, Sydney and Brisbane. Additional to COVID-19 induced disruption, forecast increase in the frequency and severity of volatile weather events, such as floods and cyclones as a product of climate change, presents further risk to the accessibility of northern prawn farms and the northern Australian aquaculture sector to southern located diagnostic veterinary laboratories and resources. In addition to extending permission for testing, including the NATA accredited laboratories of Northern Australia within the activities of AquaPLAN, the national network of aquatic animal health or a Northern aquatic node similar to the laboratory emergency animal disease diagnosis and response (LEADDR) presents a low-cost model for government to increase national capability in prawn disease testing.

The specific challenges of isolation and regionalisation of aquaculture in Northern Australia must be considered in the development of a robust emergency disease response capability for industry. Regional capability and capacity in emergency aquatic animal disease response should be established which incorporates training of farm staff. Even if the delay to diagnosis of exotic pathogens are reduced with policy change, the ability to launch a rapid disease containment and eradication response is limited by the relatively isolated location of many of the prawn operations in Northern Australia. The location of many farms poses logistical hurdles to the rapid deployment and redirection of government staff and resources if an emergency disease response were required. The training of prawn farm staff to perform the urgent, most immediate, response to an emergency aquatic disease would present a more robust model for rapid disease response preparedness. This need is not restricted to prawn farmers and emergency aquatic animal disease response training would benefit the range of aquaculture enterprises in Northern Australia. The training of farm staff presents a very cost-effective model and equips farms to better meet their General Biosecurity Obligations under the

Biosecurity Act. The training of farm staff would enable those communities most likely to be significantly impacted by an emergency disease outbreak to be most adequately prepared to respond.

The inclusion of a Northern Australia Aquatic section in the Animal Health Surveillance quarterly would provide industry with regular updates on current aquatic pathogen detections. The prospect of a de-identified summary of detections has been floated in APFA discussions but not progressed.

9.3 Recommendation 3: Actively focus efforts on the development of domesticated *P. monodon* lines.

Short-medium-long term: *Investment and policy support.*

Pathogen targets were detected in high frequency and high quantified target number from wild captured broodstock. Domestication has been demonstrated as an effective strategy to avoid the annual introduction of a range of pathogens via wild broodstock. Although the urgency to implement domestication of Australian *P. monodon* is driven by the risk posed by the spread of WSSV beyond the current restricted movement zone, domestication will also enable industry to pursue a range of selective breeding activities to build resilience against current and future challenges. Future challenges to biosecurity will be imposed by:

- Climate change induced stress on prawn health: particularly increased temperature, increased ocean acidification and increased incidents of extreme weather events such as drought (high salinity) or severe rain/cyclone events (rapid and sustained low salinity, Nitrogen influx to ponds and algal bloom crash),

- Climate change as a driver of the emergence of bacterial pathogens including those with antimicrobial resistance or,

- Other pathogens that emerge naturally or,

- Introduction of exotic pathogens though breaches in national biosecurity.

Options in domestication include selection of specific pathogen free (SPF), specific pathogen tolerant (SPT), or specific pathogen resistant lines (SPR). This study has highlighted a range of factors for consideration in domestication, most significantly, that pathogens are highly prevalent within wild-sourced broodstock. Therefore, sourcing of an adequate number of founding SPF prawns will be a significant challenge, particularly if industry does not adopt an industry-wide collaborative approach. Potentially, targeting of SPR/SPT lines would yield greater benefit for the Australian prawn industry because;

- It would allow greater retention of candidate broodstock = more genetic variation in founder populations

- Pathogen reservoirs in pond culture will not be of consequence as prawns would be resistant/ tolerant to infections.

- Improved understanding of pathways that influence disease resistance and tolerance can be applied to understanding the prawn-pathogen-environment relationship and enable more resilience to respond to future challenges.

Although domestication has been attempted since the 1980s, programs rarely considered screening for the presence of pathogens within founder stock and programs faulted when low-level viral infections progressed to disease when prawn were reared under suboptimal conditions (Coman & Preston, 2008). If the presence of multiple endemic pathogens is not considered, it is likely future efforts in domestication will also fail.

The presence of pathogen reservoirs in the pond environment threatens the viability of SPF as pond stock are not likely to remain pathogen free during pond production. Results of this project suggest the pursuit of an exclusion strategy for management of endemic disease will also need to develop engineering approaches to prevent the entry of pathogens into prawn ponds from the environment. Although chemical treatment of incoming water may prevent the entry of carrier organisms, the widespread application of chemicals will impact on farm economics, introduce collateral issues of chemical disposal and threaten consumer acceptance of farmed product.

9.4 Recommendation 4: Increase research focus on the role of host-pathogen-environment factors in prawn health and disease.

- Short-term: Co-investment with research and funding providers to strengthen regional capability and capacity to complete aquatic animal health research particularly to investigate multi-component disease challenge and testing of efficacy of treatments to endemic pathogens.

When pathogens are invariably present in systems, biosecurity management options are directed towards asset-based protection. Asset based protection prioritises management against the most damaging pathogens and seeks to maximise the health of cultured species to avoid disease outbreaks when pathogens are invariably present. Optimisation of health requires a strong understanding of the interaction between pathogen, prawn and environment in disease expression. The lack of understanding of the host-pathogen-environment interactions is a significant knowledge gap that hinders the optimisation of prawn health and production in aquaculture. A better understanding of the dynamics and occurrence of disease in commercial prawn production systems and how this information might be integrated into domestication programs and biosecurity management strategies is required. There is a high priority need to conduct pathogen characterisation and experimental challenge using purified strains of endemic pathogens. Where purification of pathogens cannot be conducted HTS analysis should be conducted to identify the presence of co-infecting pathogens that may be present in experimental viral extracts. Attention to the strain variance in GAV and IHHNV, in particular, should be elucidated based on their high percentage of detection within this project. There is one complete sequence of GAV in the NCBI database from Australian prawns and comparative analysis on a small segment of the GAV genome indicates there is variation in the genome which may influence pathogenicity. Diagnostic and industry screening assays should be aligned to detect the pathogenic strains of endemic pathogens, noting the monitoring of other co-infecting pathogens should be incorporated into the industry biosecurity monitoring to elucidate any impact of multiple infections on prawn health. Future industry funded research must incorporate monitoring of multiple pathogens regardless of the target pathogen.

The aforementioned need to improve regional capacity in aquatic animal health also applies to research in Northern Australia. Australia is one of few developed countries, located in the Tropics, with an established prawn farming industry, yet relies heavily on the research activities of developing nations to identify, characterise and combat prawn pathogens. Exotic pathogens are not the sole disease risk to industry. Disease outbreaks have occurred historically in association with a number of endemic pathogens (Section 3.4). The emergence of endemic pathogens has caused significant impact in other Australian aquaculture industries such as Abalone Viral Ganglioneuritis (AVG) in Abalone, QX disease of Sydney rock oysters and Pilchard Orthomyxovirus in Atlantic salmon. Each of these industries are in close proximity to government veterinary diagnostic laboratories and research facilities and their recovery from disease incidents has been supported by geographic proximity to facilities with strong capability in aquatic animal health. This is a sharp contrast to the Northern Australian aquaculture industries, which has relied on disease research to be conducted by facilities in southern Australia such as CSIRO in Geelong, Brisbane and Bribie Island. The reliance on temperately located research facilities to conduct research on tropical aquatic disease is not the most efficient research model. Research outcomes to industry investigating YHV-7 and Pir-AB associated disease have been delayed years at the Australian Centre for Disease Preparedness (formerly Australian Animal Health Laboratory) due to the necessary redirection of resources to national emergency aquatic disease responses. Considerable freight costs are incurred by research projects to ship live prawns from farms in Northern Australia to southern facilities and experimental outcomes can be inhibited by limited access to experimental animals. The industry funded project investigating the potential for RNAi to knockdown GAV

levels were limited by a lack of ready-access to high load GAV animals and experiments with less than ideal experimental animals contributed to project outcomes that demonstrated knockdown of GAV in very low load animals with undetermined efficacy to knockdown high load GAV (Sellars *et al.*, 2018). Studies conducted in temperate experimental facilities may not translate to similar outcomes in the tropical conditions of prawn farming in Northern Australia. The recent publication of Noble *et al.,* (2020) noted the contrast in outcomes between laboratory studies conducted in the Bribie Island Aquaculture facility in SE QLD and analysis conducted on farm in North Queensland. Additional limitations in the completion of studies in temperature locations may presently be undetected. For example, the role of the bacterial community in health is being recognised across a range of human and animal health sectors, yet under current research arrangements, the positioning of prawn research in temperate locations does not incorporate any contribution of endemic tropical marine bacteria in prawn health. There is a need to strengthen capacity in Northern Australia to research tropical aquatic pathogens under tropical conditions to maximise the likelihood that laboratory research will be translational to Northern Australian prawn farm outcomes.

Flow on benefits to improving regional capacity for aquatic animal disease research are wide ranging including:

- Provision of foundational infrastructure that enables increased opportunity for regional institutes to access other funding eg. ARC, FRDC, etc.

- Provision of a regionally located facility to perform hands-on training to current and future industry workforce.

- Access of regional enterprises to actively partner with researchers to develop novel tropically specific therapies.

In addition to establishing capacity in prawn health research in Northern Australia, a shift in the approach considering the multicomponent nature of disease would provide insight to guide more effective disease management. Improving the understanding of the interaction between prawn-pathogen-environment necessitates a change in the way disease research in prawn aquaculture has been conducted. Industry would be better positioned to improve disease management if a multi-disciplinary approach to disease research were adopted. Multidisciplinary disease research considers the complexity of factors that contribute to disease and impacts on productivity. Recognising a need for a paradigm shift and refocussing of scientific studies and policy relating to aquatic animal health to consider the complexity of disease was an outcome from a collaborative workshop between aquatic animal health experts from United Kingdom and Thailand to address the needs of expanding and sustainable aquaculture industry to 2050 (Stentiford *et al.,* 2017). However, an impediment to this approach is that complex research proposals are often not palatable or easily aligned to the compartmentalised strategic research goals of industry or funding providers. Although complex systems research has previously involved a high level of risk, such risks could be reduced by applying the rapid advancements in HTS, machine learning and biotechnology.

9.5   Recommendation 5: Increased Education in Prawn health and Biosecurity

Short-medium term: development and delivery of prawn health and aquatic biosecurity training modules for industry and education providers. Partnership between Industry and training providers. FRDC AAHS training subprogram

Sustained profitable prawn farming in the presence of pathogens requires effective management of prawn health to maximise survival and growth. There is inadequate robust knowledge about how to optimise prawn health, including reproduction, to limit disease risk factors in farm production and achieve optimal harvest weights with a high level of consistency in prawn pond production. This is reflected by the increased frequency of lower annual growth of production in the past 9 seasons (Figure 3) and the variation in survival (26 to 100%) and FCR (1.7 to 2.34) from the subject 55 ponds of this project. The variations in survival and food conversion ratio impact considerably on farm profitability. The

variation in pond productivity between the best and worst performing ponds, observed in this project, assuming a stocking density of 50/m^2 of 26% v 100% survival equates to $78,000 gross farm gate sale v $300,000 per hectare pond (assuming harvest weight of 30g and $20/kg). Similarly, the difference in consumption of feed due to the different FCRs (assumed stocking density of 50/m^2) based on 100% survival leads to increased feed consumption of 25,500kg feed to 35,700 kg per hectare resulting in a feed cost differential of $30,600 per hectare (assuming feed cost of $3/kg) (Table 10). Improved knowledge in the management of factors that optimise health, growth and survival will translate to significantly improved farm profitability.

Although the need for a national curriculum in aquatic animal health was raised in 2015, little progress has been made. The delivery of a relevant national curriculum will be difficult due to the fragmentation and diverse nature of the major aquaculture industries across Australia. Tasmanian and South Australian based aquaculture produced 76% of Australia's gross value of aquaculture in 2018 (ABARES, 2019). A National Curriculum focussed on the production of temperate species would deliver the most relevant training to the majority of Australian aquaculture, but contribute very little to support the training of employees in tropical species aquaculture in Northern Australia. The study of prawn health, culture and disease, particularly *P. monodon*, is a niche topic that is not well considered or relevant within the development of broad national curriculums in veterinary science or aquaculture. Training programs that specialise in prawn or tropical crustacean health would address the more immediate needs of training the 825 employees required by 2030 and support the forecast expansion of the Northern Australian prawn farming industry (Cobcroft *et al.*, 2020). Industry specific modules should be developed and present the most recent and relevant technologies to increase the awareness and exposure of industry to recent technologies and enable industry to make more informed decisions in the direction of industry investment and research. The application of guided biotics to manage bacterial diseases, machine learning to predict pond performance and identify risk factors in disease or sub-optimal production and the application of HTS to study the complexity of the host-pathogen-environment interaction are some of many emerging technologies that may yield benefit to industry. Industry is advised to collaborate with Northern Australian universities and training providers to develop industry relevant and specific training that could be delivered directly to the prawn farming industry and integrated with relevant general aquatic biosecurity modules if a National Aquatic animal health and biosecurity curriculum is developed. Increased education of industry in the topics of prawn health and aquatic biosecurity would strengthen the ability of industry to; develop industry-lead innovation to drive farm productivity, more actively meet their General Biosecurity obligations and, potentially reduce the impact of isolation and regionalisation raised in 7.2.2 to 7.2.4.

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11 Appendices: Final Report

11.1 Appendix A: Checklist of Biosecurity practises present including level of risk to biosecurity posed by absence of practise and relative costs to implement.

Item	Description	Number of Hatcheries With item/practise present	Level of Risk to Biosecurity posed by absence	Cost to implement
	Restricted access to Facilities (Human)			
	Restricted entry to property	4	Low	Moderate
	Restricted entry to facility	2	Low	Moderate
	Vehicle wash at property	1	Low	Moderate
	Biosecurity signage at property	3	Low	Low
	Anti-room at entry to hatchery	3	Moderate	Moderate
	Visitor register	4	Low	Low
	Biosecurity induction of visitors	1	Moderate	Low
	Biosecurity Questionnaire at entry	2	Moderate	Low
	Ethanol wash/Footbaths at entry	4	Moderate	Low
	Water intake			
	Fishing activity within 400m of intake	2	Moderate-High	High
	Intake within 3km of boat ramp/jetty	1	Moderate	High
	Remote location	2	Moderate	High
	Settlement/storage ponds	4	High	High
	Chlorination of storage ponds	1	Moderate	Moderate/High
	UV treatment	4	High	Moderate

	Ozone treatment	4	High	High
	Sand and 0.1um treatment	4	High	Moderate
	Heating	4	Moderate	Moderate
	Discharge treatment	1	Moderate-High	High
Algal cultures				
	Ethanol at entry	4	Moderate	Low
	Footbaths at entry	4	Moderate	Low
	In separated areas	4	Moderate	Moderate/High
	Insect contamination issue (verbal)	3	Moderate-High	Moderate/High
	Insect contamination visible	2	Moderate-High	Moderate/High
	Bacterial counts conducted	2	Moderate	Low
	Concentration of algae to feed	4	Moderate	Low/Moderate
Artemia cultures				
	Ethanol at entry	4		Low
	Footbaths at entry	4		Low
	In separated area	4		Moderate
	Disinfection of cysts	4		Low-Moderate
	Removal of cysts via magnets	4		Low-Moderate
	Concentration of Artemia to feed	4		Low-Moderate
	Freezing of Artemia	2	Low-Moderate	Low
	Defrost in fridge	1	Low-Moderate	Low
	Bacterial counts performed	1	Moderate	Low
Staff Movement				

	Separation of tasks by staff	2	Moderate	Moderate
	Separation of tasks by time-space	4	Moderate	Low-Moderate
Hatchery signage/facility				
	Biosecurity Zones	1	Low	Low
	Wash your hands	2	Low-Moderate	Low
	Close the door	1	Moderate	Low
	One laboratory area for analysis	1	Moderate	Moderate
	Multiple laboratory areas for analysis	1	Low	Moderate/High
	One office	3	Moderate	Moderate
	Multiple office areas	1	Low	Moderate/High
Biosecurity Response				
	SOPS for activities	4	Moderate	Low
	SOP for Biosecurity: disinfection of tanks	3	Moderate	Low
	Disinfection chemical in all areas	1	Moderate	Low
	Detailed Facility Lay out present	4	Low	Low
	Satellite facility image present	1	Low	Low
	Routine disinfection schedule	2	Moderate	Low
Broodstock Intake				
	NT wild	4	Moderate	High
	EC wild	4	Moderate	High
	Screened as per translocation (QLD)	3	Moderate	High
	Screened as per translocation (NSW)	1	Moderate	High
	Screened beyond translocation	3	Moderate	High

	Sampled at intake to facility	0	Moderate	High
	Submitted for testing in batch	4	Moderate	High
	Broodstock individually identified	3	Moderate	High
	Broodstock individually held	0	Moderate	High
	Multiple maturation sheds in facility	4	High	High
	Screening between maturation tanks	0	Moderate	Low
	Spawning individually identified	1.5	Low	Low
	Gamma irradiated wild feed	4	Moderate-High	Moderate
	Imported components in feed	4	Moderate	High
	Feed regularly routinely screened	0	Moderate	Moderate
	Bacterial culture performed	1	Low	Moderate
	Tank mortality recorded	4	High	Low
	Tank mortalities retained	4	Low	Low
	Tank mortalities submitted to testing	2	Low	Low
	Surface disinfected on receipt	4	High	Moderate
	Surface disinfected into spawning tank	1	High	Low
	Marking of testing results on tank	0	Moderate	Low
Eggs and Nauplii				
	Counts performed	4		
	Survival recorded	4		
	Surface disinfection	4		
	Separate batches	4		
	Antibiotic routine	0		

	Probiotic routine	2		
	Aerosol sheet between tanks	0		
	Monitoring of aerosol in shed (bacterial)	0		
Mysis-Post Larvae				
	Health assessed (microscope)	1		
	Health assessed by feed rate	3		
	Health assessed by survival	4		
	Bacterial plating performed: weekly	0		
	Pathogen screening conducted	3		
	Pathogen screen results applied	1		
	Pre-stocking stress test conducted	1		
	Nursery Stage	1		
	Pond-side acclimation	1		
	Health criteria for stocking applied	2	Moderate	Moderate
Mortalities				
	Recorded	2		
	Stored frozen	4		
	Stored separate to feed freezers	3		
	Disposal: incineration	2		
	Disposal: land fill	2		
	Tested: Translocation (NSW)	1		
	Tested: Farm practise always	1		
	Tested: Farm practise variable	3		

Pathogen target	DNA/ RNA targeting	Component	Sequence (5'-3')	Reference
WSSV	DNA	Forward	CCGACGCCAAGGGAACT	CSIRO unpublished
		Reverse	TTCAGATTCGTTACCGTTTCCA	
		Probe	CGCTTCAGCCATGCCAGCCG	
Pir-A toxin gene	DNA	Forward	TTGGACTGTCGAACCAAACG	(Han *et al.*, 2015)
		Reverse	GCACCCCATTGGTATTGAATG	
		Probe	AGACAGCAAACATACACCTATCATCCCGGA	
IHHNV	DNA	Forward	CCTAAAGAAAACAGTGCAGAATAT	(Cowley *et al.*, 2018)
		Reverse	TCATCGTCAAGTTTATTGACAAGTTC	
		Probe	CTCCAACACTTAGTCAAA	
HDV	DNA	Forward	CTACTCCAATGGAAACTTCTGAGC	(Owens *et al.*, 2015)
		Reverse	GGCACTTCCTGTATTTTTCCCG	
		Probe	TACCGCCGCACCGCAGCAGC	
YHV-1	RNA	Forward	AGTCTACAGTGCTCTGATCT	CSIRO unpublished
		Reverse	GATTCTTGAAGCGCATGAGT	
		Probe	TCTCATGTGTCATGATATTCTCAAGCGAGT	
YHV-7	RNA	Forward	CAT CCA ACC TAT CGC CTA CA	(Cowley *et al.*, 2015)
		Reverse	TGT GAA GTC CAT GTG AAC GA	
		Probe	CAA CGA CAG ACA CCT CAT CCG TGA	
When-2	RNA	Forward	GGC TCT TTAGCC TGA CTT TAT CT	Unpublished
		Reverse	GCA GAG GAC AGG AAG TGA TTA	
		Probe	ACC TCA CTG TCT GAG TTC TGC ACA	
GAV	RNA	Forward	GGG ATC CTA ACA TCG TCA ACG T	(De La Vega et al., 2004)
		Reverse	AGT AGT ATG GAT TAC CCT GGT GCA T	
		Probe	TCA GCC GCT TCC GCT TCC AAT G	
Dicer-1	RNA	Forward	TGG TAC CAA AGT CAC CCA TTA G	(Su *et al.*, 2008)
		Reverse	ACC TTC CCA TCA ACA AGA CGT T	
		Probe	AAC CAG AAA CAG CCA AAT	
putative RTX toxin		Forward	TTTGTGACTGGAGGAGGTAATG	JCU Unpublished
		Reverse	GTGGTCAGATGCAGGGTAATAG	
		Probe (F)	ATCAAGCGGAGACGGACACAGTTG	
ZON		Forward	CCCGATTGGCAACAGATAGA	JCU Unpublished
		Reverse	CGGTTTACGCTTAGCAAAGAAA	
		Probe (F)	TAGCTCAATCAAGGCGTGTGCTGA	
hemolysin D		Forward	CAGTCTGAGAGCGGTATGAAAG	JCU Unpublished
		Reverse	GTGGACTGACTACAATGGCTAAA	
		Probe (R)	TACCAACGAAGACGATAGCCGCC	

11.3 Appendix C: Interpretation of qPCR results

To assist in the understanding of the results some general knowledge of qPCR is useful. This section describes the terms used to express the results of qPCR analysis.

11.3.1 Why use qPCR?

Quantitative polymerase chain reaction (qPCR) is the optimal assay for detecting prawn pathogens. The assay is very sensitive (detects low copies of target) and specific (only detects the specified target). A qPCR can also be easily applied to a large number of samples to attain a result in a short amount of time, within 2 hours of sample receipt (high throughput, low turnaround).

11.3.2 What does a qPCR detect?

A qPCR copies and detects genomic material (nucleic acid). The detection of genomic material does not mean the target detected is infectious and viable (alive). Although it has not been determined for prawn pathogens, viral genome has been detected in other animal health settings when the animal immune response has killed viable virus and no longer subject to effects of the virus. For this reason, the results are discussed as detection of "targets" rather than virus or bacteria.

11.3.3 What is the qPCR result?

A qPCR result is expressed as a number called the cycle threshold (Ct) value. Because the majority of prawns in a cohort will often be positive for the detection of a target, qPCR is superior to PCR (presence/absence detection only) for managing biosecurity risk of disease. For example, in this project, 90% of the samples analysed were positive for the detection of GAV. The quantity of the pathogen target detected is useful for identifying individuals of highest risk or those animals more likely to suffer adverse growth or survival in association with a pathogen detection. By applying qPCR, the level of risk posed by each individual can be quantitatively estimated and the effect of treatment or any other management strategy to reduce pathogen quantity can also be assessed.

11.3.4 What is Cycle threshold (Ct)?

When a qPCR analysis has been activated it completes a cycle that consists of 2 steps. During the first step in the cycle the reaction components bind to the target and the second step copies and detects the target. The 2 steps of the analysis are repeated for 40 (endemic targets) or 45 (exotic targets) times. The point during the analysis when the target is detected as positive (exceeds the threshold value) is reported against the number of qPCR cycles (cycle), referred to as the cycle threshold value (Ct). The Ct value is out of a maximum of 40 or 45 cycles. The step in the qPCR cycle that detects the target takes between 10 to 45 seconds. The percentage of time taken within the 10 to 45 seconds to detect a target is indicated by decimal values e.g. 36.**213**. Because amplification is more efficient in some stages of the qPCR, a decimal portion of the Ct value is included in the results. The qPCR machine expresses Ct value to 8 decimal places. Rounding of the Ct value to 1 or 2 decimal places does not greatly impact on the interpretation of the result. The higher the number of copies of target present in a sample, the sooner it is detected by the qPCR, and the lower the Ct value i.e. low Ct indicates high concentration of target.

11.3.5 What is calculated copy number?

A validated qPCR detects the target with a known level of performance, whereby a certain Ct value is known to detect a specified concentration (number of copies) of a positive standard. The qPCR machine analyses the positive standard Ct

values against the log of the number of copies of target of the positive standards as a linear equation. By applying the linear equation to the test samples, the Ct value can be used to calculate the number of copies of the target. Because the number of copies is not directly counted, such as for bacteria on a culture plate, the number is reported as the **calculated** copy number. **Error! Reference source not found.** below provides a generalized illustration of the relationship between Ct value and calculated copy number whereby low Ct equates to high copy number which generally presents as a high risk to disease (red square in **Error! Reference source not found.**)

11.3.6 Categorization of pathogen load

Each pathogen has a different concentration at which it will cause cellular changes and disease in a prawn. The concentrations differ between pathogens, prawn life stage and environmental conditions. As qPCR is a relatively new technology applied to prawn disease, the concentration at which any individual pathogen will cause disease, as determined by a qPCR result, is difficult to define. Until further data is acquired, JCU AquaPATH applies the above model as a general guide of the relationship between Ct value, calculated copy number and pathogen load. Data to support the general categories of high, moderate and medium load is described in the discussion section of this report and was presented in Report 1: Hatchery Component of this project.

11.3.7 How are qPCR results discussed in this report?

This report discusses qPCR results in terms of Ct value <u>and</u> in some cases calculated copy number. The average Ct, minimum Ct, maximum calculated copy number and standard deviation are discussed.

11.3.8 Why use Ct value?

Ct values can be a useful index because they are more easily visually assessed compared to calculated copy number (

Table 12). Each qPCR assay has slightly different performance characteristics so comparison of Ct value between assays is generally, but not exactly applicable. Ct values are also not suitable for comparison with other research studies and statistical analysis unless laboratories have specifically aligned when implementing assays. A degree of comparison of Ct values between laboratories can occur if laboratories have harmonized by using the same brand of qPCR machine and assay reagents. JCU AquaPATH has, where possible, harmonized with AFDL AAHL in the detection of WSSV and Pir-A. qPCR analysis to detect YHV-7 have produced similar Ct values. National proficiency testing is conducted for WSSV and YHV-1 to support NATA accreditation to demonstrate competency in the detection of the target pathogens. There is no proficiency testing for pathogens that are currently declared as endemic to Australia.

11.3.9 Average/mean Ct value

The average/mean Ct value of a sample group provides a general indication of the whole cohort. When there is little variation in a cohort, for example, when all prawns are very healthy or very sick, comparison of average Ct values is a useful metric. Compared to average copy number, Ct value is less skewed by the presence of a small number of individuals with a high calculated copy number (Refer to Cohort 1 and 2

Table 12).

11.3.10 Minimum Ct value

Animals with high pathogen load (lowest Ct) pose the greatest immediate biosecurity risk. In the absence of a disease outbreak, there are very few low Ct value positives in a single cohort. Hence, the average Ct is not always a good indicator of biosecurity risk. Minimum Ct value is an indicator of the earliest detections of target by qPCR analysis i.e. it is reflective of highest copy number and a more accurate indicator of biosecurity risk (Refer to Cohort 3

Table 12).

11.3.11 Calculated copy number (CCN)

Ct value is affected by a range of variables including the different brands of reagent, different qPCR machines and different tissue processing protocols. Ct values should not be stringently compared between different laboratories as variance of 1-3 Ct values will generally occur. The statistical relationship between Ct value and copy number is less affected by variations in laboratory protocols. For comparison with other studies/laboratories calculated copy number is more appropriate; however, as the unit for expression of calculated copy number is not standardized across laboratories, calculated copy number comparisons are not always appropriate either. CCN expressed in this report refers to the number of copies present per ul of the total nucleic extract. Other laboratories report CCN as copies per ng of Total Nucleic Acid, copies per mg of tissue and copies per 400ng of Total nucleic acid. Regardless of the CCN calculation, individuals that have a high copy number disproportionately increase the mean and the standard deviation of their entire cohorts' result (e.g. Sample 15 in Table 12), IHHNV Ct of ~15.85 will have a copy number approximately 1.2×10^8 which markedly increases the cohort mean to 3.3×10^7 (High risk) rather than 6.4×10^4 (Moderate risk) which is the average of same cohort of animals only samples 11 to 14 are considered).

11.3.12 Interpreting qPCR results to determine Biosecurity risk

Wild caught broodstock are typically introduced into a hatchery as a batch. In the absence of mass mortality, there is no single metric that provides an indication of the biosecurity risk posed by the introduction of a broodstock batch. Rather hatchery managers should consider a range of criteria. 12, provides a number of examples of how qPCR Ct value and calculated copy number can be considered to support risk-management decisions. The example provided is applicable to IHHNV analysis on a broodstock cohort whereby a cohort of high load individuals with presents a high risk Levels of risk vary between lifestage, stocking density and pathogen. There is very little, industry wide data available to support the implementation of specific actions in relation to qPCR analysis. Presently, the most common application of qPCR to determine biosecurity risk is to determine freedom from WSSV, YHV-1 and AHPND.

Table 12 Guide to Interpretation of qPCR results indicating the results of three hypothetical cohorts of test animals. Cohort 1 typifies the result of a healthy cohort whereby all animals have a high Ct value (close to 40) which equates to a low calculated copy number. Low standard deviation (less than 1.5) indicates the Average Ct of the cohort is representative of the whole cohort and overall level of risk posed by this cohort is low. Cohort 2 is typical of a diseased cohort whereby all individuals have a low Ct and corresponding high CCN and overall high risk of disease and mortality. Cohort 3 is typical of a mixed cohort whereby some individuals have low Ct and low-moderate CCN, yet a single individual has a low Ct and high CCN. The standard deviation of 6.5 of Cohort 3 indicates the average Ct and CCN is not representative of the cohort and biosecurity management to remove the high-risk animal will reduce the risk of the cohort from High to moderate to express disease.

Guide to Interpretation of qPCR results						
Visible "health" status	Individual qPCR Analysis Results			Summary of Analysis of each cohort		Level of Risk
	Prawn Id.	Ct value	Calculated copy No. mL^{-1}			
Cohort 1: "Healthy"	1	34.4	7.01E+03	Average Ct value	Average copy No. mL^{-1}	Low
	2	35.5	3.24E+03	35.90	2.94E+03	
	3	36.9	1.37E+03			
	4	36.7	1.54E+03	Minimum Ct value	Maximum copy No. mL^{-1}	Low
	5	36.0	2.35E+03	34.36	7.01E+03	
	Std.dev.	0.9	2.06E+03	low s.d. compared to mean. average is indicative of cohort		
Cohort 2: "Diseased" expect mass mortality in tanks	6	18.7	1.82E+08	Average Ct value	Average copy No. mL^{-1}	High
	7	19.2	1.31E+08	17.2	5.33E+08	
	8	15.6	1.34E+09			
	9	17.0	5.45E+08	Minimum Ct value	Maximum copy No. mL^{-1}	High
	10	15.6	1.34E+09	15.6	1.34E+09	
	Std.dev.	1.5	5.34E+08	low s.d. compared to mean. average is indicative of cohort		
Cohort 3: "Variable" expect one mortality with possible spread and further mortalities	11	35.4	3.3E+03	Average Ct value	Average copy No. mL^{-1}	Moderate / High
	12	35.4	3.2E+03	30.62	3.3E+07	
	13	28.7	2.4E+05			
	14	34.9	4.5E+03	Minimum Ct value	Maximum copy No. mL^{-1}	High
	15	18.7	1.7E+08	18.7	1.7E+08	
	Std. dev.	6.5	6.65E+07	high s.d. compared to mean. average is not indicative of cohort		

Note: The risk ratings above are generalised. The ratings of low-moderate-high are variable with each target. E.g a Pir-A Ct of 28 would be considered higher risk than at GAV Ct of 28.

Low Risk	Moderate-Low Risk	Moderate-High Risk	High Risk

12 Progress Report: CRC NA Project: Biosecurity in northern Australia prawn aquaculture.

CRC NA Project: Biosecurity in northern Australian prawn aquaculture.

Project Reference Number: A.3.1718113

Project progress Report: Farm Component: Detection of endemic pathogens from prawn hatchery-systems

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Project Reference Number: A.3.1718113

Project progress Report: Hatchery component: Detection of endemic pathogens from prawn hatchery systems

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13 Acknowledgements: Hatchery component

14 Executive summary: Hatchery component

This report provides a summary to the Australian Prawn Farmers Association of Component I: Detection of endemic pathogens from prawn hatchery systems of the CRC NA project "Improving Biosecurity in northern Australian prawn aquaculture" (Ref No. A.3.1718113). The aim of Component 1 was to attain a "snap-shot" assessment of the pathogens that are present at the starting Component of prawn production, namely, the hatchery system.

All samples tested in Component 1 were collected from four commercial hatcheries that supply over 80 % of the post larvae to the Australian prawn farming industry. Nine hundred and sixty-seven pleopod samples collected from wild-caught *Penaeus monodon* broodstock were analysed for the presence of WSSV, YHV-1, IHHNV, GAV, YHV-7, HDV, When-2, Pir-A, and the *P. monodon* Dicer-1 gene. Details of the geographic source (East Coast Queensland or Northern Territory), health status (culled, moribund, dead, dead on arrival and jumped from the tank), production status (pre- or post-spawn) and date of sample collection were provided. No date of sample collection is included in this report. Data that is potentially commercially sensitive, has been withheld until the harvest of the current crop but will be incorporated into the final report.

There was no detection of WSSV or YHV-1 from any of the prawns sampled. Dicer-1 was detected from 99 % of the samples analysed. A total of 896/967 (93 %) samples were positive for the detection of an endemic pathogen target. Only 71/967 (7 %) of samples were negative for all of the targeted pathogens. GAV and IHHNV were the most frequently detected pathogen targets. The majority of samples, 695/967 (72 %) analysed were positive for the detection of more than one of the target pathogens. There were very few individuals positive for the presence of a single pathogen target. From a total of 967 prawns the single-target detections included GAV (17 %) IHHNV (1.7 %), HDV (0.6 %) and When-2 (0.3 %).

The overall prevalence and severity of infection were comparable between the East Coast wild monodon (ECWM) and Northern Territory wild monodon (NTWM) sourced broodstock. However, there were some variance between the geographic sources for individual pathogen targets. Most notably, ECWM collected broodstock displayed a higher prevalence of When-2 (46 %) compared to NTWM (18 %). Pir-A was only detected from NTWM (8 %). The range of hatchery variables between ECWM and NTWM were not standardised. Thus, the data presented is not a robust representation of the pathobiome (pathogen community) present in the animals at the point of collection from the wild. Rather, it is an indication of pathogens that are present, following typical conditions in the hatchery.

There were differences in the proportion of positive detections of qPCR pathogen targets when geographic source was further divided into a) prawn spawn status and b) prawn health status. The proportion of positive detections for HDV, YHV-7 and Pir-A was increased in the post-spawn cohort compared to pre-spawn. The proportion of When-2 positives was lower in the post-spawn cohort of ECWM but higher in the post-spawn cohort of NTWM. When prawn health status at the time of sample collection was considered there were some individuals within the "poor" health cohort with very high levels of pathogen load; however, there was no single dominant pathogen detected, nor was the average levels of detection very different from that of levels of detection of prawns' pre-spawn.

From the total number of samples, GAV (3 %), IHHNV (3 %) and When- 2 (8%) were the only pathogen targets detected in high calculated copy number, greater than 10^6 mL^{-1}. GAV, IHHNV and When-2 were detected in high calculated copy number both pre-spawn and post-spawn.

Component 1 has achieved the overarching aim to improve the understanding of pathogen populations present in the hatchery production Component. The analysis conducted in Component 1 of the NA CRC is the most comprehensive and extensive study presented to date that examines endemic viruses in Australian prawn hatchery systems. Component 1 activities have collected samples and data that will contribute to Components 2 and 3. The next progress report is scheduled for delivery in September 2019.

15 Background: Hatchery component

During 2016/2017 prawn farms located in the Logan River Catchment in South East Queensland were severely impacted by Whitespot syndrome disease (WSD). Although Whitespot syndrome Virus (WSSV) has not been detected beyond the movement restriction zone in SE QLD, the potential establishment of WSSV in the environment or spread of WSSV outside the Moreton Bay poses high risk to prawn farming activities in Queensland.

The increased risk posed by the presence of WSSV has led to an increased priority on improved biosecurity systems in Australian prawn farms. However, the paucity of data about the pathobiome in the various stages of production in Australian prawn farming makes evidence-based management decisions very difficult. The CRC for Developing Northern Australia (CRC NA), Australian Prawn Farmers Association (APFA), Fisheries Resource and Development Co-operation (FRDC) and James Cook University (JCU), in recognition of a need to improve biosecurity management and recreate a more resilient prawn farming industry, has developed the CRC NA Project "Improving Biosecurity in Australian Prawn Farms".

While WSSV is the most economically significant viral infection affecting prawn aquaculture, some pathogens that are endemic to Australia, namely Yellowhead Virus strain-7 (YHV-7) and bacteria that host the Photorhabdus insect-related toxin like-gene (Pir-AB), can also cause substantial losses to farm productivity. Additional viruses that are endemic to Australia, including, Infectious Hypodermal Hematopoietic Necrosis Virus/Penaeid stylirostris Densovirus (IHHNV/PstDV), Hepatopancreatic Necrosis Virus/ Hepandensovirus (HPV/HDV), and Yellowhead virus strain-2/Gill Associated Virus (GAV), although being less severe, can also cause economic loss when culture conditions are sub-optimal. Analysis for the detection of endemic viruses serves as a good indicator of the effectiveness of current industry practices to prevent the entry of pathogens. Such information is useful to consider how to improve biosecurity management protocols.

A combination of factors determine the level of biosecurity risk faced by the prawn aquaculture industry:

a) The likelihood of introduction of a pathogen (i.e. the number of animals that enter the production system that are infected with a pathogen).

b) The level/severity of infection that is being introduced in animals that are infected (i.e. the calculated copy number of the pathogen).

c) The impact of the infection on health/productivity in the hatchery (i.e. does the infection cause acute disease outbreaks or have chronic effects on health/productivity).

d) The dynamics involved in the expression of disease and spread of the pathogen (i.e. what causes animals to display disease outbreaks rather than be persistent carriers of the pathogen).

e) The ability to manage the culture environment, through genetics, nutrition and water quality to favour prawn health (i.e. can the factors that lead to disease outbreaks be managed?).

The NA CRC project will gather information towards a better understanding of topics a-e. The project will operate through to June 2020. The project is a five-component program consisting of:

a) Component 1: Detection of endemic pathogens from prawn hatchery systems.

b) Component 2: Detection of endemic pathogens from prawn pond grow-systems.

c) Component 3: Detection of emerging pathogens from prawn production systems.

d) Component 4: Evidence-based assessment of enterprise and industry level biosecurity.

e) Component 5: Improving biosecurity in Australian prawn farms.

16 Aim: Hatchery component

The overarching aim of Component 1 is to improve understanding of the pathobiome, which is the community of pathogens, within the hatchery production Component under typical hatchery conditions. Component 1 of the project investigates the prevalence and calculated copy number/severity of infection by pathogens in broodstock from hatchery collected samples. Analysis relating to post larvae testing has been withheld to protect commercially sensitive information and to allow the data to be presented as a more complete picture with the pond collected samples.

The data presented in this report should not be used to guide broodstock source selection decisions. Analysis conducted at JCU AquaPATH indicates there is significant temporal (season/monthly) variation in the pathobiome of ECWM and NTWM broodstock (unpublished data). The results presented in this report do not accurately reflect the temporal-geographic variation patterns. A structured surveillance system is required to gather data to support broodstock source selection decisions. The project did not standardize the number of days the broodstock were held prior to sample collection. Some of the positive detections of target will be a result of transmission of the pathogen within the hatchery system.

17 Method: Hatchery component

17.1 Sample collection

Four hatcheries participated in the project. Each hatchery initially provided a maximum of 160 samples for analysis. The source of samples was not standardized across the hatcheries. Hatchery management were directed to select samples that would investigate the biosecurity topic they considered a priority for their hatchery. The following topics were common across the four hatcheries:

a) Pathobiome of wild East Coast Queensland and Northern Territory sourced *P. monodon* broodstock. Herein referred to as ECWM and NTWM respectively.
b) Changes in the broodstock pathobiome from time of collection in wild, time of sample collection for translocation testing (pre-spawn) and to end of spawning (post-spawn).
c) Effect of pooling on pathogen detection.
d) Pathobiome of larval and Post-larval *P. monodon.*

Samples collected in Component I have been retained and will be applied to Component 3: "Detection of emerging pathogens from prawn production systems". At industry request, an additional 310 samples were collected.

17.2 Target tissues, species, and the number of samples

A total of 967 *P. monodon* broodstock samples were analyzed. Although some hatcheries provided whole frozen prawns, all analysis reported for Component 1 was conducted on one pleopod from each animal. Frozen whole prawns have been retained for Component 3 analysis. The number of broodstock sourced from East Coast Queensland (n= 489) and Northern Territory (n= 475) was approximately equal.

17.3 Analysis of samples by qPCR

Samples were analysed by the standard operating protocols used in the JCU AquaPATH laboratory. Briefly, tissue samples were extracted using the MagMAX Core nucleic acid extraction protocol on a Kingfisher Flex 96 well extraction robot. Total nucleic acid was analysed by qPCR using Bioline and/or Applied Biosystems qPCR mixes. More detailed outline of qPCR protocols is available on request and will be provided in the project final report. The assays targeted the detection of:

- Whitespot Syndrome Virus (WSSV), (Exotic virus)
- Yellowhead Virus-1 (YHV-1), (Exotic virus)
- Yellowhead Virus-7 (YHV-7), (Endemic virus)
- Gill Associated Virus (GAV), (Endemic virus)
- Penaeid stylirostris Densovirus (IHHNV/PstDV), (Endemic virus)
- Hepandensovirus (HDV/HPV), (Endemic virus, some strains Exotic)
- Whenzhou Shrimp Virus-2 (When-2) (Endemic virus, presently uncharacterized, discovered in 2018)
- Pir-A toxin gene (Endemic bacterial toxin gene)
- *Penaeus monodon* Dicer-1 gene (proposed integrity control)

18 Results of Analysis: Hatchery component.

There was no detection of WSSV or YHV-1 from 967 broodstock samples. In addition to this report, each hatchery was provided with their respective individual results. The project reports on the combined data from all hatchery analysis. Some commercially sensitive data will be withheld from this report and provided in the 12-month project progress report after the current crop cycle is complete. To align the results with the factors that can be used to determine biosecurity risk, the results are presented in the following sections:

- Overview of endemic targets with geographic source (ECWM and NTWM).
    - Endemic target summary
    - Number of detections and percentage of positive detections
    - qPCR results: Ct value
    - qPCR results: Calculated copy number
- Individual endemic target results.
    - *P. monodon* Dicer-1 gene
    - HDV
    - IHHNV
    - GAV
    - YHV-7
    - When-2
    - Pir-A

Because the sample collection was not a balanced design, robust statistical analysis of the data is not possible. However, trends in detection are presented in the following sections:

- Trends by prawn activity status (if known).
    - Pre-spawn v Post-spawn.
- Trends by prawn health status (if known).
    - Collected at sea, dead on arrival (DOA), dead in hatchery, died by jumping from tank, live for health assessment (pre-spawn), culled post spawn (post-spawn).
- Trends by sample collected at sea v collected at hatchery.
- Trends in the detection of multiple infections.

19 Overview of Results: Hatchery component

All of the endemic pathogen targets were detected. Within this report, if results are provided as a Figure, the tabulated data is contained in the Appendix.

19.1 Endemic target overview

Overall summary of endemic targets:

- An endemic pathogen was detected from 896/967 samples (93 %).
- The Dicer-1 gene was detected from 959/967 samples (99 %).
- Dicer-1 was detected in higher <u>average</u> copy number than any of the pathogen targets.
- There were 71/967 samples with no pathogen detected (7 %). Five of the samples were also negative for the detection of Dicer-1 indicating they were not suitable for analysis by qPCR.
- GAV (89 %) and IHHNV (47 %) were the most prevalent viruses detected.
- There were 196/967 single target detections (20 %).
- There were 343/967 dual target detections (36 %).
- There were 298/967 triple target detections (31 %).
- There were 51/967 quadruple target detections (5.3 %).
- There were 3/967 broodstock positive for 5 targets; all were post-spawn collected samples (0.5 %).
- YHV-7 (11 %) and Pir-A (8 %) were detected in low prevalence.
- IHHNV, GAV and When-2 were the only targets detected with a <u>maximum</u> calculated copy number exceeding 10^8 copies mL^{-1}.

A summary of the endemic agents is presented in three sections namely:

1. Prevalence of detection (number of positive detections; proportion of positives detected/total number analysed).
2. qPCR results of detection (Ct value).
3. qPCR results of detection (mL^{-1}).

19.2 Prevalence of detection of targets

The number and percentage of broodstock with a detectable pathogen target is a useful metric to consider the likelihood of introduction of a pathogen. Table 13 displays a summary of the number of positive detections with reference to geographic source of broodstock. The target pathogens were detected in 90% of the samples analyzed. GAV and IHHNV were the most frequently detected pathogen targets. HDV, IHHNV, GAV and When-2 were detected more frequently in ECWM compared to NTWM.

Table 13: Number of positive detections of targets from ECWM and NTWM broodstock.

Source	Total tested	Number of positive detections of each target						
		HDV	IHHNV	GAV	YHV-7	When-2	Pir-A	Dicer-1
ECWM	489	115	293	467	52	226	0	489
NTWM	475	78	162	393	57	84	81	470
Total	964*	193	455	860	109	310	81	959
% Positive		20%	47%	89%	11%	32%	8%	99%

*Three samples were analyzed in the project with no geographic source information.

Figure 24 displays the percentage of samples positive for the detection of targets by geographic source of broodstock. IHHNV and When-2 were approximately 26 % more prevalent in the ECWM compared to NTWM. Pir-A was only detected from NTWM (17%). The detection of Dicer-1 and YHV-7 was approximately equal in ECWM and NTWM.
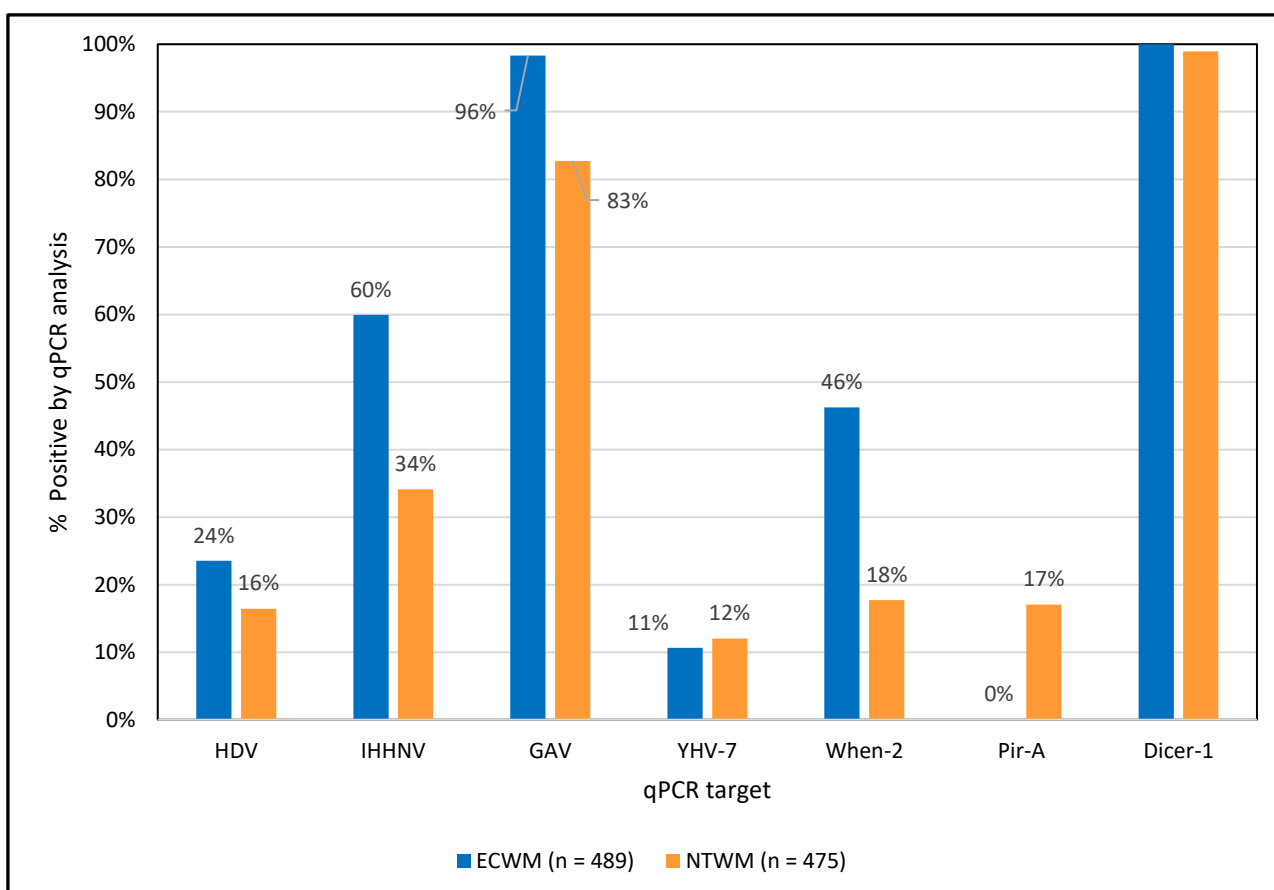
Figure 24: Percentage of samples positive for the detection of each target from ECWM and NTWM broodstock.

19.3 qPCR results of detections (Ct value)

19.3.1 Average Ct value

The average Ct value of each pathogen target falls within the low to moderate category illustrated in Table 1 page 7. Table 14 indicates the average Ct value of positive detections for each target summarized by geographic source. The average Ct of positive detections was comparable between the geographic sources. *P. monodon* Dicer-1 had the lowest average Ct value. All the average Ct values of pathogen targets are greater than 30.

Table 14: Average Ct value of qPCR detection of each target from ECWM and NTWM broodstock.

Source	Total tested	Average Ct value of qPCR detection of each target						
		HDV	IHHNV	GAV	YHV-7	When-2	Pir-A	Dicer-1
ECWM	489	39.16	34.45	33.98	35.74	30.58	nd	**28.61**
NTWM	475	38.39	32.06	31.44	35.77	32.58	35.00	27.96
Total	964	38.85	33.60	32.81	35.76	31.10	35.00	28.29

*nd= not detected

19.3.2 Standard deviation

Table 15 summarises the standard deviation in Ct of the positive detections of each target. When-2, IHHNV and Pir-A were detected with SD greater than that of the Dicer-1 gene. The larger standard deviations indicate large variation in the Ct values.

Table 15: Standard deviation of Ct value of the positive detections of each target.

Source	Total tested	Standard deviation of Ct value of qPCR detection of each target						
		HDV	IHHNV	GAV	YHV-7	When-2	Pir-A	Dicer-1
ECWM	489	1.43	**3.38**	2.21	1.20	**5.68**	nd	**2.30**
NTWM	475	2.18	**5.20**	2.97	1.10	2.40	**3.00**	2.88
Combined	964	1.81	4.27	2.87	1.14	5.09	3.00	2.62

Note: A cohort with very little variance in the analysis result will have a low s.d. Cohorts that are all very healthy or all very sick will have a low standard deviation. Broodstock collected from the wild tend to have a greater amount of variation and higher SD

19.3.3 Minimum Ct value

Table 16 summarises the minimum Ct for each target. IHHNV, GAV and When-2 were detected with minimum Ct values that fall within the High load category (less than 20). In addition, the Ct values were also lower than those of Dicer-1. Comparison with Dicer-1 is preliminary and requires further investigation. Because Dicer-1 is a prawn gene, the detection of any pathogen target, earlier than that of host genes is theoretically an appropriate index to indicate potential impacts on prawn health. The project will continue to monitor the usefulness of comparisons between pathogen targets and Dicer-1.

Table 16: Minimum Ct value of qPCR detection of each target from ECWM and NTWM broodstock.

Source	Total tested	Minimum Ct value of qPCR detection of each target						
		HDV	IHHNV	GAV	YHV-7	When-2	Pir-A	Dicer-1
ECWM	489	35.53	17.63	13.88	33.00	15.64	nd	**23.48**
NTWM	475	32.00	15.85	20.04	33.56	21.78	27.66	21.86
Combined	964	32.00	15.85	13.88	33.00	15.64	27.66	21.86

Note: Minimum Ct value is an indicator of the earliest detections of target by qPCR i.e. is an index of high copy number. The lower the Ct value, the sooner the target was detected because a high number of copies of target were present. Ct values less than 20 (/40 cycles) are indicative of detection of very high numbers of target copies.

19.3.4 Average calculated copy number (mL^{-1})

The average copy number of HDV and IHHNV was increased in NTWM compared to ECWM. Conversely the average copy number of GAV and When-2 was higher in ECWM. The differences in copy number detected were approximately 10 fold between sources. Refer to Figure 25.

Some of the increased proportion of positive detections and calculated copy number trends observed between the geographic sources may be related to the status of the animal when the sample was collected rather than true geographic trends of target detections. The status of the broodstock along with geographic source are discussed further in sections that incorporate the estimated health and activity status at the time of sample collection.
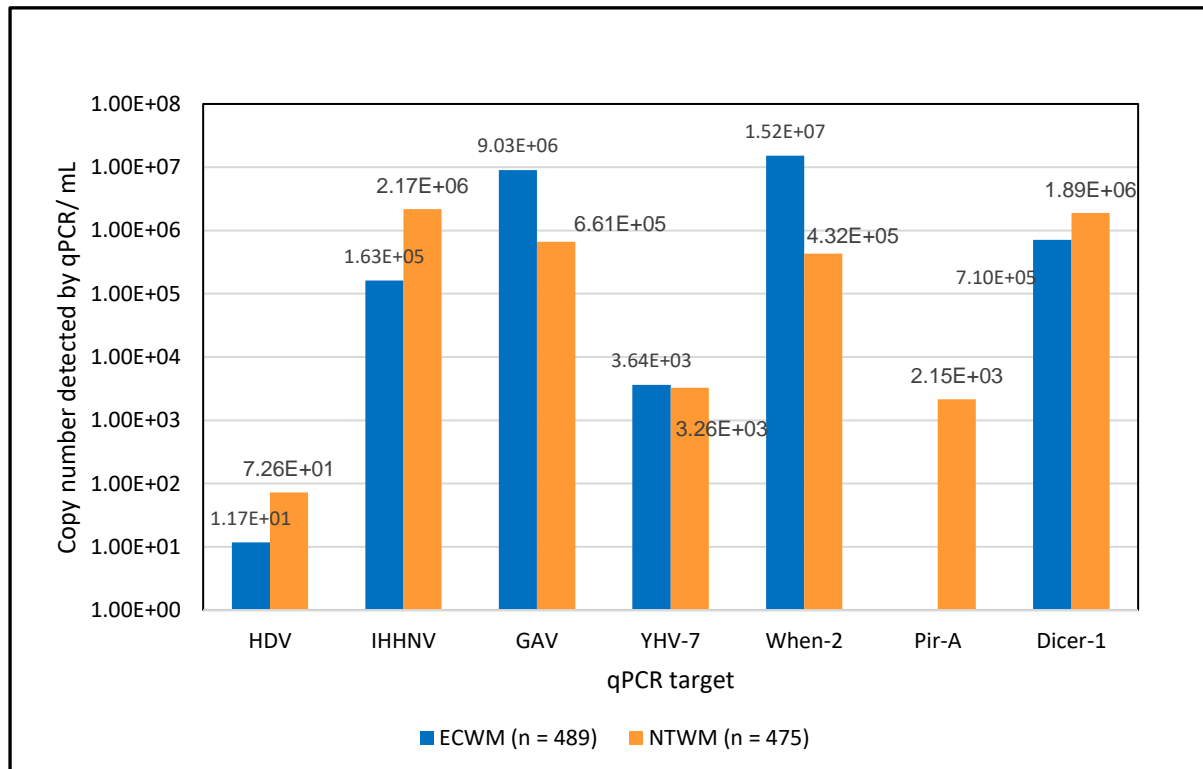
Figure 25: Average calculated copy number of each qPCR target detected from ECWM and NTWM broodstock.

19.3.5 Maximum calculated copy number

Maximum calculated copy number displayed a similar trend to average calculated copy number. Refer to Figure 26.
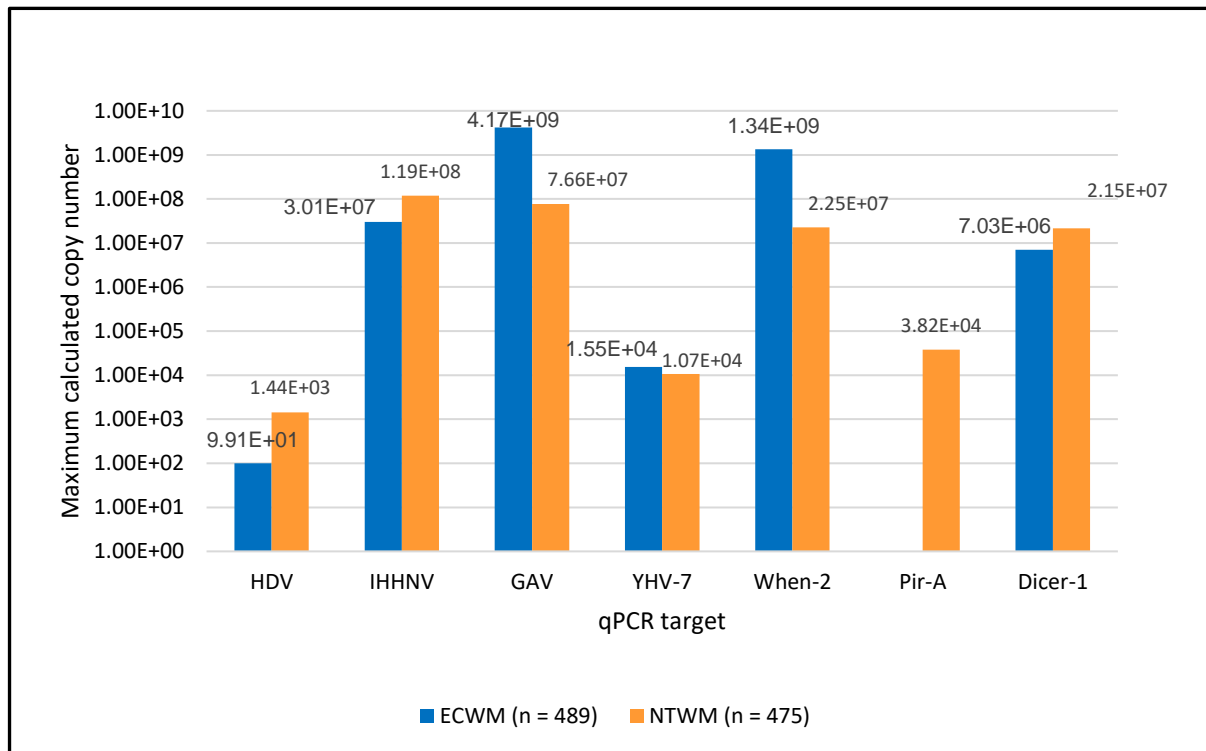
Figure 26: Maximum calculated copy number of each target detected from ECWM and NTWM broodstock.

19.4 Endemic Targets: Individual target summaries

A summary of detection of each of the individual targets is listed:

19.4.1 P. monodon Dicer-1 gene

- Dicer -1 was detected in 962 (99 %) samples.
- The 5 samples negative for Dicer-1 were also negative for all qPCR targets.
- Dicer-1 was detected with the lowest average Ct value (28.61).
- Average copy number for Dicer-1 was 1.29 x 10^6 copies mL^{-1}.
- Scatter plots indicate Dicer-1 Ct does not correlate with any of the target Ct values across the broad range detected in this component. (Refer to Appendix 1).
- Some comparison between Dicer-1 and low Viral Ct values are provided in the individual target sections.
- Only IHHNV, GAV, and When-2 were detected with maximum higher copy number than Dicer-1.
- Only IHHNV and When-2 were detected with a maximum higher copy than Dicer-1 <u>without</u> another target being present in high copy number.

19.4.2 Gill Associated Virus (GAV)

- GAV was detected in 863 samples (89 %).
- The average Ct value of GAV was 32.81.
- The average load of GAV was 5.2 x 10^6 copies mL^{-1}.
- The highest load of GAV was detected in ECWM and was 4.17 x 10^9 viral copies mL^{-1}.
- GAV was detected in higher occurrence in ECWM (96 %) compared to NTWM (83 %).
- GAV was detected as single, dual, triple, quadruple and quintuple target detections.
- When GAV was detected with Ct values less than 24, Dicer-1 Ct's were higher than GAV Ct. i.e. there was more GAV detected than the prawn Dicer-1 gene (data provided in Appendix 1).

19.4.3 Penaeus stylirostris Densovirus (IHHNV/PstDV)

- IHHNV was detected in 445 samples (46 %).
- The average Ct value of IHHNV was 33.60.
- The average load of IHHNV detected was 8.7 x 10^5 copies mL^{-1}.
- The highest load of IHHNV was detected in NTWM and was 1.2 x 10^8 copies mL^{-1}.
- IHHNV was detected in higher occurrence in ECWM (60 %) compared to NTWM (34 %).
- IHHNV had a lower occurrence in broodstock that were subjected to cold stress compared to not cold stressed.
- IHHNV was detected as single, dual, triple, quadruple and quintuple target detections.
- In all cases where IHHNV was detected with Ct values less than 24, the Ct value of Dicer-1 was higher than that of IHHNV i.e. there was more IHHNV detected than the prawn Dicer-1 gene.

19.4.4 Hepandensovirus (HPV/HDV)

- HDV was detected in 193 samples (20 %).
- The average Ct value of HDV was 38.85.
- The average load of HDV was 3.6 x 10^1 copies mL^{-1}.
- The highest load of HDV was detected in NTWM and was 1.4 x 10^3 copies mL^{-1}.
- HDV was rarely detected as dual infection with IHHNV (0.1% occurrence).

- HDV had similar prevalence of detection in ECWM (24 %) and NTWM (16 %) however, ECWM had a higher prevalence by 8 %.
- HDV was detected as single, dual, triple, quadruple and quintuple target detections.
- There were no cases where the HDV Ct value was lower than the Dicer-1 value.

19.4.5 Pir-A toxin gene (Pir-A)

- Pir-A was detected from 81 samples (8 %).
- The average Ct value of Pir-A was 35.00.
- The average load of Pir-A was 2.15×10^3 copies mL^{-1}.
- Pir-A was only detected from NTWM.
- The highest load of Pir-A was detected in NTWM and was 3.82×10^4 copies mL^{-1}.
- The proportion of Pir-A positive detections increased dramatically from pre-spawn (5 %) to post-spawn (56 %).
- There were no cases when the Pir-A Ct value was lower than the Dicer-1 Ct value.
- Levels of detection were generally low, with an average of 2.2×10^3 copies mL^{-1} and maximum detection of 3.8×10^4 copies mL^{-1}.
- The optimal tissue for detection of Pir-A, for disease diagnostic testing is hepatopancreas and stomach.
- The relationship between the detection of Pir-A from a pleopod sample and disease risk has not been established.
- Although levels of detection were low from pleopod tissue, levels of Pir-A in hepatopancreas and stomach may be much higher.
- Pir-A was only detected in dual, triple, quadruple and quintuple target detections.

19.4.6 Whenzhou Shrimp Virus-2 (When-2)

- When-2 was detected in 311 samples (32 %).
- The average Ct value of When-2 was 31.10.
- The average load of When-2 was 1.12×10^7 copies mL^{-1}.
- The highest load of When-2 was detected in ECWM and was 1.34×10^9 copies mL^{-1}.
- When-2 was detected in higher average load (1.5×10^7 copies mL^{-1}) than the other targets. By comparison, GAV was detected with the next highest load of 8.1×10^6 copies mL^{-1}.
- When-2 was detected more frequently in ECWM (46%) compared to NTWM (18%) but, positive detections in NTWM were mainly observed only after prawns had been in the hatchery for a period of time. NTWM stocks may have a much lower prevalence of When-2 in their natural stocks than is indicated by this report. Nonetheless NTWM are susceptible to When-2.
- When-2 was detected as single, dual, triple, quadruple and quintuple target detections.
- In cases where When-2 was detected with a Ct less than 27 the corresponding Dicer-1 Ct was higher than the When-2 Ct i.e. there were more copies of When-2 virus detected than Dicer-1 prawn gene.

19.4.7 Yellowhead Virus-7 (YHV-7)

- Yellowhead Virus-7 was only detected in 111 samples (11 %).
- The average Ct value of YHV-7 was 35.76.
- The average load of YHV-7 was 3.43×10^3 copies mL^{-1}.
- The highest load of YHV-7 was detected in ECWM and was 1.55×10^4 copies mL^{-1}.

- The distribution of the positive detections were approximately equal in each source ECWM (11 %), NTWM (12 %).
- <u>YHV-7 was only</u> detected from cold stressed animals.
- YHV-7 was not detected as a single target infection.
- HDV was detected as a dual, triple, quadruple and five target detections.
- There were no cases when the YHV-7 Ct value was lower than the Dicer-1 value.

19.5 Multiple infections

The majority of samples were positive for the detection of multiple pathogen targets (Refer to Table 17). Eighty-seven percent (87 %) of the broodstock samples were positive for the detection of between 1 and 3 pathogen targets. Dual infections were the most common. Dual detections accounted for the highest proportion of multiple pathogen target positives (35.5 %), along with triple (30.8 %) single (20.3 %) and finally quadruple (5.3 %). There was negligible difference between ECWM and NTWM except for triple infections. For samples in the triple detection category NTWM had 49.5 % less number of positives, despite both stocks having close to 300 (±20) prawns tested from each, and triple infections accounting for 30.8 % of all cases.

GAV and IHHNV were the most common dual pathogen target detected occurring in 13.9 % of the samples, When-2 and GAV was the next highest dual detection (9.2 %). Cases that were positive for 3-5 pathogen targets in a single prawn were less common with an overall prevalence of 36.4 %. The most common triple pathogen target detection consisted of IHHNV and GAV were present with another virus target i.e. When-2 which was detected in combination with IHHNV & GAV 144 times (15 % of population). Cases were all five viral targets were detected from an individual prawn occurred in 0.3 % of the population which is lower than prawns that were negative for all target pathogens which comprised 7.3 % of the population, or 71 prawns out of a total 967.

Table 17: Summary of number of multiple detections of qPCR targets in the same sample.

Number of Targets detected in the same sample.	Targets detected	HDV	IHHNV	GAV	YHV7	When-2	Pir-A tox	Count	Occurrence (%)	Total Occurrence (%)
0	No pathogen detected							71	7.4%	7.3%
1	HDV	6						6	0.6%	20.3%
	IHHNV		16					16	1.7%	
	GAV			**171**				171	**17.8%**	
	When-2					3		3	0.3%	
2	HDV		1					1	0.1%	35.5%
	HDV			47				47	4.9%	
	IHHNV			**134**				134	**13.9%**	
	IHHNV					**6**		6	**0.6%**	
	GAV				56			56	5.8%	
	GAV					**89**		89	**9.2%**	
	GAV						7	7	0.7%	
	YHV-7					1		1	0.1%	
3	HDV IHHNV			54				54	5.6%	30.8%
	HDV GAV				21			21	2.2%	
	HDV GAV				16			16	1.7%	
	IHHNV GAV				8			8	0.8%	
	IHHNV GAV					**142**		142	**14.7%**	
	IHHNV GAV						48	48	5.0%	
	GAV YHV-7					7		7	0.7%	
	GAV When-2						8	8	0.8%	
4	HDV IHHNV			6	6			6	0.6%	5.3%
	HDV IHHNV			19		19		19	2.0%	
	HDV IHHNV			10			10	10	1.0%	
	HDV GAV				8	8		8	0.8%	
	HDV GAV					1	1	1	0.1%	
	IHHNV GAV				1	1		1	0.1%	
	IHHNV GAV					6	6	6	0.6%	
5	HDV IHHNV GAV When-2						3	3	0.3%	0.4%
	HDV IHHNV GAV When-2				1			1	0.1%	
6	All targets							0	0.0%	0.0%
Total Positive		193	445	863	111	311	81	967	100.0%	
Percentage of Total Positive		20%	46%	89%	11%	32%	8%	93%		

19.6 Trends by prawn activity status (pre-spawn v post-spawn)

A summary of the percentage of positive detections in broodstock from each source collected at pre-spawn and post-spawn are provided in Figure 27 and Figure 28. A summary of the average copy number detected from broodstock from each source collected at pre-spawn and post-spawn are provided in Figure 29 and Figure 30. Trends are skewed by the lower number of samples analysed in the ECWM post-spawn cohort and also because there were moribund and dead animals sampled in every cohort. The number of moribund and dead animals in each group was not equal. The data is more accurately represented when prawn health status is considered. There appears to be an increase in the detection of Pir-A and YHV-7 from pre-spawn to post-spawn in NTWM. When-2 detection decreased in prevalence pre-spawn to post-spawn cohorts in both ECWM and NTWM. However, not all broodstock that died were provided to the project. Appendix 2 displays the average and range of Ct values of each target by pre-spawn and post-spawn.
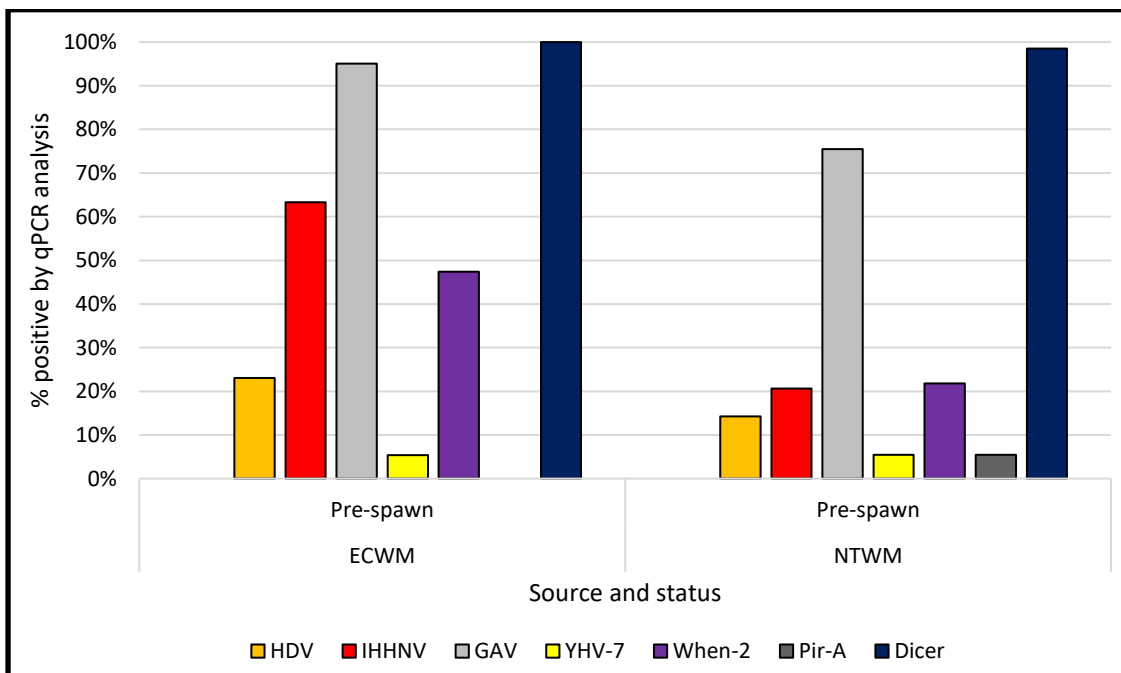
Figure 27: Percentage of positive detections from ECWM and NTWM at Pre-spawn.
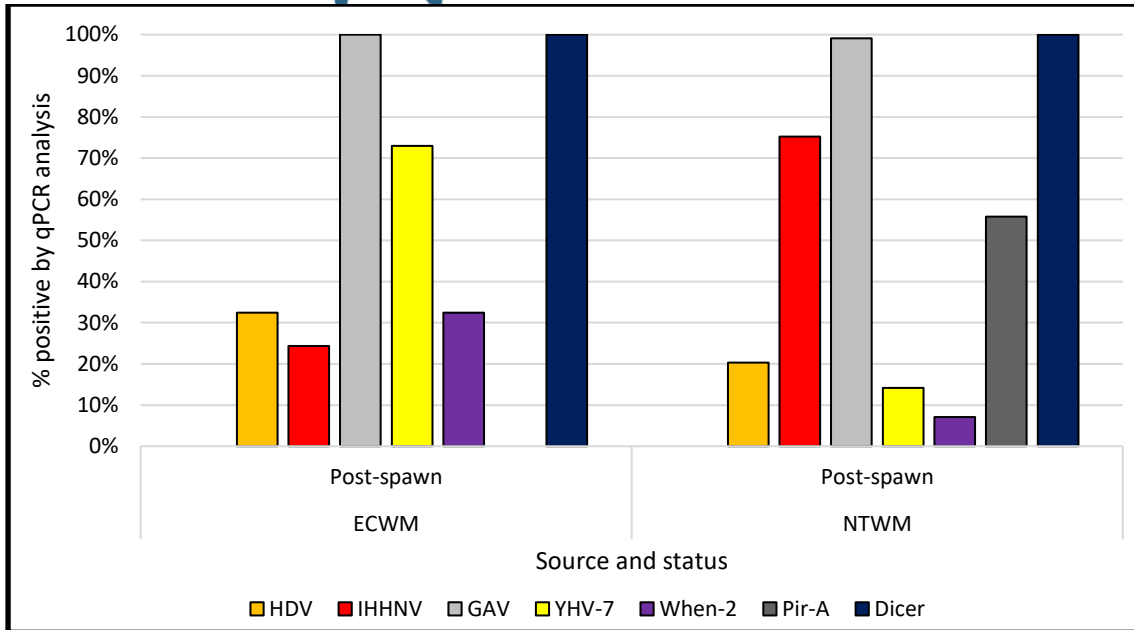
Figure 28: Percentage of positive detections from ECWM and NTWM at Post-spawn.
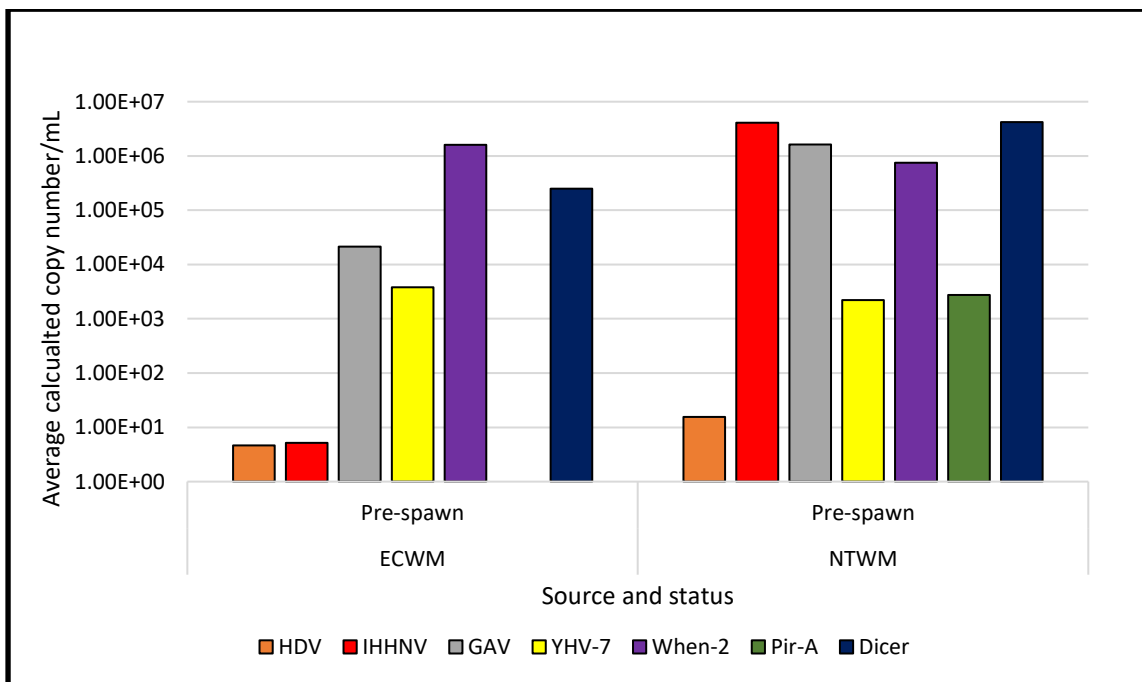
Figure 29: Average calculated copy number of positive detections from ECWM and NTWM Broodstock at Pre-spawn.
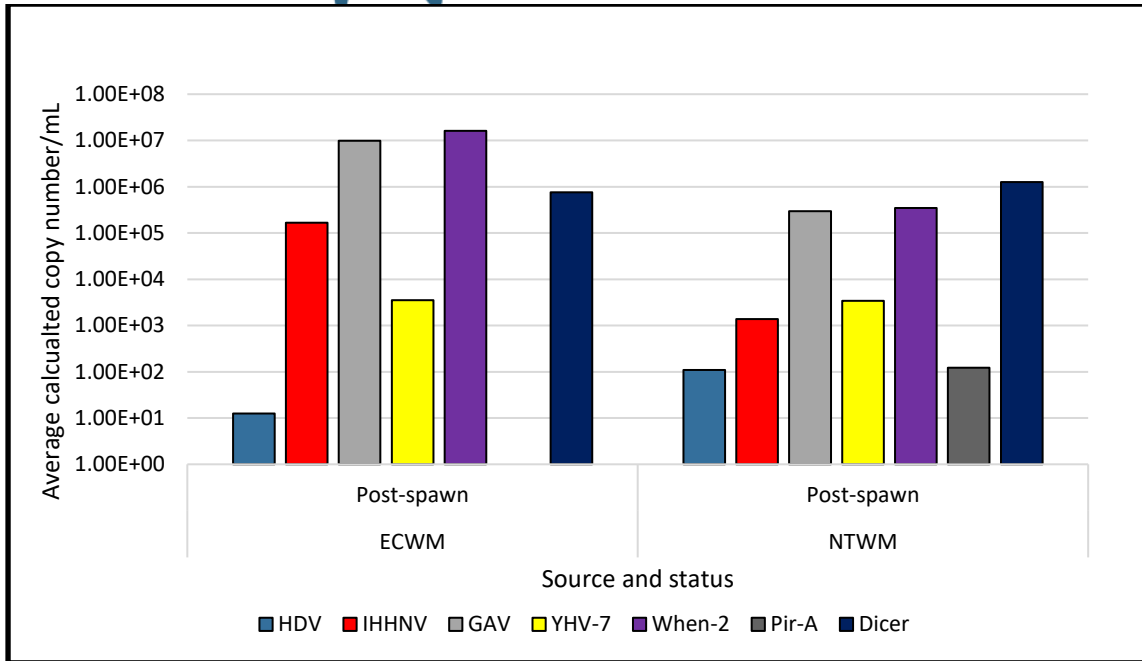
Figure 30: Average calculated copy number of positive detections from ECWM and NTWM broodstock at Post-spawn.

19.7 Trends by prawn health status (if known)

Due to the unbalanced design comparison of the number of positive detections of each health status is not practical (Table 18). As an alternative, relative percentage of positive detection of each qPCR target, linked with prawn health status is provided in Figure 31; incorporating geographic source and status Figure 32.

The relative percentage of Dicer-1 (dark blue) and GAV (grey) was approximately equal for each prawn status. The relative percentage of IHHNV, YHV-7, Pir-A and When-2 varied greatly with prawn health status, but no single pathogen target dominated in any of the groups that would generally be considered poor health (dead, dead on arrival, moribund and post spawn). All of the pathogen targets present in dead, post spawn, dead on arrival and moribund, which are presumably the poorer health cohorts, were also detected in the pre-spawn collected samples.

Table 18: Number of Positive detections with geographic source and prawn health status.

Source	Status	Total	HDV	IHHNV	GAV	YHV-7	When-2	Pir-A	Dicer-1
ECWM	Total	489	115	293	467	52	226	0	489
ECWM	DOA	26	15	2	25	18	8	0	26
ECWM	Jumper	5	0	1	6	1	2	0	6
ECWM	On-Trawler	93	19	83	88	0	48	0	93
ECWM	post	37	12	9	37	27	12	0	37
ECWM	Survey	330	69	198	314	8	157	0	330
NTWM	Total	475	78	162	393	57	84	81	470
NTWM	Dead	74	11	71	74	2	6	59	74
NTWM	DOA	13	6	4	12	6	0	0	13
NTWM	Jumper	30	8	9	30	21	4	0	30
NTWM	Moribund	3	2	3	3	0	0	1	3
NTWM	post	38	10	11	37	16	2	3	38
NTWM	Survey	317	41	64	237	12	72	18	312
	Total	967	193	455	863	111	311	81	962

	Dead	DOA	Jumper	Moribund	On-Trawler	post	Survey	Grand Total
Dicer-1	74	39	36	3	93	75	642	962
Pir_A	59	0	0	1	0	3	18	81
When-2	6	8	6	0	48	14	229	311
YHV7	2	24	22	0	0	43	20	111
GAV	74	37	36	3	88	74	551	863
IHHNV	71	6	10	3	83	20	262	455
HDV	11	21	8	2	19	22	110	193
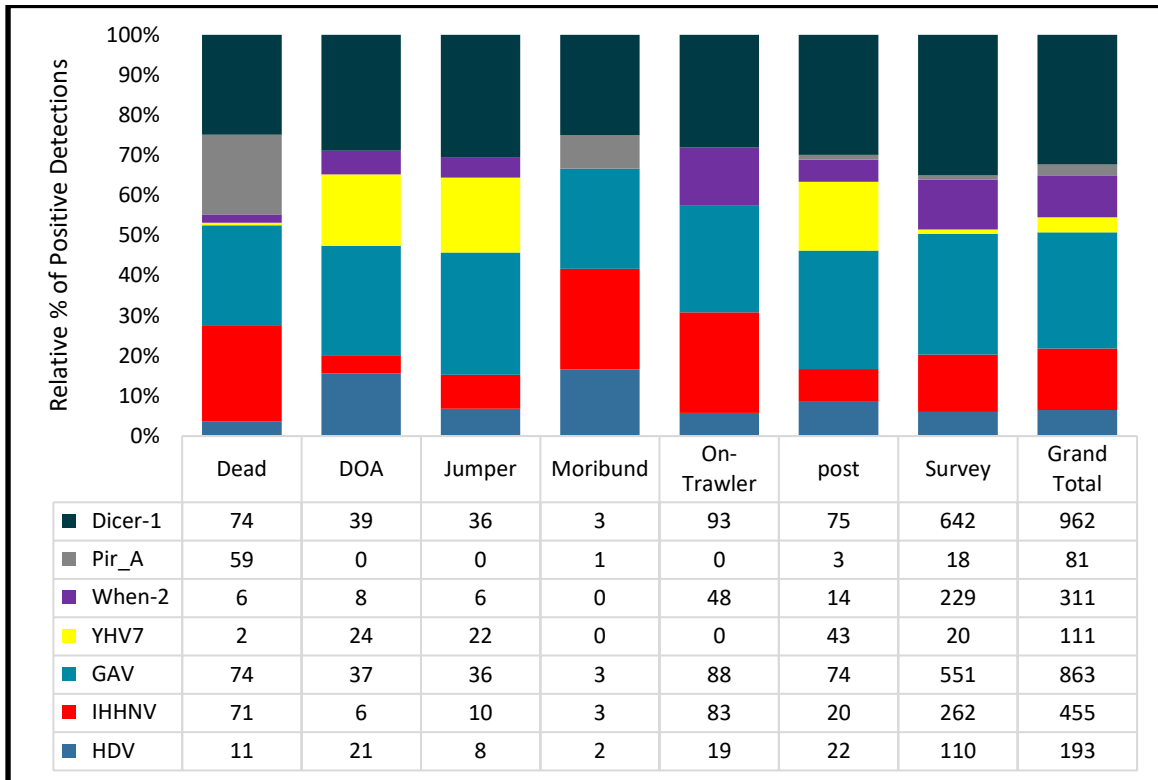
Figure 31: Relative percentage of positive detections of each target with condition at sample collection.

	Dead NTWM	DOA ECWM	DOA NTWM	Jumper ECWM	Jumper NTWM	Moribund NTWM	On-Trawler ECWM	Survey ECWM	Survey NTWM
Dicer-1	74	26	13	5	30	3	93	328	312
Pir_A	59	0	0	0	0	1	0	0	18
When-2	6	8	0	2	4	0	48	156	72
YHV7	2	18	6	1	21	0	0	6	12
GAV	74	25	12	5	30	3	88	312	237
IHHNV	71	2	4	1	9	3	83	198	64
HDV	11	15	6	0	8	2	19	69	41
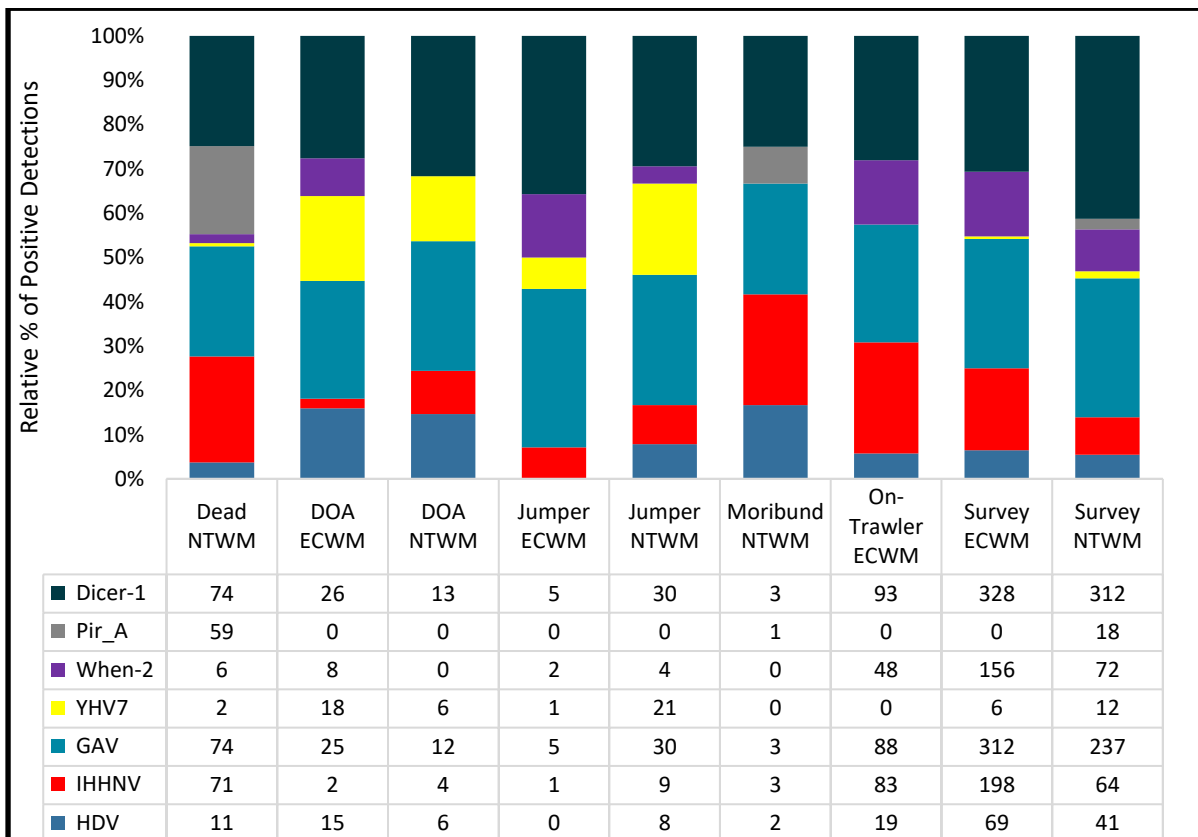
Figure 32: Relative percentage of positive detections of targets for each condition at sample collection divided into geographic source of broodstock.

Investigating the levels of detection of the qPCR results, also indicate that no single pathogen dominated the poorer health cohorts. Refer to Table 19 and Table 20, average Ct value and minimum Ct value are presented for each status. Whilst there were some detections of IHHNV, GAV and When-2 with minimum Ct values below Dicer-1 and within the moderate-high load range were detected, the number of samples with low Ct weren't prevalent often enough to greatly reduce the average of the cohort. It is possible that a presently unknown pathogen is present within the "poor health" group. Further analysis applying next generation sequencing will be applied to the samples to investigate the presence of other pathogens.

Table 19: Average Ct of positive detections linked with prawn activity.

Activity	Total	HDV	IHHNV	GAV	YHV-7	When-2	Pir-A	Dicer-1
Dead	74	37.56	29.94	28.42	37.10	31.89	34.23	25.06
DOA	39	40.33	38.10	34.53	36.25	31.41	nd	28.82
Jumper	36	39.74	37.41	32.93	35.33	31.04	nd	30.87
Moribund	3	38.37	31.66	28.61	nd	nd	38.76	28.56
On-Trawler	93	**40.42**	**34.64**	**34.56**	**nd**	**31.97**	**nd**	**27.66**
Post spawn	75	39.80	30.38	32.61	35.82	31.30	35.56	28.80
Pre-spawn	647	**38.17**	**34.28**	**33.05**	**35.37**	**30.88**	**37.22**	**28.52**
Total	967	38.85	33.60	32.81	35.76	31.10	35.00	28.29

Table 20: Minimum Ct of positive detections linked with prawn activity.

Activity	Total	HDV	IHHNV	GAV	YHV-7	When-2	Pir-A	Dicer-1
Dead	74	36.58	15.85	20.04	37.00	23.93	27.66	21.86
DOA	39	37.49	33.72	27.19	33.00	25.10	0.00	25.10
Jumper	36	39.02	35.12	29.33	33.75	24.05	0.00	27.99
Moribund	3	36.96	28.30	27.73	0.00	0.00	38.76	23.60
On-Trawler	93	39.12	17.63	28.91	0.00	19.21	0.00	25.37
Post-spawn	75	37.12	17.27	23.69	33.34	22.84	34.55	22.25
Pre-spawn	647	32.00	21.46	13.88	33.29	15.64	31.83	22.78
Total	967	32.00	15.85	13.88	33.00	15.64	27.66	21.86

20 Discussion: Hatchery component

20.1 Exotic Targets: WSSV and YHV-1

There was no detection of WSSV or YHV-1 in any of the project samples. A national survey for the detection of WSSV, YHV-1 and GAV by PCR was conducted in 2005 (East 2005). The survey did not detect WSSV (n=3081), or YHV-1 (n=1006) from a variety of crustacean species.

Following the WSSV incursion into SE QLD in December 2016, continuous monitoring of wild captured crustaceans has occurred from sites along the Eastern QLD coast. Approximately 35,750 wild-captured crustaceans have been analysed for WSSV using qPCR. The only positive detections were reported from the Logan River, immediately adjacent to affected farms in Dec 2016 and in the Northern Moreton Bay area in March 2017 and March 2018. At the time of preparation of this report the current round of surveillance sampling is underway (pers. comm. Biosecurity QLD).

WSSV surveillance data can be accessed online:

https://www.google.com/maps/d/u/0/viewer?mid=1Vzbe2ip8kR2Nboost_45-Mlt4vTt-y8f&ll=-27.41204885141302%2C153.1691395042419&z=10

20.2 Endemic Target: Prevalence of detection

The project detected at least one prawn pathogen target in 93 % of the project samples. There have been very few surveys applying qPCR to detect a range of prawn pathogens conducted in Australia. Cowley *et. al.* (2015) reported qPCR analysis for the detection of GAV and YHV-7 from ECWM and NTWM (held in EC hatchery). Of 271 NTWM broodstock, prevalence of detection from 3 hatcheries ranged from 10 to 98 % (GAV) and 4 to 39 % (YHV-7). Prevalence of detection from the 155 ECWM ranged from 32 to 93 % for GAV and 2 to 3 % for YHV-7. This project has detected GAV at a very high prevalence in both ECWM (96 %) and NTWM (83 %). Unlike the report of Cowley *et. al.* (2015) the prevalence of detection of YHV-7 was approximately 11 % for both ECWM and NTWM. In another study, Moody *et. al.* (2010) reported prevalence of detection of IHHNV ranging from of 1.4 to 5.4 % (n=998) from QLD farm-collected samples during 2010.

It cannot be determined how the reported prevalence in any of these studies compare to wild prawns sampled at the time of capture. Analysis from a small cohort of prawns that were collected at sea in this project (n=93) indicated the presence of prawns with multiple and high load of pathogen targets present in wild populations.

The majority of cases that detected multiple pathogen targets came from pre-spawn *P. monodon.* Pre-spawn animals are highly valuable and any pathogen outbreak in pre-spawn broodstock can limit the success of spawning, larval production and subsequently farm stocking. The current results indicate that in a typical spawning season approximately 28 in every 200 broodstock (5.6 %) will harbor four or more viruses. This may present a high risk to all cohabitating broodstock. Nonetheless the highest risk prawns, containing all five viruses only accounted for a minute portion of the population. It is possible, prawns infected with five viruses in the wild are more likely to die and not enter the hatchery production system.

The most desirable prawns from a biosecurity standpoint are those with no pathogen targets detected. This occurred in less than 10 % of the samples analysed in the project, indicating that most wild captured prawns in the hatchery will be harboring at least one virus (also important to consider in the establishment of future specific-pathogen-free SPF breeding lines). The most common viruses involved in multiple infection cases are GAV and

IHHNV. IHHNV has been associated with runt deformity syndrome in *P. monodon* causing economic losses (Primavera & Quinitio, 2000) and its high prevalence in dual infections highlights the need to address biosecurity issues in the hatchery to minimize the risk of viral transferal from broodstock to Nauplius. When-2 combined with IHHNV and GAV comprised 15 % of cases where multiple infections were detected. The risk to *P. monodon* farm productivity posed by IHHNV has been studied (Lightner et al, 1983; Primavera & Quinitio, 2000; Sellars *et. al.,* 2019); however, When-2 is a new and uncharacterized virus with no knowledge of its effect on production. This gain in knowledge should be a point to initiate further investigation.

In all categories of multiple infection cases the difference between ECWM and NTWM was negligible, except the triple infection category where ECWM was much more prominent compared to NTWM. This is most likely linked to the high prevalence of When-2 in ECWM (46 %) compared to NTWM (18 %) which is why we see ECWM having a much higher portion of triple infections with the most prevalent triple infection being between IHHNV, GAV and When-2 (15 % of total population).

20.3 Endemic Targets: Understanding the impact of calculated copy number/viral load on hatchery production

The successful production of many millions of post larvae by the participating hatcheries indicates the presence of a pathogen target does not invariably lead to disaster and disease. However, it is very likely viral loads will impact on broodstock performance and consequently affect the efficiency of hatchery and farm operations. Considering industry relies on the yearly intake of wild captured broodstock, factors that impact on the efficient production of post-larvae should be considered a priority for industry. Understanding the levels at which pathogens impact on productivity will help prioritize investment in biosecurity. Understanding factors that lead to an increase in viral load in prawns will improve the ability to manage the impact of pathogens. Unfortunately, there is a significant gap in the understanding of both topics. There are very few survey investigations conducted for either Australian or for *P. monodon* more widely, that discuss qPCR analysis for the detection of prawn pathogens. The majority of published reports apply qPCR to disease investigations i.e. the interpretation of high load/mass mortality scenario is fairly easily identifiable but there is a paucity of evidence to interpret qPCR results and guide management decisions outside of disease outbreak scenarios.

20.3.1 Yellow head complex
Members of the yellow head complex of viruses, GAV and YHV-7 were the most prevalent viral targets detected in this Component of the project. However, they were generally detected in low load. The biosecurity risk of GAV and YHV-7 at low load has not been studied. In previous studies, prawns were collected during disease outbreaks on farms. During a disease outbreak, positive detections of YHV-7 Ct values were in the range of 15.7 to 21.8 (Cowley 2015). Similar Ct values (12.9 to 22.6) were reported from a cohort of YHV-7 experimentally challenged *P. monodon* that suffered ~ 60 % cumulative mortality after inoculation with YHV-7 (inoculum Ct 13.4) (Moody & Crane 2015). Interestingly, in the same study similar Ct values were reported from *P. merguiensis*, with minor cumulative mortality (Moody & Crane 2015). Within the JCU AquaPATH laboratory systems, Ct values 12.9 to 22.6 for YHV-7 equate to calculated copy numbers ranging from 7.2×10^9 to 1.3×10^7.

20.3.2 Whenzhou shrimp virus-2
Whenzhou shrimp virus-2, is a newly discovered, uncharacterized pathogen. Interpretation of Ct value of When-2, with respect to risk to prawn health has not been determined. Comparison with Dicer-1 may provide some indication of the calculated copy number required before prawn. If this were an accurate index, applying the target Cts indicated in Appendix H whereby pathogen targets exceeded Dicer-1 detection, threshold for the minimum Ct values linking with effects on productivity for When-2 are approximately Ct 27 which equates to a

calculated copy number in the range of 8.3×10^5 mL^{-1}. Further investigation into the significance of Whenzhou shrimp virus on prawn health is required.

20.3.3 Pir-A

Han (2015) reported a calculated copy number of 1.8×10^3 to 4.7×10^6 of plasmid hosting the PIR-AB toxin gene from tissues from moribund/dead shrimp in laboratory bioassays investigating Acute Hepatopancreatic Necrosis Disease (AHPND). Samples collected from AHPND disease outbreaks on farms reported copy numbers of 5.8×10^5 (Vietnam strain) and 1.5×10^4 (China strain). Hepatopancreas and stomach samples are the optimal tissue for the detection of the Pir-AB toxin gene. Average calculated copy number of 2.15×10^3 of Pir-A were detected in this project. Notably, the majority of those prawns were dead at the time of sample collection.

20.3.4 IHHNV

IHHNV was considered a significant risk to the prawn farming industry during the 1990s. The switch to *L. vannamei*, which is far less susceptible to IHHNV, by the majority of the world's shrimp producers has led to IHHNV being poorly studied during the period that qPCR has emerged as an analytical tool. Sellars *et. al.* (2019) noted a significant increase in IHHNV load ($\times 10^7$) in the time of sampling from some *P. monodon* females from the time of arrival until ~6 weeks later after spawn. Following the performance of crops stocked with post larvae of varying loads of IHHNV indicated that a stocking ponds with IHHNV-low lead to an extrapolated increase in farm gate value of \$67 000 per hectare (Sellars *et. al.* 2019). The authors linked the IHHNV-high post-larvae to broodstock females. The females entered the hatchery production run with calculated copy number ranging from 11 to 6.71×10^5. At the time of spawning the calculated copy number had increased to 1.83×10^5 and 1.10×10^9. Other female broodstock, had similar low-level detections of IHHNV on arrival, but did not display similar increase in calculated copy number at spawning. As the study did not conduct analysis for other prawn pathogens such as HPV or When-2 the role of IHHNV in the poor growth was not definitively proven to be caused by IHHNV but rather associated with high copy number detection.

20.4 Effect of multiple infections

The trends discussed in this section provide a good framework for future studies that aim to reduce the biosecurity risk of prawn farming in the hatchery stage of production by understanding the risk of broodstock to spawning success and larval rearing. In any case the absolute message is that the majority of prawns analysed carried between 1-3 pathogens and were in the pre-spawn stage meaning the risk they pose is quite high to other valuable broodstock as well as exposing pathogens to their progeny which may affect larval stages.

20.5 Dicer-1 as an integrity control

The selection of an integrity control when studying disease can be difficult because many genes are affected by tissue type, developmental stage, nutritional status, environmental conditions and bacterial and viral pathogens. Ideally an integrity control should not display great variation across the range of samples that it supports. The use of *P. monodon* Dicer-1 as a prawn integrity control is a novel application.

Dicer-1 is an important gene for the regulation of prawn genes. Dicer-1: Su *et. al.* (2008) reported Dicer-1 levels were not correlated with the levels of detection of GAV or Mourilyan Virus (MoV) in naturally infected *P. monodon*. Although Dicer-1 levels did not align with viral detection levels, the knockdown (turning off) of *P. monodon* Dicer-1 resulted in more rapid mortalities and higher viral loads. Similarly, knockdown of Dicer-1 from *Marsupenaeus japonicus* prevented the expression of the shrimp microRNA, miR-7, which prevents the

production of WSSV gene 477 (wss477) and by extension, WSSV replication (Huang & Zhang 2012). Refer to Appendix 3: image from Huang & Zhang that illustrates the process.

20.6 Disclaimer: limitations of the data

Robust interpretation of geographic distribution of targets is not possible with the current data. A feature of these results is that the time a prawn spent in the hatchery before it is sampled was not standardized. It is possible broodstock held in the hatchery became infected in the hatchery. The sample collection was not standardised because the overarching aim of the project is to improve understanding of the population of targets that are present within the hatchery production Component under typical operational conditions, rather than guide broodstock source selection decisions.

Likewise, the unbalanced design of the sample collection does not allow for statistical analysis to determine the significance of any particular virus with respect to impact on prawn health. Rather, the data illustrates, that the myriad of variables that broodstock may be exposed to in the wild and at the hatchery, leads to a similar variation in pathobiome of broodstock in the hatchery system.

It is unlikely the prevalence and calculated copy number of targets detected in this project are a dramatic increase to those which the Australian Prawn Farm industry has managed during the past 10 years. However, the more frequent detection of previously exotic or novel strains of pathogens, namely IHHNV (2008), YHV-7 (2012), Pir-AB (2015), and WSSV (2016) from Australian farmed prawns indicates the Australian prawn farming industry may be facing a more frequent and evolving suite of challenges with respect to disease. Fortunately, the development of molecular detection and genome sequencing technology has also improved at a rapid rate. Farmers now have access to more superior analytical tools than have ever been present. Application of those tools (including next generation sequencing methodologies) will be directed to the remaining Components of the project to help assess the potential for spread and the impact of the hatchery pathobiome into the larval and grow out stages.

20.7 Further Project Activities

Component 2, 3 and 4 of the CRC NA project are underway. Component 2 will investigate the prevalence and levels of detection of the same endemic pathogen targets applying the same qPCR analysis as Component 1 to farm collected samples. Application on farm collected samples will extend upon the outcomes of Component 1 by allowing:

a) Tracking to determine how effectively pathogens are transferred from broodstock through to pond Component culture.
b) Greater assessment of the impact of the detected pathogen targets on productivity.
c) More robust statistical analysis of the qPCR results.

Component 3 involves studies to detect novel, presently undescribed pathogens in the hatchery and farm collected samples. This Component applies whole genome next generation sequencing technology to screen project samples. Component 4 applies further qPCR analysis of the project samples to determine prevalence and calculated copy number of a novel pathogen in hatchery and farm collected samples.

21 References: Hatchery component

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Han J., Tang K., Pantoa C., White B., and Lightner D (2015) qPCR assay for detecting and quantifying a virulence plasmid in Acute Hepatopancreatic necrosis disease (AHPND) due to pathogenic *Vibrio parahaemolyticus*. Aquaculture 442(1): 12-15.

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Lightner, D. V., Redman, R. M., & Bell, T. A. (1983). Infectious hypodermal and hematopoietic necrosis, a newly recognized virus disease of penaeid shrimp. *Journal of Invertebrate Pathology, 42*(1), 62-70. doi:10.1016/0022-2011(83)90202-1

Sellars, M. J., Cowley, J. A., Musson, D., Rao, M., Menzies, M. L., Coman, G. J., & Murphy, B. S. (2019). Reduced growth performance of black tiger shrimp (*Penaeus monodon*) infected with infectious hypodermal and hematopoietic necrosis virus. *Aquaculture, 499*, 160-166. doi:10.1016/j.aquaculture.2018.09.032

22 Appendices: Progress Report 1: Hatchery component

22.1 Appendix D: Scatter plots of Ct pf qPCR targets v Dicer-1 Ct

APPENDIX 1: Scatter plot of Dicer-1 Ct v Pathogen target Ct.

Indicates detection of Pathogen target occurred prior to P.monodon Dicer-1 gene
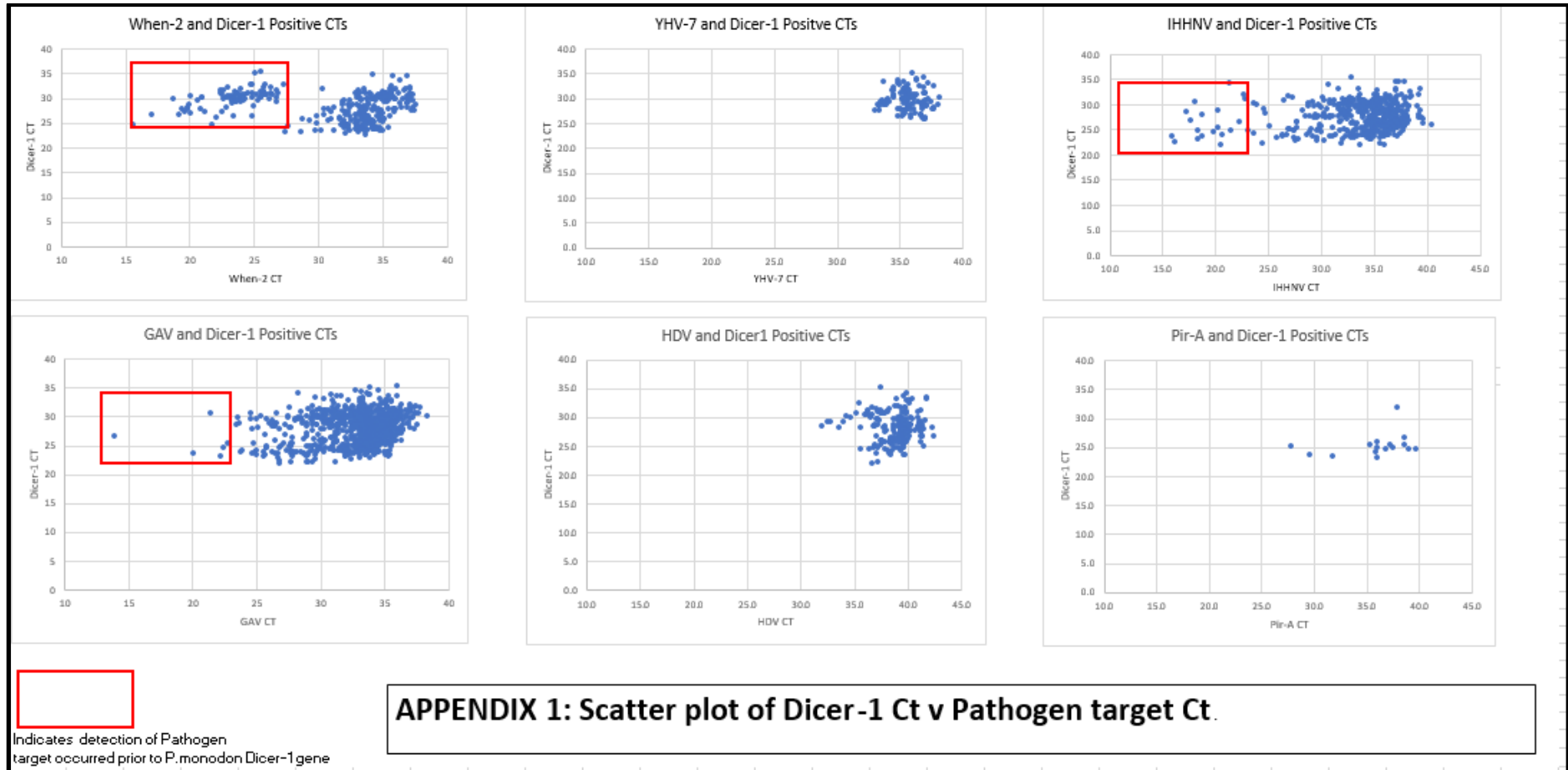
22.2 Appendix E: Box and Whisker plots of Ct values of qPCR targets Pre-spawn and Post-spawn

APPENDIX 2: Average Ct values of each target at Pre - and Post-spawn
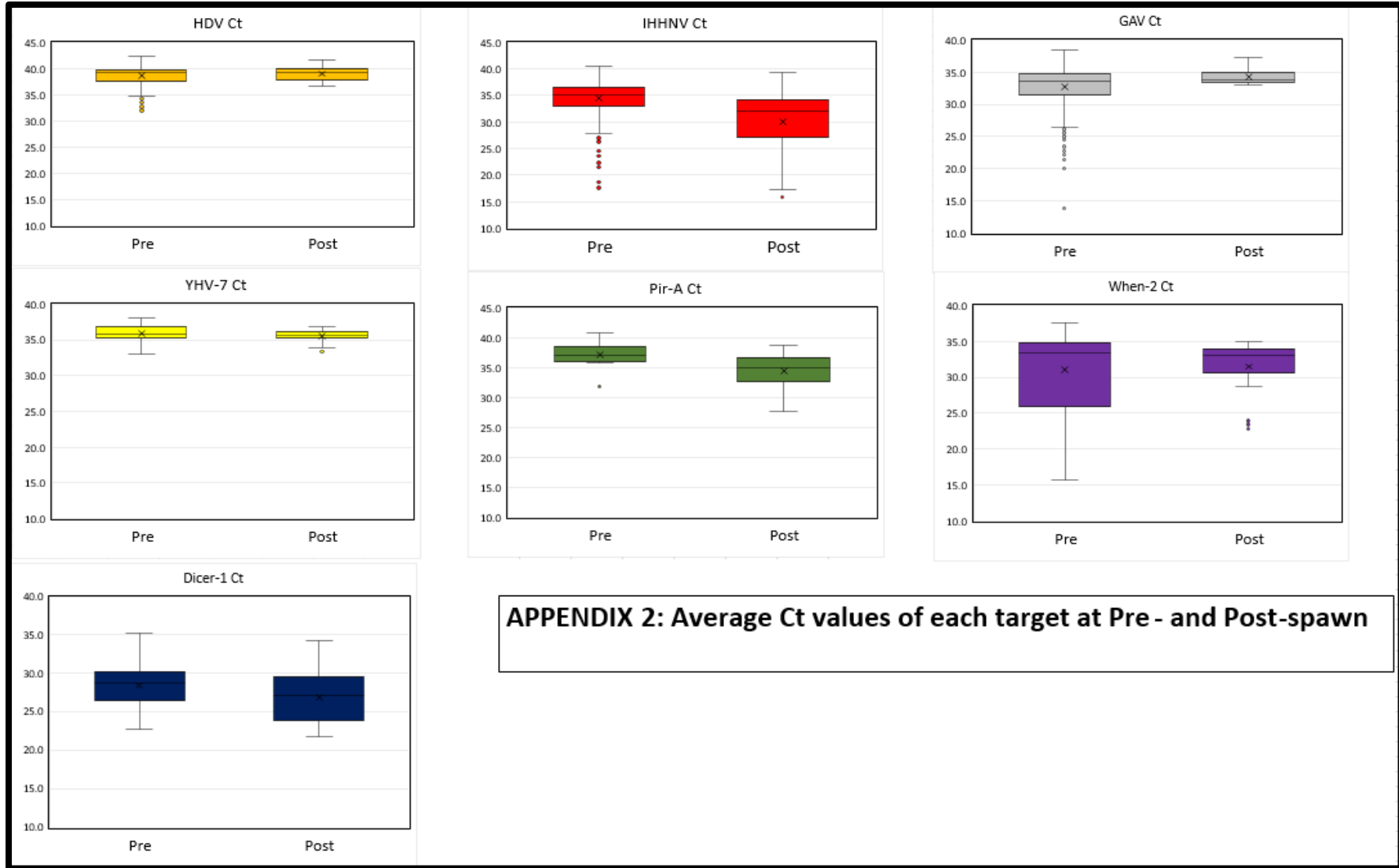
22.3 Appendix F: Image from Huang and Zhang (2012)

In the
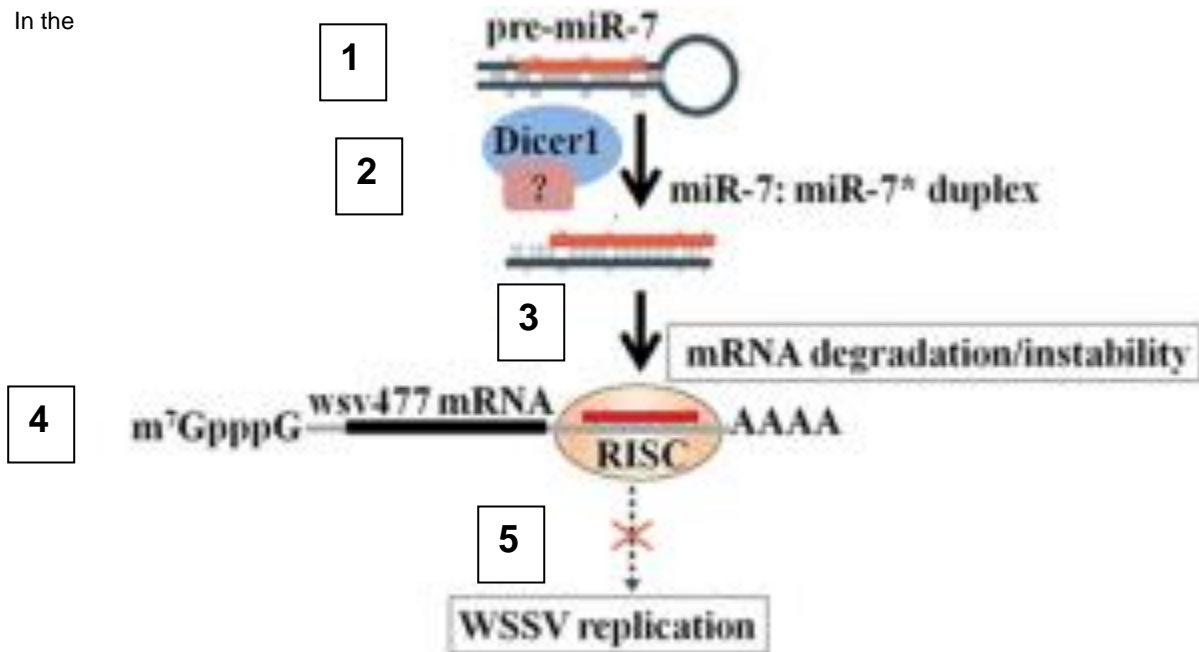
image above,:

1. The cell produces pre-mirR-7 in response to WSSV infection.

2. Dicer-1 through some unknown mechanism, cleaves pre-mir-7 into its smaller active form (miR-7).

3. miR-7 binds to RNA from the WSSV genome (a gene referred to as 477).

4. The miR-7 is loaded into the RNA silencing complex (RISC) which destroys any RNA sequence with homology to miR-7.

5. If WSSV gene 477 is required for viral replication, reproduction of WSSV does not occur.

22.4 Appendix G Tabulated data of Project results.

Percentage of positive detections of targets from ECWM and NTWM broodstock.

Source	Total tested	qPCR Target						
		HDV	IHHNV	GAV	YHV-7	When-2	Pir-A	Dicer-1
ECWM	(n = 489)	24%	**60%**	96%	11%	**46%**	0%	100%
NTWM	(n = 475)	16%	34%	83%	12%	18%	**17%**	99%
Total	100%	20%	47%	89%	11%	32%	8%	99%

Average calculated copy number of targets detected from ECWM and NTWM broodstock.

Source	Total tested	Average calculated copy number of qPCR target (mL^{-1})						
		HDV	IHHNV	GAV	YHV-7	When-2	Pir-A	Dicer-1
ECWM	(n = 489)	1.17E+01	1.63E+05	9.03E+06	3.64E+03	1.52E+07	nd	7.10E+05
NTWM	(n = 475)	7.26E+01	2.17E+06	6.61E+05	3.26E+03	4.32E+05	2.15E+03	1.89E+06
Total		3.63E+01	8.73E+05	5.19E+06	3.43E+03	1.12E+07	2.15E+03	1.29E+06

Maximum calculated copy number of each target detected from ECWM and NTWM broodstock.

Source	Total tested	Maximum calculated copy number detected for each qPCR target (mL^{-1})						
		HDV	IHHNV	GAV	YHV-7	When-2	Pir-A	Dicer-1
ECWM	(n = 489)	9.91E+01	3.01E+07	4.17E+09	1.55E+04	1.34E+09	0.00E+00	7.03E+06
NTWM	(n = 475)	1.44E+03	1.19E+08	7.66E+07	1.07E+04	2.25E+07	3.82E+04	2.15E+07

Percent of Positive detections of each qPCR target with geographic source and spawn status.

Source	Status	Target							Total tested
		HDV	IHHNV	GAV	YHV-7	When-2	Pir-A	Dicer	
ECWM	Pre-spawn	23%	63%	95%	5%	47%	0%	100%	447
ECWM	Post-spawn	32%	24%	100%	73%	32%	0%	100%	37
NTWM	Pre-spawn	14%	21%	75%	5%	22%	5%	98%	330
NTWM	Post-spawn	20%	75%	99%	14%	7%	56%	100%	113

Average calculated copy number of each qPCR target with geographic source and spawn status.

Sourc e	Status	Target							Total tested
		HDV	IHHNV	GAV	YHV-7	When-2	Pir-A	Dicer	
ECWM	Pre-spawn	4.64E+00	5.14E+00	2.13E+04	3.78E+03	1.60E+06	nd	2.50E+05	447
ECWM	Post-spawn	1.26E+01	1.67E+05	9.92E+06	3.53E+03	1.61E+07	nd	7.55E+05	37
NTWM	Pre-spawn	1.56E+01	4.08E+06	1.64E+06	2.20E+03	7.47E+05	2.73E+03	4.19E+06	332
NTWM	Post-spawn	1.10E+02	1.37E+03	2.94E+05	3.43E+03	3.48E+05	1.23E+02	1.26E+06	113

Relative percentage of positive detections linked with source and health status.

Source	Status	Total	HDV	IHHNV	GAV	YHV-7	When-2	Pir-A	Dicer-1
ECWM	Average	489	24%	60%	96%	11%	46%	0%	489
ECWM	DOA	26	58%	8%	96%	69%	31%	0%	26
ECWM	Jumper	6	0%	20%	100%	17%	33%	0%	6
ECWM	On-Trawler	93	20%	89%	95%	0%	52%	0%	93
ECWM	post	37	32%	24%	100%	73%	32%	0%	37
ECWM	Pre	330	21%	60%	95%	2%	48%	0%	330
NTWM	Average	475	16%	34%	83%	12%	18%	17%	470
NTWM	Dead	74	15%	96%	100%	3%	8%	80%	74
NTWM	DOA	13	46%	31%	92%	46%	0%	0%	13
NTWM	Jumper	30	27%	30%	100%	103%	13%	0%	30
NTWM	Moribund	3	67%	100%	100%	0%	0%	33%	3
NTWM	post	38	26%	29%	97%	42%	5%	8%	38
NTWM	pre	317	13%	20%	75%	4%	23%	6%	312
	Total	967	193	455	863	111	311	81	962

Average Ct Value of Positive detection linked with source and prawn status.

Source	Status	Total	HDV	IHHNV	GAV	YHV-7	When-2	Pir-A	Dicer-1
ECWM	**whole cohort**	**489**	**39.16**	**34.45**	**33.98**	**35.74**	**30.58**	**nd**	**28.61**
ECWM	Jumper	5	nd	38.40	33.10	35.82	33.39	nd	30.93
ECWM	Post	37	39.71	35.42	33.78	35.59	31.24	nd	29.36
ECWM	Pre	447	39.09	34.41	34.00	35.91	30.52	nd	28.52
ECWM	Jumper	1	nd	nd	32.95	nd	nd	nd	30.76
ECWM	Pre	2	nd	nd	31.75	35.93	24.89	nd	30.31
NTWM	**whole cohort**	**475**	**38.39**	**32.06**	**31.44**	**35.77**	**32.58**	**35.00**	**27.96**
NTWM	dead	2	nd	nd	29.39	37.10	nd	nd	29.79
NTWM	Jumper	30	39.74	37.30	32.90	35.31	29.86	nd	30.86
NTWM	Post	113	38.65	29.52	29.40	36.21	31.83	34.36	26.15
NTWM	Pre	330	38.03	34.53	32.20	35.77	32.81	37.22	28.31
	Total	967	38.85	33.60	32.81	35.76	31.10	35.00	28.29

Minimum Ct Value of Positive detection linked with source and prawn status.

Source	Status	Total	HDV	IHHNV	GAV	YHV-7	When-2	Pir-A	Dicer-1
ECWM	**whole cohort**	**489**	**35.53**	**17.63**	**13.88**	**33.00**	**15.64**	**0.00**	**23.48**
ECWM	Jumper	5	0.00	38.40	32.56	35.82	33.19	0.00	30.11
ECWM	Post	37	38.66	30.21	29.72	33.34	22.84	0.00	26.37
ECWM	Pre	451	35.53	17.63	13.88	33.00	15.64	0.00	23.48
ECWM	Jumper	1	0.00	0.00	32.95	0.00	0.00	0.00	30.76
NTWM	**whole cohort**	**475**	**32.00**	**15.85**	**20.04**	**33.56**	**21.78**	**27.66**	**21.86**
NTWM	dead	2	0.00	0.00	25.72	37.00	0.00	0.00	29.69
NTWM	Jumper	30	39.02	35.12	29.33	33.75	24.05	0.00	27.99
NTWM	Post	113	36.58	15.85	20.04	35.08	23.93	27.66	21.86
NTWM	Pre	330	32.00	26.82	21.39	33.56	21.78	31.83	22.78
	Total	967	32.00	15.85	13.88	33.00	15.64	27.66	21.86

23 Progress Report: CRC NA Project: Progress Report: Pond component

CRC NA Project: Biosecurity in northern Australian prawn aquaculture.

Project Reference Number: A.3.1718113

Project progress Report: Farm Component: Detection of endemic pathogens from prawn pond grow-systems

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25 Project Participants

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25.2 Peer Review Statement

The CRCNA recognises the value of knowledge exchange and the importance of objective peer review. It is committed to encouraging and supporting its research teams in this regard.

The author(s) confirm(s) that this document has been reviewed and approved by the project's steering committee and by its program leader. These reviewers evaluated its:

- originality
- methodology
- rigour
- compliance with ethical guidelines
- conclusions against results
- conformity with the principles of the Australian Code for the Responsible Conduct of Research (NHMRC 2018),

and provided constructive feedback which was considered and addressed by the author(s).

26 Executive summary: Pond component

This report provides a summary to the Australian Prawn Farmers Association of the Pond component: Detection of endemic pathogens from *Penaeus monodon* of the CRCNA project "Improving Biosecurity in Northern Australian Prawn Aquaculture" (Ref No. A.3.1718113). The aim of this aspect of the project was to enhance the understanding of the pathobiome in commercial *P. monodon* aquaculture ponds in Northern Australia. This component of the project extends upon the Hatchery component of the project which was previously reported. This component of the project investigates the pathobiome present in pond collected samples and will contribute to the final project outcomes which tracks the progression of pathogens from initial broodstock sources to Post larvae to ponds.

P. monodon samples for this project component were provided by four commercial growout farms that contribute a significant proportion to the overall production volume of *P. monodon* aquaculture in Queensland (QLD). Fifty-five *P. monodon* growout ponds were analysed for the presence of Whitespot syndrome virus (WSSV), yellowhead virus-1 (YHV-1), Infectious hypodermal and hematopoietic necrosis virus/Penaeus stylirostris Densovirus (IHHNV/PstDV), Gill associated virus (GAV), Yellowhead virus-7 (YHV-7), Hepatopancreatic Necrosis virus/Hepandensovirus (HDV), Whenzhou Shrimp Virus-2 (When-2), Component A of Photrhabdus insect related toxin gene (Pir-A), and the *P. monodon* Dicer-1 gene. Additionally, three bacterial toxin genes were also included in the pathogen screen; Zot occludens (Zot), Repeat toxin (RtX) and Hemolysin D (Hemo). A total of 666 samples were tested over the 55 ponds. Each sample comprised of three pooled pleopods originating from three *P. monodon* individuals sampled from the same pond. The geographic origin of the broodstock that contributed to each pond was provided to enable comparisons between East Coast and Northern Territory wild monodon stocks (ECWM and NTWM, respectively).

There were no detections of YHV-1 or WSSV from any of the samples submitted. Integrity of the sample collection process was indicated by the 100% detection of the technical assay control gene *P. monodon* Dicer-1. Endemic viral targets were detected in each of the 55 ponds. The average number of viral targets detected per pond was two, however, the number of viral targets detected from individual samples ranged between zero and four. The vast majority (665/666 or 99%), of all samples were positive for the detection of at least one viral target. Only one sample reported a negative detection of all viral targets. Approximately one quarter (24%) of all samples were positive for the detection of a single viral target and the majority of samples (48%) were positive for the detection of two viral targets. GAV (98%) and IHHNV (62%) were the most prevalent viral targets detected across the pond component analysis. Dual detection of GAV and IHHNV from individual samples was the most common multiple infection detected (18.6%). Only GAV, IHHNV and When-2 were detected with a calculated copy number exceeding 10^6 mL^{-1} in pond collected samples.

There was no detection of Pir-A from any of the samples submitted. This document reports, for the first time, the prevalence, Ct and copy number of three other bacterial toxin genes in a large-scale analysis

of *P. monodon* growout ponds. Toxin gene targets were detected in 50/55 (91%) ponds, and 411/666 of the samples tested (62%). The average number of toxin gene targets detected per pond was approximately one, however, the number of toxin gene targets detected from individual samples ranged between zero and three. Zot toxin prevalence was the lowest of the three toxin gene targets (21%), with 99% of the detections above a Cycle threshold (Ct) value of 30. RtX was the most prevalent toxin gene target detected (57%) and also had the lowest average Ct (34.86 Ct). Hemo was detected in 23% of samples, with the majority (97%) of positive detections above a Ct value of 35. Hemo and RtX were detected at a calculated copy number exceeding 10^6 mL^{-1}.

The Pond component endemic viral data, as well as the emerging toxin gene data, was compared between growout ponds based on the geographic origin of broodstock that contributed post larvae to each respective pond (ECWM or NTWM). Generally, the prevalence and average Ct of detection of viral targets were similar between the two stock sources. However, most notably, the prevalence of IHHNV was 37% higher in ECWM stock (85% prevalence overall) compared to NTWM stock (48% prevalence overall). Additionally, the minimum positive detection of IHHNV from ECWM stock (min Ct 18.6) was markedly lower than from NTWM stock (min Ct 27.7). The toxin gene targets were very similar in prevalence and average Ct between the two geographic sources, with the exception of RtX, which was 15% more prevalent in NTWM (63% overall prevalence) compared to ECWM (48% overall prevalence). Considering pond conditions were not standardized, the extent to which this data can definitively confirm stock source differences, and trends related to stock source is limited. Bacterial community structure is known to be related to pond preparation and this sampling and analysis strategy does not incorporate pond preparation. The results of this analysis should be used as an indication of the pathogens present in *P. monodon* commercial ponds and as a **potential indicator** of trends. A more rigorous experimental design would be required to scientifically confirm trends and differences.

Glass shrimp (*Acetes spp.*) were also collected from ponds where possible and tested (n = 39) for the same pathogen suite as the *P. monodon* samples. The glass shrimp samples were positive for the detection of HDV, IHHNV, When-2, Zot, RtX and Hemo. The most prevalent pathogen detected in the glass shrimp was When-2 (49%), however, individual viral detection patterns were highly variable between farms. This variability was likely derived from the unequal and relatively low quantity of samples submitted by each farm due to sample collection constraints and the availability of glass shrimp in ponds.

The analysis and findings presented, meet the overarching aim of the project, namely, increasing understanding of pathogen presence throughout *P. monodon* production. This pond component activity represents the most comprehensive large- scale study examining endemic viruses and bacterial toxins in *P. monodon* growout systems to date.

27 Introduction: Pond component

During 2016/2017 prawn farms located in the Logan River Catchment in South East Queensland were severely impacted by Whitespot syndrome disease (WSD). Although White Spot Syndrome Virus (WSSV) has not been detected beyond the movement restriction zone in South-East (SE) QLD, the potential establishment of WSSV in the environment or spread of WSSV outside Moreton Bay poses high risk to prawn farming activities in Queensland.

Following the 2016-17 outbreak of WSSV, the Australian prawn industry was reduced from ~22 farms to 16 farms in 2017-18, with an associated drop in production volume from 4264 t to 3921 t (QLD Department of Agriculture and Fisheries, 2018). Despite this apparent reduction, the average production volume per unit farm remained relatively steady throughout this period, increasing by approximately 20% from 2016-17 to 2017-18.

The increased risk posed by the presence of WSSV has led to an increased priority on improved biosecurity systems in Australian prawn farms. However, the paucity of data about the pathobiome in the various stages of production in Australian prawn farming makes evidence-based management decisions very difficult. The Cooperative Research Centre for Developing Northern Australia (CRCNA), Australian Prawn Farmers Association (APFA), Fisheries Resource and Development Co-operation (FRDC) and James Cook University (JCU), in recognition of a need to improve biosecurity management and recreate a more resilient prawn farming industry, has developed the CRCNA Project "Improving Biosecurity in Australian Prawn Farms".

While WSSV is the most economically significant viral infection affecting prawn aquaculture, some pathogens that are endemic to Australia, namely Yellowhead Virus strain-7 (YHV-7) and bacteria that host the Photorhabdus insect-related toxin-like-gene (Pir-AB), can also cause substantial losses to farm productivity. Additional viruses that are endemic to Australia, including, Infectious Hypodermal Hematopoietic Necrosis Virus/Penaeid stylirostris Densovirus (IHHNV/PstDV), Hepatopancreatic Necrosis Virus/ Hepandensovirus (HPV/HDV), and Yellowhead virus strain-2/Gill Associated Virus (GAV), although being less severe, can also cause economic loss when culture conditions are sub-optimal. Analysis for the detection of endemic viruses serves as a good indicator of the effectiveness of current industry practices to prevent the entry of pathogens. Such information is useful when considering how to improve biosecurity management protocols.

A combination of factors determines the level of biosecurity risk faced by the prawn aquaculture industry:

a) The likelihood of introduction of a pathogen (i.e. the number of animals that enter the production system that are infected with a pathogen).

b) The level/severity of infection that is being introduced in animals that are infected (i.e. the calculated copy number of the pathogen).

c) The impact of the infection on health/productivity in the hatchery (i.e. does the infection cause acute disease outbreaks or have chronic effects on health/productivity).

d) The dynamics involved in the expression of disease and spread of the pathogen (i.e. what causes animals to display disease outbreaks rather than be persistent carriers of the pathogen).

e) The ability to manage the culture environment, through genetics, nutrition and water quality to favour prawn health (i.e. can the factors that lead to disease outbreaks be managed?).

The CRCNA project will gather information towards a better understanding of topics a-e. The project will operate through to June 2020. The project is a five-component program consisting of:

a) Hatchery component: Detection of endemic pathogens from prawn hatchery systems (reported). b) Pond component: Detection of endemic pathogens from prawn pond grow-systems (this report).

c) Emerging pathogen: Detection of emerging pathogens from prawn production systems.

d) Industry assessment: Evidence-based assessment of enterprise and industry level biosecurity.

e) Final report: Improving biosecurity in Australian prawn farms

28 Aim: Pond component

Following from the Hatchery component of this project, the overarching aim for the Pond component was to improve our understanding of the pathobiome in north Australian *P. monodon* aquaculture ponds. This aspect intended to provide a comprehensive baseline data set of the pathobiome during the growout stage; it is the largest scale analysis of its type conducted on Australian prawn farms. This study intends to fill data and knowledge gaps surrounding the pathobiome of *P. monodon* growout systems through pathogen screening (7 viral targets and 4 toxin gene targets) from 55 *P. monodon* growout ponds collected across four farms. The ponds sampled were selected based on the geographic source of broodstock which contributed the post-larvae of each pond, enabling comparisons between East Coast Wild Monodon sourced stocks (ECWM) and Northern Territory Wild Monodon sourced stocked (NTWM) ponds.

The results from the Farm component will contribute to further activities of this CRCNA project which combines all aspects to provide a complete picture of viral prevalence and load throughout the production cycles of *P. monodon.*

29 Method: Pond component

29.1 Sample Collection

Four farms participated in the project; each farm provided samples from between 11 and 16 ponds for analysis. The ponds sampled were selected based on the genetic source of the broodstock, which contributed post-larvae to the pond, allowing for comparisons between progeny originating from ECWM and NTWM ponds. Farmers were directed to collect whole prawns and transport them on ice directly to JCU AquaPATH for analysis, or where that was not practical, sample 33 prawns from each pond and submit samples as 11 pooled samples of pleopods from three individual prawns. Farmers were also requested to collect 'glass shrimp' (*Acetes spp.;* n = >5 per pond) providing they were present in the pond, which were tested in conjunction with the *P. monodon* samples. Additionally, specific samples were selected for continuation through to whole genome sequencing for novel pathogens. The findings presented in this report are representative of a pathobiome associated with an average production year, which indicates relevance as baseline data to inform improved biosecurity management protocols.

29.2 Target tissues, species and number of samples.

A total of 55 ponds (ECWM = 22, NTWM = 33) were sampled across four farms, equating to 666 samples in total (ECWM = 262, NTWM = 404). Thirty-three *P. monodon* were sampled from each pond and were delivered to JCU AquaPATH whole on ice. One pleopod was excised from each prawn and submitted for analysis as a pool from 3 individuals (≈11 samples per pond). Glass shrimp were collected at quantities ranging from 5-30 per pond. Whole glass shrimp were tested as pools of three individuals, originating from the same pond, per sample, yielding a total of 59 glass shrimp samples submitted for analyses.

Analysis of samples by quantitative Polymerase Chain Reaction (qPCR)

Samples were analysed by the standard operating protocols used in the JCU AquaPATH laboratory. Tissue samples were extracted using the MagMAX Core nucleic acid extraction protocol on a Kingfisher Flex 96 well extraction robot. Total nucleic acid was analysed by qPCR using Bioline and/or Applied Biosystems qPCR mixes. More detailed outline of qPCR protocols is available on request and will be provided in the project final report. The assays targeted the detection of:

- Whitespot Syndrome Virus (WSSV), (Exotic virus)
- Yellowhead Virus-1 (YHV-1), (Exotic virus)
- Yellowhead Virus-7 (YHV-7), (Endemic virus)
- Gill Associated Virus (GAV), (Endemic virus)
- Penaeid stylirostris Densovirus (IHHNV/PstDV), (Endemic virus)
- Hepandensovirus (HDV/HPV), (Endemic virus, some strains Exotic)

- Whenzhou Shrimp Virus-2 (When-2) (Endemic virus, presently uncharacterized, discovered in 2018)
- Pir-A toxin gene (Endemic bacterial toxin gene)
- *Penaeus monodon* Dicer-1 gene (proposed integrity control)
- Zonula occludens toxin gene
- Repeats in toxin (RTX) gene
- Hemolysin D toxin gene

Dicer-1 is an important gene for the regulation of the prawn immune response and is used in assays as a technical control for quality of genetic material. Theoretically, comparison of the levels of target detected with Dicer-1 could provide an indication of the effect of the pathogen target on prawn health.

Three toxin-encoding genes, namely Zonula occludens toxin (Zot), Repeats in toxin (RTX) and Hemolysin D (hemoD) toxin gene have been included in the list of qPCR targets. The prevalence of the toxin genes were included in the scope of potentially "unknown pathogens". The toxin genes were identified through High Throughput Sequencing (HTS) analysis on post-larval samples collected from hatchery tanks displaying mass mortality. These three toxins are included in a range of factors that are linked with virulence/pathogenicity in gram negative bacteria, particularly members of the *Vibrio* spp. Briefly, the Zot, RTX and hemolysin toxins cause changes that lead to the distribution of host cell structure/integrity, particularly of gut epithelial. The present report focuses on determining the frequency of detection of these genes from prawn pond systems. Detailed discussion on the potential impact of the toxin genes on prawn production systems will be presented in the final project report.

30 Results of Analysis: Pond component

There was no detection of YHV-1, WSSV or Pir-A in any of the pond samples tested (n = 666). The results are presented here as the combined data of all ponds across the four farms involved in the analysis. In addition to this report, each farm was provided with their respective results. To align the results with the factors that can be used to determine biosecurity risk, the results are presented in the following sections:

- o Overview of **viral** targets with geographic source (ECWM and NTWM).
  - ➢ Endemic viral target overview
  - ➢ Number of detections and percentage of positive detections
  - ➢ qPCR results: Ct value
  - ➢ qPCR results: copies mL^{-1}
- o Individual **viral** target results.
  - ➢ *P. monodon* Dicer-1 gene
  - ➢ GAV
  - ➢ IHHNV
  - ➢ HDV
  - ➢ When-2
  - ➢ YHV-7
  - ➢ WSSV
- o Overview of **toxin** targets with geographic source (ECWM and NTWM).
  - ➢ Endemic toxin target overview
  - ➢ Number of detections and percentage of positive detections
  - ➢ qPCR results: Ct value
  - ➢ qPCR results: copies mL^{-1}
- o Individual **toxin** target results.
  - ➢ Pir-A
  - ➢ Zot
  - ➢ RtX
  - ➢ Hemo
- o Multiple infections.
- o Cohabiting organisms in *P. monodon* production ponds (*Acetes spp.*).
- o Post Larval Results.

31 Endemic Viral Target Results: Pond component

31.1 Endemic viral target overview

Overall summary of endemic viral targets:

- An endemic pathogen was detected from 665/666 samples (99%).
- There was only one sample which was negative for the detection of all pathogen targets (<1%).
- The Dicer-1 gene was detected in 666/666 samples (100%) indicating the sample collection and analysis process was conducted on samples of appropriate quality for qPCR analysis.
- The average Ct of Dicer-1 was the lowest detected across all targets (26.81 Ct);
- When-2 was detected at the lowest average Ct (28.66 Ct) and therefore highest loading of all viral targets.
- GAV was the most prevalent viral target detected (98%).
- YHV-7 was the least prevalent viral target detected (<1%)
- Amongst the viral targets there were 161/666 single target detections (24%).
- Amongst the viral targets there were 338/666 dual target detections (51%).
- Amongst the viral targets there were 154/666 triple target detections (23%).
- Amongst the viral targets there were 12/666 quadruple target detections (2%).
- Six samples (6 of 149 positive) were positive for the detection of When-2 at calculated copy number exceeding 10^8 copies mL^{-1}.

To follow is a summary of the endemic agents presented in three sections:

4. Prevalence of detection (number of positive detections; proportion of positives detected/total number analysed).
5. qPCR results of detection (Ct value).
6. qPCR results of detection (mL^{-1}).

31.2 Prevalence of detection of targets

Table 21 summarises the number of positive detections between ECWM and NTWM for pond samples. An endemic pathogen target was detected in 99% of samples submitted. The prevalence of viral targets varied between ECWM and NTWM sourced *P. monodon* (Figure 33Figure 33. Percentage of samples positive for the detection of each viral target from ECWM and NTWM stocked ponds.). IHHNV & HDV were detected at a higher prevalence in ECWM, whereas, GAV and When-2 were detected at a higher prevalence in NTWM (Figure 33).

Table 21. Number of positive detections of targets from ECWM and NTWM ponds and the percentage of positive detections overall for each viral target and the *P. monodon* Dicer-1 gene.

Source	Total Tested	HDV	IHHNV	GAV	YHV-7	When-2	Dicer-1
ECWM	262	58	222	252	0	49	262
NTWM	404	71	193	400	1	100	404
Total	666	129	415	652	1	149	666
Total (% Positive)	666	19%	62%	98%	<1%	22%	100%
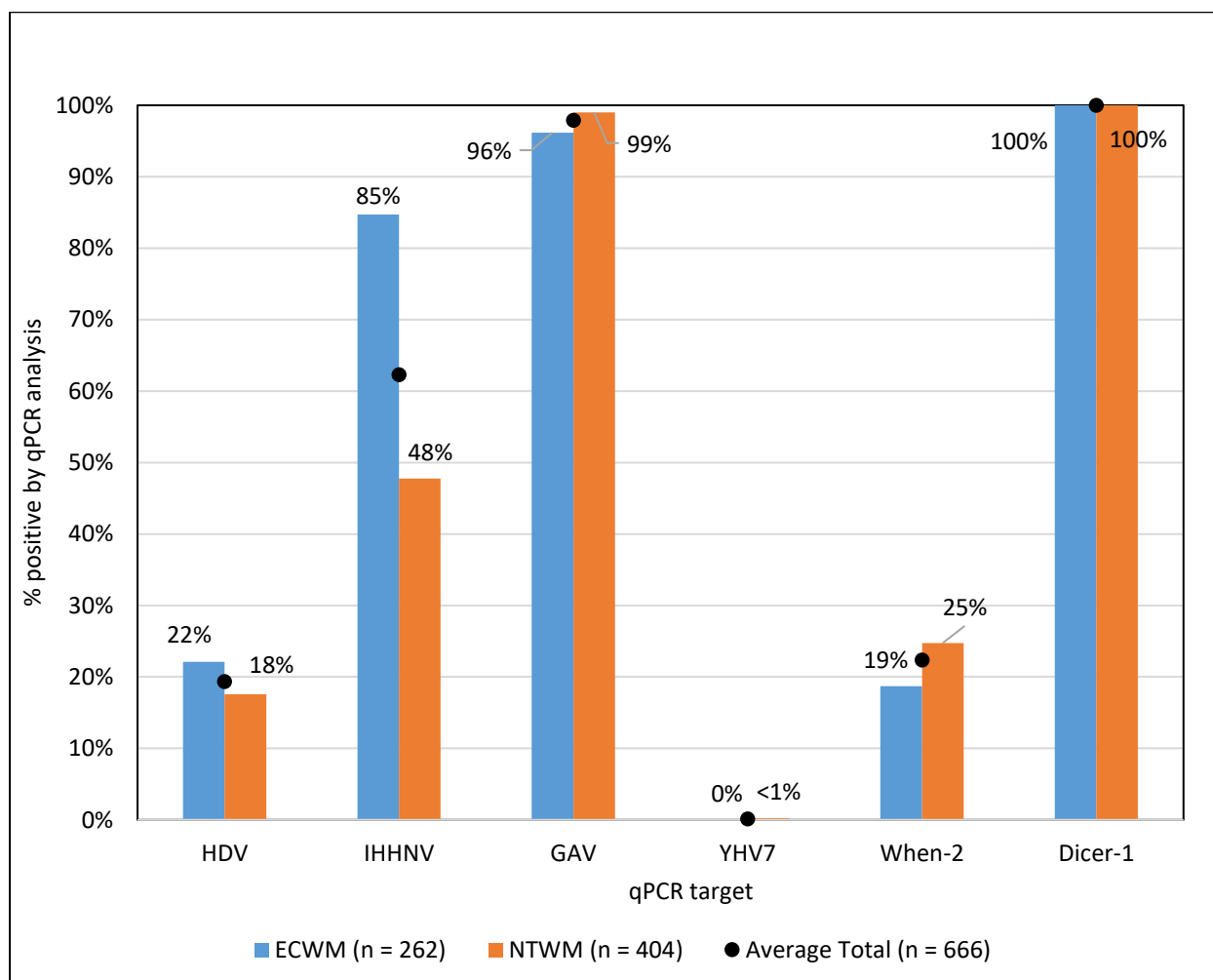
Figure 33. Percentage of samples positive for the detection of each viral target from ECWM and NTWM stocked ponds.

31.3 qPCR results of detections: cycle threshold (Ct) value

Information to assist with interpretation of qPCR results was described in the project Hatchery component report. Explanation to assist with interpretation of the follow qPCR results are provided in Appendix B. Briefly the cycle threshold (Ct) value is an indicator of the quantity of the viral or bacterial gene. Ct values in this range from 18.44 to 41.65 with the lower number being an indicator of high copy number. Results of qPCR analysis are discussed as the average Ct ± Standard deviation (SD) and maximum calculated copy number of the genes detected.

Average Ct value

The *P. monodon* Dicer-1 gene was detected with the lowest average Ct of all qPCR targets (26.81 Ct). The majority of positive detections were above a Ct value of 30. IHHNV and When-2 were the only viruses detected at a Ct value below 20. When-2 was detected at the lowest average Ct of the viral targets and was the only viral target detected with an average Ct below 30 (28.66 Ct). The majority of positive When-2 detections were between a Ct value of 20 to 30 (48%). There was no detection of YHV-1, WSSV or Pir-A in any of the samples submitted. HDV and the *P. monodon* Dicer-1 gene had standard deviations (SD) <2 Ct which indicates narrow variation in the level of detection across samples. When-2 had the highest variation in Ct values (SD = 6.02 Ct) which was 3.13 Ct (a factor of 10x more in viral copy numbers) above the second highest SD observed across the viral targets (IHHNV, SD = 2.83 Ct).

Table 22. Viral target summary statistics expressed as Ct values for *P. monodon* pond samples broken down by genetic source ECWM & NTWM as well as the combined Total statistics.

		HDV	IHHNV	GAV	YHV-7	WSSV	YHV-1	When-2	Pir-A	Dicer-1
NTWM	Mean ± SD	37.99 ± 1.44	32.81 ± 1.82	32.59 ± 3.00	34.84 ± 0.00	nd	nd	28.75 ± 6.07	nd	26.53 ± 1.68
(n = 404)	Min-Max	34.80 - 43.40	27.70 - 36.40	24.65 - 39.33	34.84 - 34.84	nd	nd	18.55 - 39.67	nd	22.32 - 30.15
ECWM	Mean ± SD	38.56 ± 1.29	31.98 ± 3.52	32.59 ± 2.51	nd	nd	nd	28.47 ± 5.92	nd	27.23 ± 1.62
(n = 262)	Min-Max	35.79 - 41.65	18.55 - 37.73	23.83 - 37.74	nd	nd	nd	21.63 - 38.12	nd	23.62 - 30.39
Total	Mean ± SD	38.25 ± 1.40	32.37 ± 2.89	32.59 ± 2.83	34.84 ± 0.00	nd	nd	28.66 ± 6.02	nd	26.81 ± 1.69
(n = 666)	Min-Max	34.80 - 43.40	18.55 - 37.73	23.83 - 39.33	34.84 - 34.84	nd	nd	18.55 - 39.67	nd	22.32 - 30.39
	+ Detections Ct >30	100%	82%	81%	100%	nd	nd	47%	nd	1%
	+ Detections 30> Ct >20	0%	18%	19%	0%	nd	nd	48%	nd	99%
	+ Detections < Ct 20	0%	1%	0%	0%	nd	nd	5%	nd	0%

31.4 qPCR results of detection (calculated copy number (mL-1))

With the exception of IHHNV, there were small differences in copy number detected for each target between ECWM and NTWM (Figure 34 and Figure 35). The average and maximum copy number for IHHNV was higher in ECWM compared to NTWM, (Figure 34 and Figure 35).

Average calculated copy number (mL^{-1})
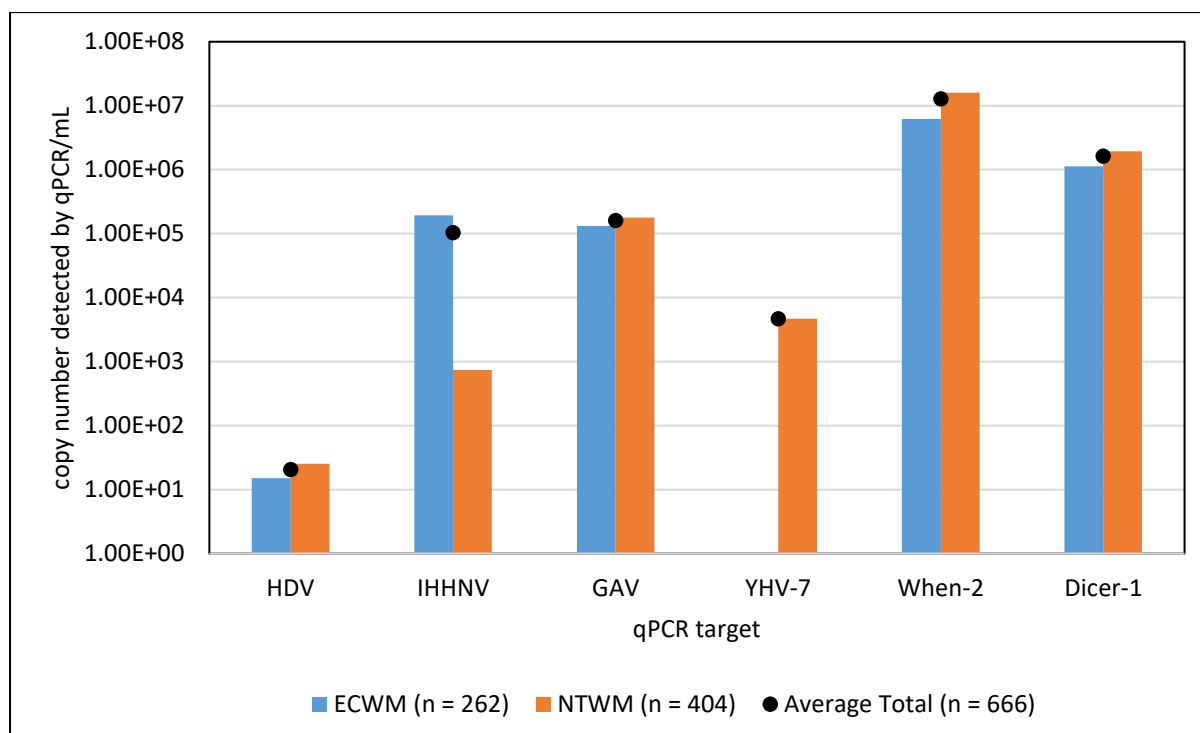
Figure 34. Average calculated copy number of each viral target partitioned into ECWM and NTWM as well as total.

Maximum calculated copy number

When-2 was detected at the highest copy number in a single sample (1.8×10^8 copies mL^{-1}). The samples with the highest 10 detections (copy number) of HDV and GAV were distributed between both geographic sources of broodstock (Table 23). The highest 10 detections of IHHNV were all from ECWM-sourced stocks. In contrast, the highest 10 detections of When-2 were all from NTWM-sourced stock (Table 23Figure 35).

The 10 highest detections of each pathogen target commonly all originated from a single farm (with the exception of HDV) (Table 24). However, in these cases, each farm only held the highest 10 detections for a single pathogen target (i.e. No one farm held the top 10 detections for more than one pathogen target). The 10 samples with the highest detected copy numbers of When-2 all originated from a single pond on Farm B. The 10 samples with the highest detected copy numbers of IHHNV were distributed over two ponds from Farm D. The 10 samples with the highest detected copy numbers of GAV were distributed over five ponds from Farm C.

Table 23. Quantity of samples from each stock source that contributed to the top 10 highest copy number detections for the viruses HDV, IHHNV, GAV, When-2 and P. monodon Dicer-1 gene.

	HDV	IHHNV	GAV	When-2	Dicer-1
ECWM (n = 262)	1	10	2	0	0
NTWM (n = 404)	9	0	8	10	10

Table 24. Quantity of samples from each farm source that contributed to the top 10 highest copy number detections for the viruses HDV, IHHNV, GAV, When-2 and the P. monodon Dicer-1 gene.

	HDV	IHHNV	GAV	When-2	Dicer-1
Farm A	2	0	0	0	0
Farm B	1	0	0	10	6
Farm C	7	0	10	0	4
Farm D	0	10	0	0	0
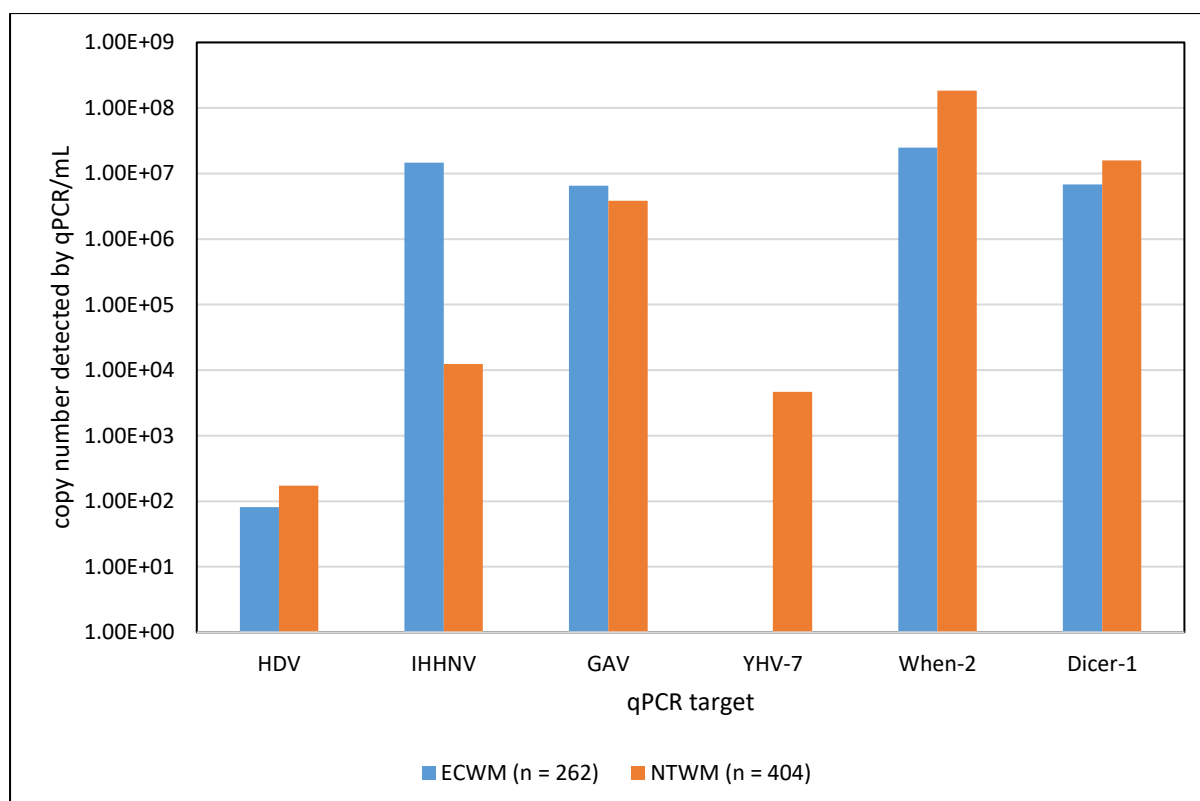
Figure 35. Maximum calculated copy number of each viral target detected from ECWM and NTWM ponds.

31.5 Endemic Targets: Individual target summaries

31.5.1 P. monodon Dicer-1 gene

- **Dicer -1** was detected in 666 (100%) samples.
- **Dicer-1** was detected with the lowest average Ct value across all targets (26.81 Ct).
- Average copy number for **Dicer-1** was 1.6 x 10^6 copies mL^{-1}.
- Only **IHHNV, GAV** and **When-2** were detected with a maximum copy number higher than Dicer-1 ie. The 3 viral targets were detected in higher quantity than the prawn Dicer-1 gene.

31.5.2 GAV

- **GAV** was detected in 652 samples (98%).
- The average Ct value of **GAV** was 32.59 Ct.
- The average load of **GAV** was 1.6 x 10^5 copies mL^{-1}.
- The single highest copy number detection of **GAV** was in an **ECWM** sample (6.52 x 10^6 viral copies mL^1).
- Eight of the 10 highest load detections were from **NTWM** samples.
- **GAV** was detected at high prevalence in both **NTWM** (99%) and **ECWM** (96%).
- **GAV** was detected in single, double, triple, quadruple, quintuple and sextuple target detections (multiple infections).

31.5.3 IHHNV

- **IHHNV** was detected in 415 samples (62%).
- The average Ct value of **IHHNV** was 32.37 Ct.
- The average load of **IHHNV** was 1.0 x 10^5 copies mL^{-1}.
- The highest load of **IHHNV** was detected in an **ECWM** sample (1.5 x 10^7 viral copies mL^{-1}).
- All of the 10 highest load detections were from **ECWM** samples.
- **IHHNV** was detected at a higher prevalence in **ECWM** (85%), compared to **NTWM** (48%).
- **IHHNV** was detected in double, triple, quadruple, quintuple and sextuple target detections (multiple infections).

31.5.4 HDV

- **HDV** was detected in 129 samples (19%).
- The average Ct value of **HDV** was 38.25 Ct.
- The average load of **HDV** was 2.1 x 10^1 viral copies mL^{-1}.
- The highest load of **HDV** was detected in an **NTWM** sample (1.7 x 10^2 viral copies mL^{-1});
- Nine of the 10 highest load detections were from **NTWM** samples.
- **HDV** was detected at a marginally higher occurrence in **ECWM** (22%) compared to **NTWM** (18%).
- **HDV** was detected in double, triple, quadruple, quintuple and sextuple target detections (multiple infections).

31.5.5 When-2

- **When-2** was detected in 149 samples (22%).
- The average Ct value of **When-2** was 28.66 Ct.
- The average load of **When-2** was 1.3×10^7 viral copies mL^{-1}.
- The highest load of **When-2** was detected in an **NTWM** sample (1.8×10^8 viral copies mL^{-1})
- All of the 10 highest load detections were from **NTWM** samples.
- **When-2** was detected at a marginally higher prevalence in **NTWM** (25%), compared to **ECWM** (19%).
- **When-2** was detected in double, triple, quadruple, quintuple and sextuple target detections (multiple infections).

31.5.6 YHV-7

- **YHV-7** was detected in 1 sample (<1%).
- The Ct value of the **YHV-7** detection was 34.84.
- The load of the **YHV-7** detection was 4.7×10^3 viral copies mL^{-1}.
- The detection occurred from a **NTWM** sample.
- **YHV-7** was detected as a triple infection with GAV and RtX.

31.5.7 YHV-1

- **YHV-1** was not detected in any of the CRCNA pond samples submitted.

31.5.8 WSSV

- **WSSV** was not detected in any of the CRCNA pond samples submitted.

31.6 Toxin Gene Target Results

31.6.1 Toxin gene target overview

Overall summary of toxin gene targets:
- Toxin genes were present in varied prevalence in grow out ponds.
- A toxin gene was detected in 411/666 samples (62%).
- Pir-A was not detected in any of the samples (0%)
- RtX was the most prevalent endemic toxin gene target detected (57%).
- RtX was detected at the lowest average Ct (34.86 Ct) of all toxin gene targets.
- Zot was the least prevalent endemic toxin gene target detected (21%).
- There were 255/666 samples which were negative for the detection of all toxin gene targets (38%).
- There were 189/666 single toxin-gene target detections (28%).
- There were 177/666 dual toxin-gene target detections (27%).
- There were 45/666 triple toxin-gene target detections (7%).
- One sample was positive for the detection of RtX at a copy number exceeding 10^8 copies mL^{-1}.

A summary of the toxin gene targets is presented in three sections namely:

1. Prevalence of detection (number of positive detections; proportion of positives detected/total number analysed).
2. qPCR results of detection (Ct value).
3. qPCR results of detection (mL^{-1}).

31.6.2 Prevalence of detection of toxin gene targets

Table 25 presents the number of positive detections between ECWM and NTWM for the bacterial toxin gene targets. RtX was the most frequently detected toxin gene (57%). RtX was detected in 34 and 36% more samples than Hemo and Zot, respectively. Variability in prevalence between stock sources was marginal for Zot and Hemo. However, RtX was 15% more prevalent in NTWM compared to ECWM (Figure 4). Prevalence of detection of the toxin genes was variable between different farms (Table 26).

Table 25. Number of positive detections of targets from ECWM and NTWM ponds and the percentage of positive detections overall for each toxin target.

Source	Total Tested	Zot	RtX	Hemo
ECWM	262	52	126	61
NTWM	404	90	254	95
Total	666	142	380	156
Total (% Positive)	666	21%	57%	23%
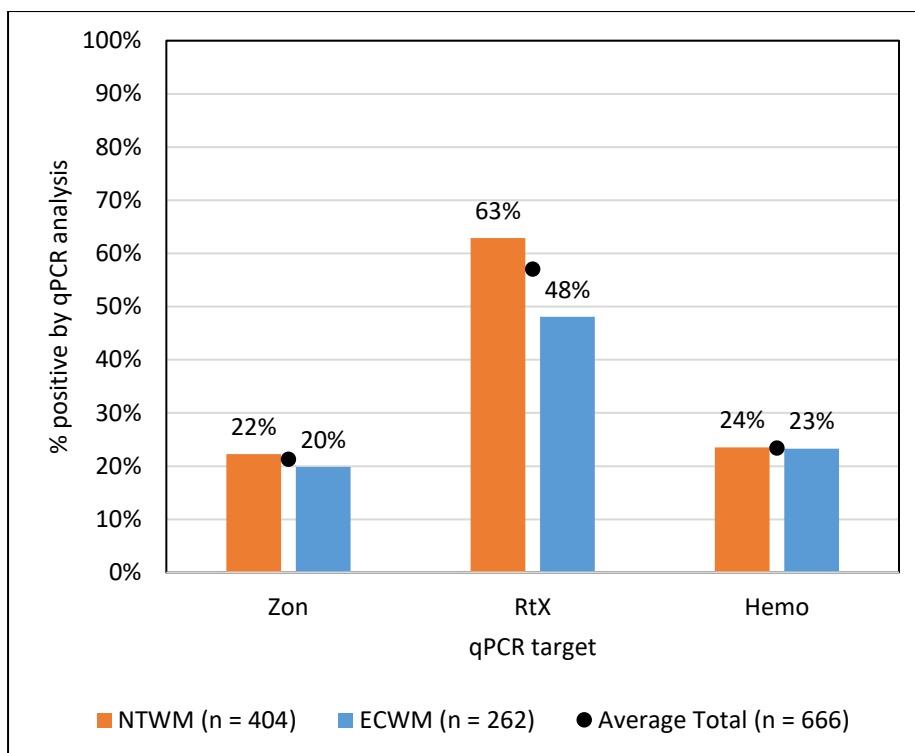
Figure 36. Percentage of samples positive for the detection of each toxin target from ECWM and NTWM ponds.

Table 26: Prevalence of toxin gene detection by Pond and Farm.

Source	Unit	Total Tested	Prevalence of Positive detection		
			Zot	RtX	Hemo
Farm A	No. of samples	190	15%	62%	17%
	No. of ponds	16	50%	100%	75%
Farm B	No. of samples	144	51%	63%	3%
	No. of ponds	11	73%	100%	27%
Farm C	No. of samples	156	21%	99%	76%
	No. of ponds	12	75%	100%	100%
Farm D	No. of samples	176	5%	10%	0%
	No. of ponds	16	38%	38%	0%
Total (% Positive)	% of total samples	666	21%	57%	23%
	% of total ponds	55	56%	82%	49%
	% of total ponds	55	56%	82%	49%

31.6.3 qPCR results of detections (Ct value)

Average Ct value

RtX was detected at the lowest average Ct (34.86 Ct) and the lowest detected Ct (17.28 Ct). The lowest detected Ct for RtX was observed in a NTWM sample. This was the only toxin gene detection at a Ct value below 20. Overall, >97% of positive toxin gene target detections were above a Ct value of 30 (Table 27).

Table 27. Toxin target summary statistics expressed as Ct values for *P. monodon* pond samples subdivided by genetic source ECWM & NTWM as well as the combined Total statistics.

		Zot	RtX	Hemo
NTWM	Mean ± SD	36.9 ± 2.26	34.91 ± 2.86	35.54 ± 2.8
(n = 404)	Min-Max	29.24 - 39.96	17.28 - 39.83	20.77 - 39.57
ECWM	Mean ± SD	38.12 ± 1.12	34.75 ± 1.73	36.47 ± 1.38
(n = 262)	Min-Max	35.74 - 39.99	32.26 - 39.91	34.22 - 39.92
Total	Mean ± SD	37.35 ± 2.01	34.86 ± 2.54	35.9 ± 2.39
(n = 666)	Min-Max	29.24 - 39.99	17.28 - 39.91	20.77 - 39.92
	+ Detections Ct >30	99%	97%	97%
	+ Detections 30> Ct >20	1%	2%	3%
	+ Detections < Ct 20	0%	1%	0%

31.6.4 **qPCR results of detection**

The average and maximum calculated copy number detections for all three endemic toxin gene targets were higher in the *P. monodon* spawned from NTWM broodstock (Figure 37 and Figure 38).
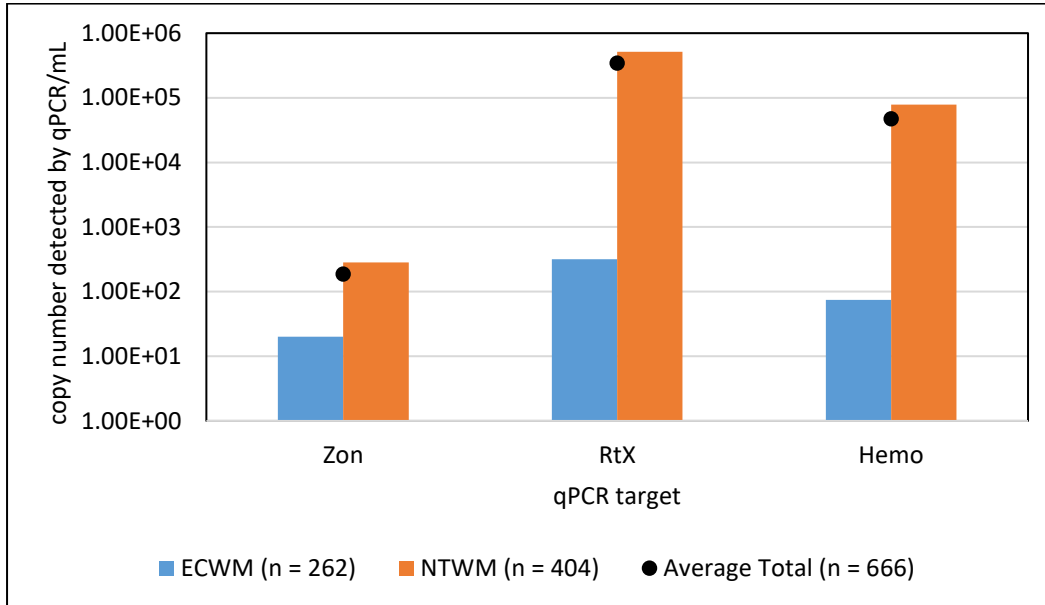
Figure 37. Average calculated copy number of each qPCR toxin target detected from ECWM and NTWM.
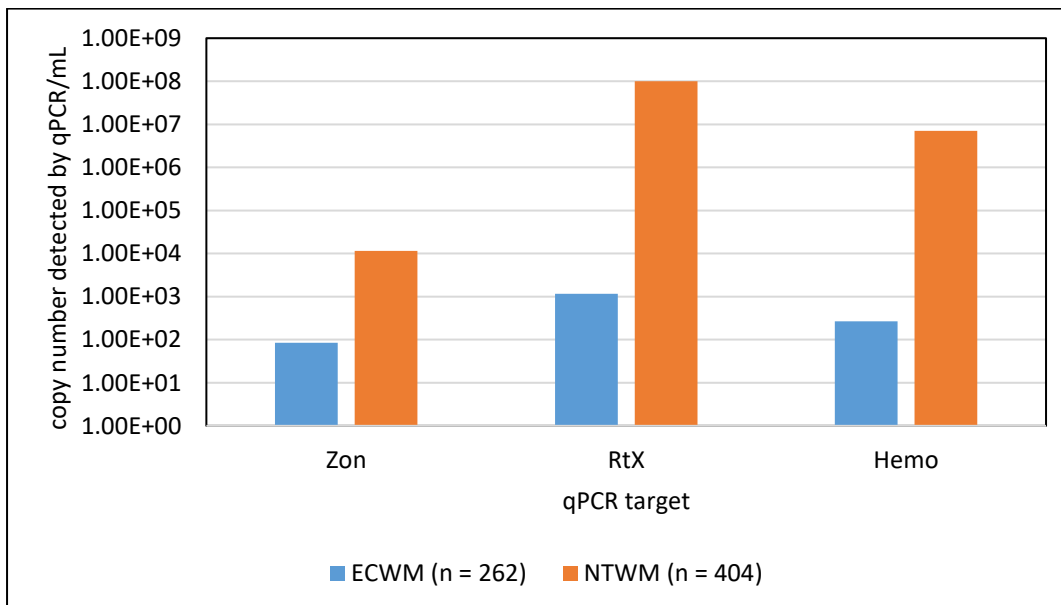
Figure 38. Maximum calculated copy number of each toxin target detected from ECWM and NTWM ponds.

31.7 Toxin Gene Targets: Individual target summaries

A positive detection summary of each of the individual toxin gene targets is listed below:

31.7.1 Photorhabdus insect-related toxin component A (Pir-A)

- **Pir-A** was not detected in any of the CRCNA pond samples submitted.

31.7.2 Zonula occludens toxin gene (Zot)

- **Zot** was detected in 142 samples (21%).
- The average Ct value of **Zot** was 37.35 Ct.
- The average load of **Zot** was 1.9×10^2 copies mL^{-1}.
- The highest load of **Zot** was detected in a **NTWM** sample and was 1.15×10^4 copies mL^{-1}.
- **Zot** was detected at a marginally higher prevalence in **NTWM** samples (22%), compared to ECWM (20%).
- **Zot** was detected in double, triple, quadruple, quintuple and sextuple target detections (multiple infections).

31.7.3 Repeat-in toxin gene (RtX)

- **RtX** was detected in 380 samples (57%).
- The average Ct value of **RtX** was 34.86 Ct.
- The average load of **RtX** was 3.5×10^5 copies mL^{-1}.
- The highest load of **RtX** was detected in a **NTWM** sample and was 1.0×10^8 copies mL^{-1}.
- **RtX** was detected at a much higher prevalence in **NTWM** samples (63%) compared to ECWM (48%).
- **RtX** was detected in double, triple, quadruple, quintuple and sextuple target detections (multiple infections).

31.7.4 Hemolysin D toxin gene (hemo)

- **Hemo** was detected in 156 samples (23%).
- The average Ct value of **Hemo** was 35.90 Ct.
- The average load of **Hemo** was 4.8×10^4 copies mL^{-1}.
- The highest load of **Hemo** was detected in a **NTWM** sample and was 7.1×10^6 copies mL^{-1}.
- **Hemo** was detected at a marginally higher prevalence in **NTWM** samples (24%), compared to ECWM (23%).
- **Hemo** was detected in double, triple, quadruple, quintuple and sextuple target detections (multiple infections).

The majority of samples were positive for the detection of multiple pathogen targets; Table 28 presents an overview of the proportion of samples with multiple detections within each geographic stock source. Table 28 also divides the targets into emerging toxin gene target data and endemic target data (including Pir-A, IHHNV, HDV, YHV-7, When-2 and GAV). Table 9 presents the proportion of samples with multiple infections divided into the different target combinations which were detected.

Only 0.2% (n = 1) samples were negative for the detection of all targets tested. The remaining samples (99.8%, n = 665) were positive for the detection of between one and six pathogen targets. Single target detections were uncommon (9%). All single detections were of GAV only. Dual and triple target detections were most common, cumulatively accounting for ~57% of all samples. GAV and IHHNV was the most common dual detection combination (18.6% of samples). Approximately one third of all samples (32%) were positive for the detection of 4 to 6 targets. There were 52 different combinations of target detections (Table 29). NTWM samples had fewer endemic target detections per sample (~ 3 targets detected per samples, on average), compared to the ECWM samples (~ 3.2 targets detected per samples, on average). One third of the NTWM samples were positive for the detection of one endemic viral target, and nearly half (45%) were positive for the detection of two endemic viral targets. In contrast, more than half of the ECWM samples were positive for two endemic targets, and nearly one third (31%) were positive for three to four endemic viral targets.

The emerging toxin gene targets (RtX, Zot and Hemo) were not detected in 38% of samples. 28% and 27% of samples tested positive for one and two emerging toxin gene targets, respectively.

Table 28. Proportion of samples which tested positive for multiple target detections. The total number of samples tested for all targets was 666; 404 samples originated from NTWM and 262 originated from ECWM.

		0	1	2	3	4	5	6
Emerging toxin gene targets	*NTWM*	35%	29%	28%	8%			
	ECWM	44%	27%	24%	5%			
	TOTAL	**38%**	**28%**	**27%**	**7%**			
Endemic targets	*NTWM*	0.2%	33%	45%	20%	2%	0%	0%
	ECWM	0%	10%	59%	29%	2%	0%	0%
	TOTAL	**0.2%**	**24%**	**51%**	**23%**	**2%**	**0%**	**0%**
All targets	*NTWM*	0.2%	10%	28%	29%	18%	12%	2%
	ECWM	0%	7%	29%	26%	21%	14%	3%
	TOTAL	**0.2%**	**9.0%**	**28.8%**	**27.9%**	**19.1%**	**12.8%**	**2.3%**

Table 29. The multiple detection patterns observed, detailing the specific pathogen targets detected, the number of targets detected, the number of samples and the relative proportion of the total number of samples that were observed to have the detection pattern.

No. of targets detected	Targets Detected	No. of samples +ve	% of total samples
0		1	0.20%
1	GAV	60	9.00%
2	RtX When-2	3	0.5%
	IHHNV HDV	1	0.2%
	GAV HDV	5	0.8%
	GAV Zot	2	0.3%
	GAV When-2	15	2.3%
	GAV Hemo	2	0.3%
	GAV RtX	40	6.0%
	GAV IHHNV	124	18.6%
3	RtX When-2 Zot	2	0.3%
	GAV When-2 HDV	1	0.2%
	GAV When-2 Zot	3	0.5%
	GAV RtX YHV-7	1	0.2%
	GAV RtX HDV	8	1.2%
	GAV RtX Zot	8	1.2%
	GAV RtX When-2	18	2.7%
	GAV RtX Hemo	29	4.4%
	GAV IHHNV HDV	28	4.2%
	GAV IHHNV Zot	14	2.1%
	GAV IHHNV When-2	16	2.4%
	GAV IHHNV Hemo	1	0.2%
	GAV IHHNV RtX	57	8.6%
4	IHHNV RtX When-2 Zot	1	0.2%
	GAV RtX Zot HDV	2	0.3%
	GAV RtX When-2 Zot	2	0.3%
	GAV RtX Hemo HDV	15	2.3%
	GAV RtX Hemo Zot	15	2.3%
	GAV RtX Hemo When-2	4	0.6%
	GAV IHHNV Zot HDV	4	0.6%
	GAV IHHNV When-2 HDV	3	0.5%
	GAV IHHNV When-2 Zot	4	0.6%
	GAV IHHNV Hemo When-2	1	0.2%
	GAV IHHNV RtX HDV	10	1.5%
	GAV IHHNV RtX Zot	15	2.3%
	GAV IHHNV RtX When-2	18	2.7%
	GAV IHHNV RtX Hemo	32	4.8%
5	IHHNV RtX Hemo When-2 Zot	5	0.8%
	GAV RtX When-2 Zot HDV	1	0.2%
	GAV RtX Hemo Zot HDV	10	1.5%

	GAV RtX Hemo When-2 HDV	2	0.3%
	GAV RtX Hemo When-2 Zot	2	0.3%
	GAV IHHNV RtX Zot HDV	3	0.5%
	GAV IHHNV RtX When-2 HDV	3	0.5%
	GAV IHHNV RtX When-2 Zot	31	4.7%
	GAV IHHNV RtX Hemo HDV	23	3.5%
	GAV IHHNV RtX Hemo Zot	3	0.5%
	GAV IHHNV RtX Hemo When-2	2	0.3%
	IHHNV RtX Hemo When-2 Zot HDV	1	0.2%
6	GAV IHHNV RtX When-2 Zot HDV	5	0.8%
	GAV IHHNV RtX Hemo Zot HDV	3	0.5%
	GAV IHHNV RtX Hemo When-2 Zot	6	0.9%

31.9 Co-habiting organisms in *P. monodon* ponds (*Acetes spp.*)

Analysis of glass shrimp, *Acetes spp.*, co-habiting the *P. monodon* ponds resulted in positive detection of HDV, IHHNV, When-2, Zot, RtX and Hemo (Table 30 and

Table 31). The viruses were not detected across all the farms. When-2 was only detected in *Acetes spp.* from Farm A, and IHHNV was only detected in *Acetes spp* from Farm D. When-2 had the lowest detected Ct (27.55 Ct), lowest average Ct (34.31 Ct) and, was also the most frequently detected target (49%). All toxin gene detections were above a Ct of 30, and no toxin genes were detected at a prevalence greater than 14%. A small proportion of the When-2 detections (7%), were below a Ct of 30, which was not observed for any other pathogen target tested in in *Acetes spp.* Table 32 and Table 33 detail the prevalence and average Ct of target detections across each farm and associated ponds, enabling comparison of detections between glass shrimp and *P. monodon* from the same pond.

Table 30. Endemic target summary statistics for *Acetes spp.* tested from *P. monodon* ponds.

		HDV	IHHNV	GAV	YHV-7	WSSV	YHV-1	When-2	Pir-A	Dicer-1
	Prevalence (%)	20%	20%	24%	0%	0%	0%	49%	0%	2%
	Total tested	59	59	59	59	59	59	59	59	59
	Mean ± SD	40.25 ± 0.95	35.29 ± 1.41	35.36 ± 1.95	nd	nd	nd	34.31 ± 1.95	nd	34.15 ± 0.00
East Coast Wild	Min-Max	39.15 - 41.75	33.06 - 38.15	31.98 - 39.59	nd	nd	nd	27.55 - 36.56	nd	34.15 - 34.15
	+ Detections Ct >30	100%	100%	100%	nd	nd	nd	93%	nd	100%
	+ Detections 30> Ct >20	0%	0%	0%	nd	nd	nd	7%	nd	0%
	+ Detections < Ct 20	0%	0%	0%	nd	nd	nd	0%	nd	0%

Table 31. Emerging toxin gene target summary statistics for *Acetes spp.* tested from *P. monodon* ponds.

		Zot	RtX	Hemo
	Prevalence (%)	5%	14%	12%
	Total tested	59	59	59
	Mean ± SD	38.65 ± 0.64	37.67 ± 1.85	37.99 ± 1.01
East Coast Wild	Min-Max	37.75 - 39.2	33.77 - 39.98	36.2 - 39.8
	+ Detections Ct >30	100%	100%	100%
	+ Detections 30> Ct >20	0%	0%	0%
	+ Detections < Ct 20	0%	0%	0%

Table 32. Prevalence of positive detection of pathogen targets from *Acetes spp.* and *P. monodon* samples from the same pond across all farms.

Glass Shrimp (*Acetes spp.*)									
Farm	Farm A			Farm B				Farm D	
Pond	G	H	I	C	D	E	F	A	B
HDV	30%	60%	20%	0%	0%	0%	0%	20%	0%
IHHNV	0%	0%	0%	0%	0%	0%	0%	100%	100%
GAV	0%	0%	0%	0%	40%	0%	0%	100%	100%
When-2	100%	100%	90%	0%	0%	0%	0%	0%	0%
Zot	10%	0%	10%	20%	0%	0%	0%	0%	0%
RtX	30%	10%	40%	0%	0%	0%	0%	0%	0%
Hemo	10%	20%	20%	20%	20%	0%	0%	0%	0%
Total tested	10	10	10	5	5	2	5	5	7
Tiger prawn (*P. monodon*)									
Farm	Farm A			Farm B				Farm D	
Pond	G	H	I	C	D	E	F	A	B
HDV	17%	nt	nt	15%	8%	0%	8%	27%	27%
IHHNV	0%	nt	nt	100%	100%	54%	100%	100%	100%
GAV	100%	nt	nt	100%	100%	100%	100%	100%	100%
When-2	17%	nt	nt	100%	100%	77%	8%	0%	0%
Zot	25%	nt	nt	100%	92%	92%	54%	0%	0%
RtX	75%	nt	nt	100%	100%	46%	15%	18%	18%
Hemo	8%	nt	nt	8%	8%	15%	0%	0%	0%
Total tested	12	0	0	13	13	13	13	11	11

Table 33. Average Ct and SD of positive target detections across farms from *Acetes spp.* and *P. monodon* samples from the same ponds.

	Glass Shrimp *(Acetes spp.)*								
Farm	**Farm A**			**Farm B**				**Farm D**	
Pond	**G**	**H**	**I**	**C**	**D**	**E**	**F**	**A**	**B**
HDV	41.1 ±0.69	39.85 ±0.94	40.49 ±1.3	nd	nd	nd	nd	39.61 ±0	nd
IHHNV	nd	nd	nd	nd	nd	nd	nd	34.33 ±1.02	35.97 ±1.39
GAV	nd	nd	nd	nd	35.78 ±5.38	nd	nd	35.76 ±1.6	34.96 ±1.39
When-2	32.76 ±2.66	35.19 ±0.78	35.07 ±0.68	nd	nd	nd	nd	nd	nd
Zot	39 ±0	nd	39.2 ±0	37.75 ±0	nd	nd	nd	nd	nd
RtX	38.89 ±1.07	38.93 ±0	36.44 ±2.09	nd	nd	nd	nd	nd	nd
Hemo	38.63 ±0	38 ±2.54	37.67 ±0.03	38 ±0	38 ±0	nd	nd	nd	nd
Total tested	10	10	10	5	5	2	5	5	7
	P. monodon								
Farm	**Farm A**			**Farm B**				**Farm D**	
Pond	**G**	**H**	**I**	**C**	**D**	**E**	**F**	**A**	**B**
HDV	36.67 ±1.32	nt	nt	38.91 ±0.03	37.42 ±0	nd	39.41 ±0	39.5 ±1.67	39.96 ±0.91
IHHNV	nd	nt	nt	32.59 ±0.39	33.58 ±0.92	34.94 ±1.5	33 ±0.55	27.6 ±4.01	29.8 ±0.66
GAV	34.31 ±1.46	nt	nt	30.01 ±2.73	31.99 ±2.41	32.51 ±0.93	33.33 ±2.14	32.69 ±2.66	34.93 ±1.35
When-2	36.13 ±2.22	nt	nt	28.31 ±4.96	28.94 ±4.88	35.94 ±1.07	35.03 ±0	nd	nd
Zot	39.06 ±0.71	nt	nt	34.09 ±0.59	35.45 ±0.44	37.8 ±1.24	37.88 ±1.13	nd	nd
RtX	36.8 ±1.21	nt	nt	34.24 ±0.68	35.21 ±1.3	37.89 ±1.88	37.84 ±0.94	36 ±1.45	36.49 ±1.12
Hemo	39.57 ±0	nt	nt	38.09 ±0	37.72 ±0	37.22 ±0.42	nd	nd	nd
Total tested	12	0	0	13	13	13	13	11	11

nt: indicates no prawn samples were collected from the pond; nd: indicates not detected.

31.10 Post-Larval Results

Due to the sensitive nature of commercial data and information, the post larvae (PL) data from the Hatchery component was previously withheld. It is now presented in this report (Table 34). There was no positive detection of YHV-7, WSSV, YHV-1 or When-2 in any of the post larval samples submitted. GAV was the most prevalent viral target detected (97%). GAV was detected at the lowest Ct value (16.72 Ct) and was the only pathogen target with its detections (59%) within a Ct range of 20 to 30. GAV was detected at extremely high copy numbers, with an average of calculated copy number of 1.12×10^{8} copies mL^{-1}. A large portion of IHHNV detections (29%) were within a Ct range of 20-30.

Table 34. Viral target summary statistics for Post Larvae.

		HDV	IHHNV	GAV	YHV-7	WSSV	YHV-1	When-2	Pir-A	Dicer-1
TOTAL	Prevalence (%)	14%	43%	97%	0%	0%	0%	0%	2%	100%
	Total tested	115	115	115	115	115	115	115	115	115
	Mean ± SD	38.37 ± 1.38	31.26 ± 2.14	21.07 ± 2.74	nd	nd	nd	nd	39.97 ± 0.47	26.44 ± 2.83
	Min-Max	36.6 - 41.27	27.6 - 36.28	16.72 - 31.66	nd	nd	nd	nd	39.5 - 40.44	22.01 - 35.65
	+ Detections Ct >30	100%	71%	1%	nd	nd	nd	nd	100%	16%
	+ Detections 30> Ct >20	0%	29%	59%	nd	nd	nd	nd	0%	84%
	+ Detections < Ct 20	0%	0%	40%	nd	nd	nd	nd	0%	0%

32 Discussion: Pond component

32.1 Exotic Targets: WSSV and YHV-1

There was no detection of WSSV or YHV-1 in any of the project samples.

32.2 Endemic Target: Prevalence of detection

The pond data reported here is the first to include six viral targets, four bacterial toxin genes and the *P. monodon* Dicer-1 gene, making it the most comprehensive investigation into the prevalence of pathogens within Australian prawn ponds to date. Previous studies report on fewer pathogen targets and are typically in response to a disease outbreak, rather than proactively sourcing samples.

At the time of stocking the pathobiome of ponds was not standardised and treatments provided to the ponds over the production period varied. Comparisons between ponds and stock sources are therefore not robust. The results are however, a representation of the pathogens present throughout the Northern Australian prawn industry during the growout period of 2018-19 and should be considered a baseline dataset of the pathogens in production ponds. The 2018-19 season represented a standard production season when the production volume on a per farm unit level is considered. From 2016 to 2018, the overall industry production tonnage decreased, however, the number of active farms also decreased relatively, yielding a consistent average production volume per farm unit (QLD Department of Agriculture and Fisheries, 2018).

The present study detected a large difference in the prevalence of IHHNV between ECWM and NTWM originated stock. ECWM stock had a substantially higher prevalence (+40%) of IHHNV, compared to the NTWM stock. Decreased productivity in ponds has been linked to the infection of IHHNV in *P. monodon* (Sellars et al, 2019; Primavera & Quinitio, 2000). This study cannot definitively determine if the difference in prevalence of IHHNV was due to the geographic origin of the stock. Nonetheless, the difference observed is substantial. Further investigation is required to test if this trend is reflected in a more robust structured surveillance and analysis.

The discovery and understanding of new or emerging pathogens is important for the establishment and maintenance of good biosecurity practices. When-2 was newly identified in *P. monodon* in China (Li et al, 2015) and has more recently been identified in *P. monodon* in Australia (Huerlimann et al., 2018). In this study, When-2 was detected in nearly half of the ponds analysed, with an overall prevalence in samples of 22%. Currently, our understanding of any impact this pathogen has on productivity is

anecdotal. Increased focus and research of this emerging pathogens is required to understand the potential impacts and effects its presence may have on the Australian prawn industry into the future.

From the findings of this report, *P. monodon* ponds in Australia can be expected to have at least one pathogen present, most likely GAV (detected in 98% of samples tested and all in ponds). The occurrence of at least one pathogen in every pond tested should highlight the importance of managing pathogens prior to stocking ponds and maintaining optimum conditions during growout to minimize stress. Stress can cause viral levels to increase and, depending on the pathogen, can impact productivity (De La Vega et al, 2004). The prevalence of pathogens observed in this study indicate that pathogens are intrinsic to *P. monodon* ponds under current industry conditions. To further understand the potential economic impact of these viruses on production, access to farm productivity data would be required.

32.3 Endemic Targets: Ct and Copy number

Information relating the average viral copy number of a pond to the occurrence of disease events in commercial farm settings is sparse, however, it is an important consideration for management decision-making on the farm. The level of viral copies required for a particular virus to impact on productivity and survival on farm is not well understood, nor is the effect that the environment and/or stress would have on this theoretical threshold. There have been studies, both in laboratory and on farm, which investigated the effect of viral load on mortality (Sellars et al, 2019; Oanh et al, 2011; De La Vega et al, 2004). Generally, as viral load increases, the likelihood of experiencing a disease event also increases. In the present study, pathogens were detected in every pond analysed, primarily due to GAV being detected in 98% of samples. The majority of positive detections for GAV and other pathogen targets were above a Ct of 30, equating to approximately <6 500 copies mL^{-1} for DNA viruses and <120 000 copies mL^{-1} for RNA viruses.

In this study we report on newly discovered pathogens such as Whenzhou shrimp virus-2 (When-2). The lack of knowledge surrounding this pathogen and its potential effects represents a large and significant gap to the industry, particularly when paired with the high prevalence levels and viral copy numbers detected from the present study (the majority of When-2 positive detections were below a Ct of 30). All remaining pathogen targets displayed a skewed distribution of Ct values towards the higher range (>80% of all positive detections were above a Ct of 30).

Samples which were positive for the detection of pathogens at extremely high levels (i.e. the highest 10 copy number detections) were generally isolated to one farm, with some cases specifically isolated to one or a few ponds (Farm D: 10 highest detections of IHHNV (from 1 pond), Farm C: 10 highest detections of GAV (from 5 ponds) and Farm B: 10 highest detections of When-2 (from 2 ponds). These results indicate that viral presence and intensity of infection can be highly variable not only across farms,

but between ponds on the same farm. The identification and monitoring of such ponds may aid in the synthesis of more focussed biosecurity management decisions.

32.4 Individual Endemic Viral Target Overview

32.4.1 YHV-7

There was a single detection of YHV-7 from the current study. The level of virus detected was at a much lower level than associated with disease events on farm (15.7 to 21.8 Ct) (Cowley, 2015) or in experimental challenges where ~ 60% cumulative mortality was observed after inoculation with YHV-7 (12.9 to 22.6 Ct) (Moody & Crane, 2015). Nonetheless, YHV-7 is an endemic virus with a history of causing mortality events on farm (81.4% mortality of Australian *P. monodon* broodstock, 12 days post stocking in hatchery; Mohr *et al.,* 2015). Thus, its presence on farms within the industry should be closely monitored. The absence of YHV-7 in 99% of samples indicates this virus is rarely present within a normal production year. In future, seasons of decreased productivity can be compared to this dataset to determine if YHV-7 is at higher prevalence and/or load.

32.4.2 GAV

GAV is extremely prevalent in Australian *P. monodon* populations (Cowley et al, 2000; Spann et al, 1995). GAV was the only viral target detected from 100% of ponds analysed in this study. Infection trials conducted by De La Vega et al (2004) and Spann et al (1997) used tissue homogenate of GAV positive prawns to induce mortality. However, in these studies, no analysis was conducted to detect the collective of viruses tested in this study, from the tissue homogenate. From the results of this study it is very unlikely that GAV will be the sole pathogen present during a disease event. The average viral copy number in single GAV infections (6.1×10^4 copies mL^{-1}) was 10fold lower than for multiple infections (1.6×10^5 copies mL^{-1}). Mortality and disease events are more likely to occur at higher viral concentrations (Sellars et al, 2019; Oanh et al, 2011; De La Vega et al, 20040); furthering the point that GAV is unlikely to be the sole pathogen present during disease events. Disease events on farm recorded for other viruses in the yellow-head complex returned Ct values in the range of 15.7 to 21.8 (Cowley, 2015). Additionally, in the present study, every pond tested was positive for the detection of multiple pathogens. Future studies should be sure to screen for the full suite of pathogens when proposing a causation of disease in *P. monodon* ponds.

In this study, the prevalence of detection of GAV was nearly 100% (98%). Previous studies on Australian *P. monodon* recorded a similarly high level of prevalence for GAV (Cowley et al, 2000 and Spann et al, 1995). These findings suggest the presence of GAV in *P. monodon* production systems is widespread and may indicate that prevalence levels and Ct values detected in the present study are common during a standard production season. However, consistent testing over time, across multiple seasons, coupled with analysis of production data, is required to confirm this trend.

32.4.3 Whenzhou shrimp virus-2 (When-2)

When-2, a newly discovered pathogen is reported here for the first time. When-2 was detected in 24 of the 55 ponds tested. In addition to the high prevalence of detection, When-2 was also detected at the lowest average Ct and, hence, the highest concentration of viral copies detected in a single sample. Currently, any information surrounding the impact of this pathogen on *P. monodon* production is based on anecdotal evidence. Research focus should be directed to characterizing this virus. Experimental challenge trials are required to enhance our understanding of the host pathogen interaction, and continued monitoring of this virus within industry surveys and biosecurity protocols should be implemented.

32.4.4 IHHNV

In the current study 46 of the 55 ponds tested were positive for IHHNV. Most of the IHHNV detections were at a Ct greater than 30, with only four ponds observed with an average detection below a Ct of 30. Considering its connection to runt deformity syndrome (RDS) in Penaeids and association with decreased economic return in IHHNV high load ponds, further investigation into IHHNV is required (Sellars et al, 2019). Although IHHNV is not a new virus, there is poor understanding of the range of strains of IHHNV in Australian prawn stocks. The absence of mortalities in many IHHNV cases concerning *P. monodon* does not negate the impact it has on productivity as stunting growth due to RDS; some common consequences include higher FCR, reduced survival and slower growth (Singhapan et al, 2004). Considering the high prevalence of detection and potential for economic loss, further resources should be directed to the study of IHHNV.

32.4.5 Effect of multiple infections

The patterns of detection observed in this study provide a basis for future studies that aim to understand the pathobiome of stock and the associated biosecurity risk of prawn farming during the growout stage of production. In the present study, the majority of samples were positive for the detection of two to four targets, and some individuals were positive for up to six targets; only one sample was negative for all pathogen targets. Multiple infections in *P. monodon* have been commonly reported in international production systems, primarily focusing on MBV, HPV, IHHNV and WSSV (Chayaburakul et al., 2004; Flegel et al., 2004; Joseph et al., 2015). However, no further understanding has been presented regarding the potential effects of multiple viral and toxin gene infections, although, it was suggested by Flegel et al. (2001) that the occurrence of multiple infections in 'grossly healthy prawns' may be related to host-viral interaction mechanisms in crustaceans. The effect and potential host mechanisms which may be involved in multiple infections is yet to be achieved and will require further and more targeted investigation.

32.4.6 Co-habitating organisms

Viruses that cause disease in *P. monodon* have been described in other non-shrimp crustaceans (OIE, 2019). Limited investigation has been conducted on the viral pathobiome of cohabitating crustaceans within *P. monodon* ponds. The samples of 'glass shrimp', *Acetes spp.* analysed in this study were collected from commercial *P. monodon* grow-out ponds. Thus, cohabitation of these species in the environment would result in contact through water, sediment and potentially via direct physical contact.

The glass shrimp samples in the present study were positive for the detection of the bacterial toxin genes Zot, RtX and Hemo*,* and the viral targets HDV, IHHNV, GAV and When-2. The detection of these pathogens was consistent with the detection observed from the *P. monodon* samples, with the exception of the YHV-7 which was only detected in a single *P. monodon* sample.

The collection of glass shrimp samples in this study was opportunistic and, as a result, not standardized by number per pond. The aim of the glass shrimp analysis was to gain an indication of the presence of viruses in organisms that that share the production environment *P. monodon.* A more rigid sampling design would be required to improve the robustness of prevalence values for each virus*.* The potential for glass shrimp to act as a vector of prawn pathogens is recorded in the WSSV AQUAVETPLAN (2013). The collection and analysis of glass shrimp from inlet canals or intake reservoirs is currently being conducted as an activity of this project.

The detection of viruses such as IHHNV that cause significant production losses in ponds (Sellars et al, 2019; Primavera & Quinitio, 2000), emerging viruses such as When-2 or, the toxin genes from the glass shrimp analysed in this study highlights the risk that cohabitating organisms in ponds potentially pose to the biosecurity of production of *P. monodon.* This study could not determine if the glass shrimp tested, carried the pathogens detected prior to their entry into the ponds or, if they acquired the pathogens via horizontal transmission from the *P. monodon* in the pond*.* Analysis of glass shrimp collected from water bodies on farm external to the ponds e.g. channels and reservoirs is on-going in this study and will indicate if the same viruses are present in glass shrimp that reside outside the *P. monodon* ponds. Glass shrimp residing in the channels or reservoirs on farm may also be acting as sources of infection, coming into the ponds via inlet pipes. Further investigation into cohabitating organisms within aquaculture ponds is a key concept for further research to understand the effects these organisms may be having on productivity and determining the cost-benefit of solutions to mitigate any issues that are discovered.

32.4.7 Disclaimer: limitations of the data

Robust interpretation of geographic distribution of targets is not possible with the current data.

The sample collection was not standardised because the overarching aim of the project is to improve understanding of the population of targets that are present within the hatchery production under typical operational conditions, rather than guide broodstock source selection decisions.

Likewise, the unbalanced design of the sample collection does not allow for statistical analysis to determine the significance of any particular virus with respect to impact on prawn health. Rather, the data reports, the high prevalence of pathogens within prawns in pond production systems.

It is unlikely the prevalence and calculated copy number of targets detected in this project are a dramatic increase to those which the Australian Prawn Farm industry has managed during the past 10 years. However, the more frequent detection of previously exotic or novel strains of pathogens, namely IHHNV (2008), YHV-7 (2012), Pir-AB (2015), and WSSV (2016) from Australian farmed prawns indicates the

Australian prawn farming industry may be facing a more frequent and evolving suite of challenges with respect to disease. Fortunately, the development of molecular detection and genome sequencing technology has also improved at a rapid rate. Farmers now have access to more superior analytical tools. Application of those tools (including next generation sequencing methodologies) will be directed to the remaining activities of the project to help assess the potential for spread and the impact of the hatchery pathobiome into the larval and grow out stages.

32.4.8 Further Project Activities

Further components of the CRCNA project are underway.

The Emerging Pathogen component involves studies to detect novel, presently undescribed pathogens in the hatchery and farm collected samples. This component applies whole genome next generation sequencing technology to screen project samples.

The Tracking component involves comparative discussion of the qPCR analysis of the Hatchery-Farm samples to determine prevalence and calculated copy number of a novel pathogen in hatchery and farm collected samples.

The Final project report will be delivered in June 2020. The aim of this report is to present the data collected from the prawns collected from pond samples. The final project report will discuss the results collected across all components of the project including, discussion transfer of pathogens from broodstock to pond systems and the application of the qPCR data to support biosecurity management decisions.

33 Strategic recommendations: Pond component

It is not appropriate to make strategic recommendations on this component of the project. Strategic recommendations to improve biosecurity in Australian Prawn farms must be consider the full and complete information gathered during the project. Strategic recommendations will be provided within the Final Project Report (June 30 2020).

34 References: Pond component

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35 Appendices: Pond component

35.1 Appendix H: Pathogen detection Ct versus Dicer-1 detection Ct

- There were no samples tested with **HDV** Ct detections less than their associated Dicer-1 Ct detection value.

- Samples with an **IHHNV** Ct detection value less than their associated Dicer-1 Ct detection value originated ECWM stock from two isolated ponds on one farm only.

- The **GAV** Ct values cluster consistently, with no distinct grouping of the samples with GAV Ct < Dicer-1 Ct.

- The only **YHV-7** detection was at a Ct value greater than the sample's associated Dicer-1 Ct value.

- The majority of **When-2** Ct < Dicer-1 Ct samples were detected from four ponds, across two farms (68% of When-2 Ct < Dicer 1 Ct samples).

- There were no samples tested with **Zot** Ct detections less than their associated Dicer-1 Ct detection value.

- There were four **RtX** detections with a Ct value lower than the sample's associated Dicer-1 Ct value. Three of the four detections originated from a single farm.

- There was two **Hemo** detections with a Ct value lower than the sample's associated Dicer-1 Ct value. These detections originated from separate farms.