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**Advancing viral detection in Australian giant black tiger
shrimp (*Penaeus monodon*) aquaculture production**

Thesis submitted by
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BAdvSc (Hons Class 1)

June 2024

For the degree of Doctor of Philosophy
in
Natural and Physical Sciences

College of Science and Engineering
James Cook University



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I declare that this thesis is my own original work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Phoebe M. Arbon

June 2024

STATEMENT OF THE CONTRIBUTION OF OTHERS

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Specific contributions of co-authors and collaborators for each chapter included in this thesis are detailed below. Each co-contributor is in agreeance with the details below and have granted permission for the inclusion of each related chapter within this thesis.

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Chapter 2	Arbon, P.M.^{1,2}, Condon, K.^{1,2}, Andrade Martinez, M.^{1,2}, Jerry, D.R.^{1,2,3,5}. (2022). Molecular detection of six viral pathogens from Australian wild sourced giant black tiger shrimp (<i>Penaeus monodon</i>) broodstock. Aquaculture 548, Part 2, 737651. Arbon, P.M.^{1,2}, Condon, K.^{1,2}, Andrade Martinez, M.^{1,2}, Jerry, D.R.^{1,2,3,5}. Finding founders: Estimating the availability of wild-sourced <i>Penaeus monodon</i> broodstock to found domestication programs adopting a Specific Pathogen Free (SPF) approach. (Poster) International Symposium on Aquaculture Genetics (ISGA) - Puerto Varas, Chile (2022)	Arbon, P.M.^{1,2} Project co-conception. Data generation. Data curation, analysis, visualisation, and interpretation. Authored initial draft of complete manuscript. Condon, K.^{1,2} Project co-conception and administration. Data generation. Funding acquisition, resources, and supervision. Manuscript review and editing. Andrade Martinez, M.^{1,2} Data generation. Jerry, D.R.^{1,2,3,5} Project co-conception. Supervision. Manuscript review and editing.
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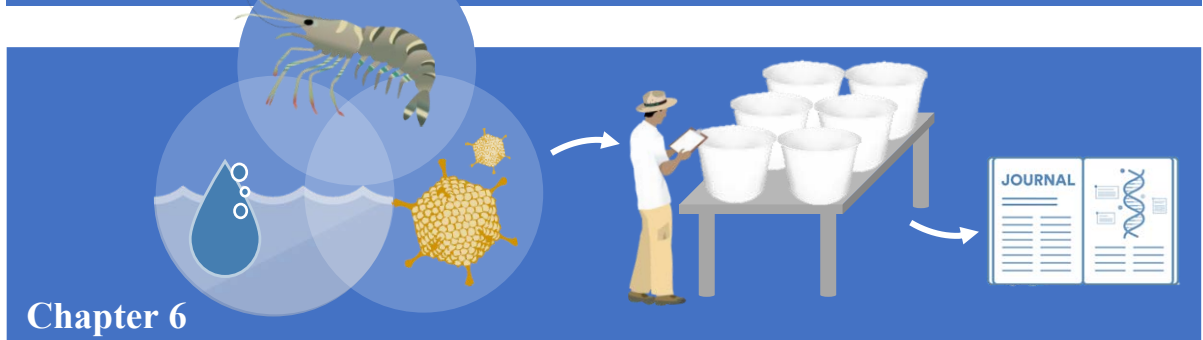
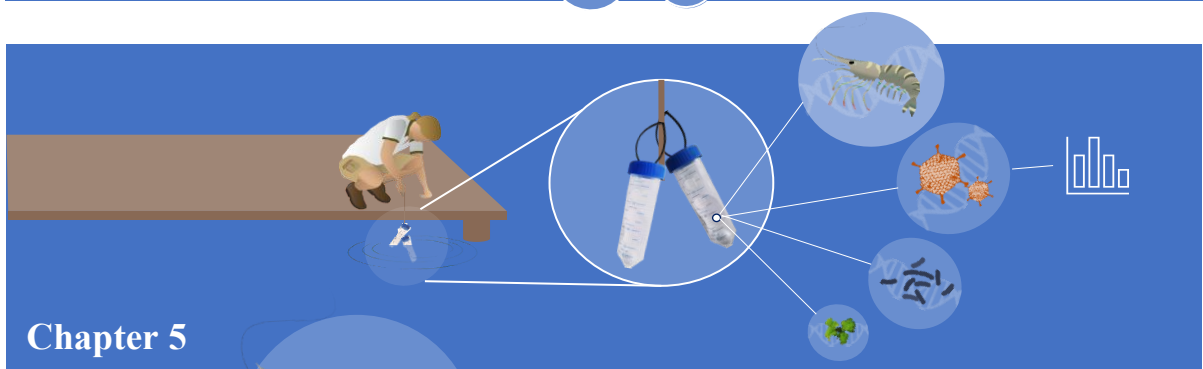
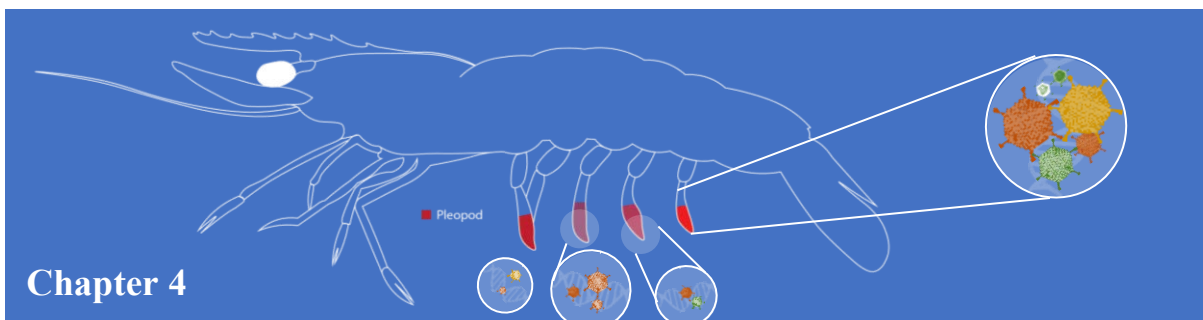
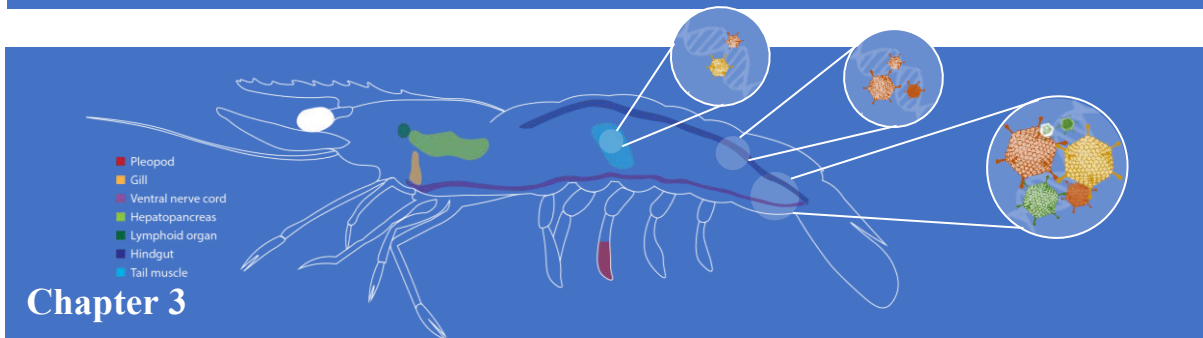
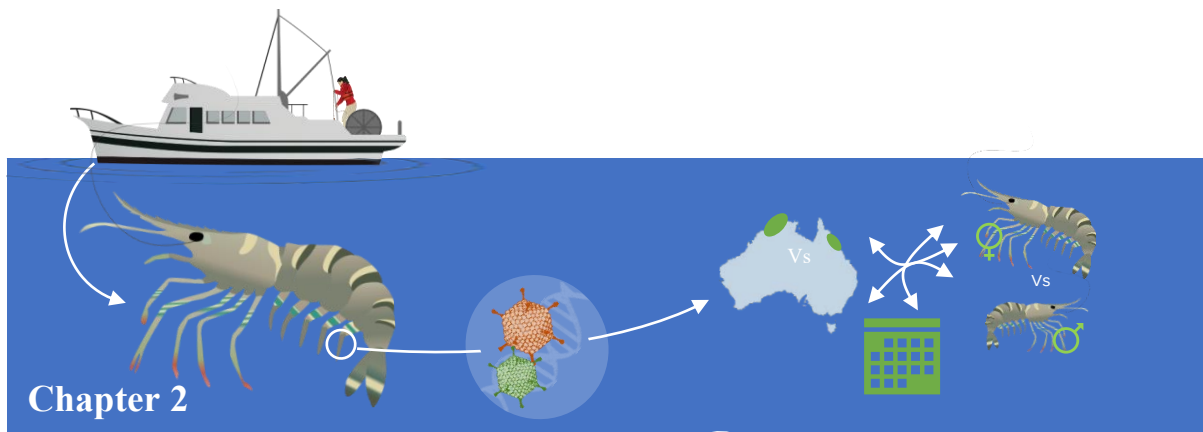
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ABSTRACT

Infectious aquatic diseases are responsible for billions of dollars in losses per year across global aquaculture. Shrimp aquaculture faces particularly severe economic and social impacts due to disease. Addressing and overcoming the challenge of disease is pivotal to facilitate the sustainable growth of the farmed shrimp industry. The tenet ‘prevention is better than cure’ applies to the control of all infectious diseases and is particularly relevant to viral disease management in shrimp aquaculture, where no effective treatments are currently available. Within Australia, a range of endemic viruses impact shrimp production, and the threat of exotic pathogen incursion is pervasive. As such, the Australian shrimp farming industry demands a strongly precautionary approach to biosecurity. Achieving effective biosecurity requires reliable, timely, accurate and cost-effective pathogen detection, in addition to a robust understanding of the host, pathogen and environmental dynamics that manifest in disease. Leveraging this capacity and understanding will support improved biosecurity and sustainable systems-based disease management. Currently, the necessary understanding and resources required to facilitate this advancement are lacking. As such, the research outlined in this thesis aimed to develop foundational knowledge and resources required to improve the detection and management of viral pathogens within Australian giant black tiger shrimp *Penaeus monodon* aquaculture. Accordingly, the research endeavour applied contemporary methods for detecting key pathogens of commercial interest to the Australian shrimp industry, to define the viral landscape of wild-sourced broodstock, modernise our understanding of viral tissue tropism to optimise tissue sampling, examine the reliability of industry-standard viral detection practices, and validate a novel environmental sampling approach with enhanced potential for large-scale pathogen surveillance and monitoring programs. Finally, the findings reported in this thesis guides future research on shrimp pathogens using challenge experiments towards improved robustness and translation to real-world advancement in shrimp disease management.

The Australian *P. monodon* aquaculture industry remains heavily reliant on wild-sourced broodstock for seedstock production, representing a large and under evaluated biosecurity risk. In the most comprehensive investigation to date, the viral landscape of wild-sourced *P. monodon* broodstock, collected from the two primary sources of supply for the Australian industry was defined. Using TaqMan quantitative polymerase chain reaction (qPCR), shrimp broodstock (N = 7472) were analysed for the detection of six commercially relevant viral pathogens. Analysis revealed nearly half of all wild-sourced broodstock were positive for the

detection of at least one viral target, and multi-virus infections were common. Two endemic pathogens associated with significant productivity losses in *P. monodon* aquaculture, namely infectious hypodermal haematopoietic necrosis virus (IHHNV) and gill-associated virus (GAV), were the most prevalent of the viruses detected (30% and 28.1%, respectively). Although at markedly lower prevalence, the highly pathogenic yellow head virus-7 (YHV-7) was also detected, demonstrating wild-sourced broodstock as a high-risk viral introduction pathway into farm systems. Geographic source, sex and year of collection were found to each significantly influence patterns of viral presence and loading, including significantly higher viral prevalence in broodstock sourced from the East coast of Queensland, compared to those from the Northern Territory. This analysis has equipped the Australian industry with evidence to adopt a more risk-averse broodstock collection strategy and support the industry's ongoing pursuit of endemic, specific pathogen free (SPF) domestication programs.

To develop understanding of host-pathogen dynamics, and critically, modernise tissue sampling strategies for optimised molecular detection, contemporary evidence of viral tissue tropism for IHHNV, GAV and YHV-7, was established. TaqMan qPCR was used to define tissue-specific distribution patterns of viral pathogens in two cohorts of naturally infected *P. monodon* ($n = 20$; 36.7 ± 7.8 g). IHHNV was found to preferentially target gill tissue, followed sequentially by hindgut, pleopod, hepatopancreas, lymphoid organ, ventral nerve cord and abdominal muscle. For the yellow head viruses (GAV and YHV-7), no significant differences in viral loading between tissues were observed. The viral distribution patterns observed here extend upon those previously described and offer updated evidence by leveraging the greatly enhanced detection capacity of modern molecular methods. Notably, the findings indicate strong detection of IHHNV from shrimp hepatopancreas, which was previously not considered an appropriate tissue for the detection of IHHNV. These findings offer clear supporting evidence for an improved tissue sampling strategy, and revisions to appropriate target tissue recommendations within global diagnostic standards (e.g. WOAHA Manual of Diagnostic Tests for Aquatic Animals).

Pathogen screening using qPCR is routinely conducted on shrimp pleopods given they are easily sampled, non-destructive and individually traceable. Despite their frequent use, few studies directly validate their suitability for accurate pathogen detection, particularly regarding the relationship of detection between pleopods and other tissues, as well as the consistency of

detection between multiple pleopods of the same animal. To address these gaps, TaqMan qPCR detection of IHHNV and GAV was compared between hepatopancreas, gill and five pleopods sampled from naturally infected *P. monodon* individuals ($n = 44$; 19.2 ± 4.9 g). Additionally, statistical sampling simulations using qPCR detection data were used to evaluate the balance between the extent of biological and technical replication and improvement in detection reliability. IHHNV detection between pleopods of the same shrimp was found to be consistent ($CV = 0.54 \pm 0.41$) with strong linear correlation to detected loading from gill ($R^2 = 0.87$) and hepatopancreas ($R^2 = 0.77$). Comparatively, detection of GAV was significantly more variable between pleopods of the same shrimp ($CV = 0.88 \pm 0.60$), and detection correlation between pleopods and gill ($R^2 = 0.39$), or hepatopancreas ($R^2 = 0.14$), were low. However, since infection progression was not controlled for, appropriate caveats should be considered when interpreting this analysis. Nevertheless, for shrimp with naturally acquired low viral titre, the results suggest that analysis of IHHNV and GAV from a single pleopod may yield false negative results. Therefore, sampling and analysis of multiple (2-3) pleopods are recommended to improve the reliability of such detections and pleopods should be tested with adequate technical replication (duplicate or triplicate tests) to overcome the Poisson distribution limitation of qPCR analysis. The balance between improved data quality and the associated increases in sampling effort and costs must be critically evaluated. Further research is needed to clarify the relationships between detected viral loads of GAV and IHHNV and their impacts on productivity and shrimp health to facilitate this evaluation.

In the pursuit of a simpler and more cost-efficient method to support large-scale on-farm pathogen surveillance and monitoring, the utility of a novel passive environmental sampling approach was investigated. Specifically, the capacity for passively collected environmental samples to detect and provide a proxy measurement of shrimp host IHHNV and GAV infections was assessed. Across a commercial farm, *P. monodon* tissue samples ($n = 39$ shrimp/pond) and environmental samples ($n = 2$ /pond) were collected from earthen shrimp production ponds ($n = 31$) for comparison. The optimal deployment duration for the passive sampling devices was also investigated using a pilot study in commercial *P. monodon* ponds ($n = 5$). Analysis using qPCR revealed that both IHHNV and GAV could be detected from passively collected environmental samples. Quantitative environmental detection of GAV did not show concordance with host tissue detection, and the possible causes for this were discussed. However, environmental IHHNV quantifications were highly correlated with those

detected from shrimp tissue collected from the same ponds ($R^2 = 0.82$). Environmental IHHNV detection indicative of host infection levels could be obtained with only 1 h of passive sampler submersion. With increasing submersion time, both IHHNV and GAV loading detected from eNA samples increased, indicating that longer submersion (24 h) of passive samplers may be more suitable for surveillance of rare or low-level infections. These findings provide strong evidence to support further application of passive environmental sample collection for efficient, accurate and cost-effective pathogen monitoring in aquaculture production systems and provides insight for optimised sampling strategies.

Finally, to support the ability of future research to make progressive advancements towards achieving ‘systems-based’ disease management, a review was conducted on key factors relating to the host (shrimp), pathogen (virus), and environment which have evidence to support their influence on disease outcomes. Borne from the host pathogen and environmental components identified, a checklist was developed. The checklist describes minimum information to consider in the design and reporting of pathogen challenge experiments to ensure key influences on disease outcomes are adequately considered and documented. Using this checklist, 186 viral challenge studies on *Penaeus monodon* published between 1997 and 2023 were critically evaluated, highlighting a concerning paucity of reported experimental detail in the existing literature. Future adoption of the checklist is envisaged to empower researchers, reviewers, and end-users to assess the internal and external validity of PCEs, facilitating critical evaluation and improved utility of findings for contemporary disease management.

In summary, the research comprising this thesis provides essential support for improved biosecurity and disease management across multiple aspects of Australian shrimp aquaculture. Critically, it delivers the most comprehensive dataset available to guide the ongoing wild sourcing of *P. monodon* broodstock, reducing the likelihood of viral introduction into production systems. This research also modernises our understanding of tissue-specific viral distribution patterns, ensuring appropriate and efficient tissue sampling for accurate detection. A major contribution of this research is the development of a novel environmental sampling approach, poised to greatly advance the scope of pathogen surveillance and monitoring within the industry. By summarising and synthesising the existing literature, a keystone set of guidelines to strengthen and support future research in the field has been provided. The

evidence, insights, tools, and resources developed and detailed herein have addressed significant gaps in our knowledge and capability related to detection and surveillance of shrimp pathogens. However, further research is required to elucidate the impact of endemic viral pathogens on *P. monodon* health and productivity, to enable biological relevant interpretation of the ever-increasing complexity of data available. Nevertheless, the research comprising this thesis advances our understanding of the detection and dynamics of viral pathogens in farmed shrimp and proposes how best to apply this understanding to advance biosecurity within both Australian and global shrimp aquaculture.

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ABBREVIATIONS

AHPND	Acute hepatopancreatic necrosis disease
ANOVA	Analysis of variance
Ct	Cycle threshold value
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOC	Days of culture
dph	Days post hatch
EC QLD	East-coast Queensland
EHP	Enterocytozoon hepatopenaei
EMS	Early mortality syndrome
g	Grams
GAV	Gill-associated virus
HDV	Hepandensovirus, synonymous with HPV
HG	Hindgut
HP	Hepatopaneas
HPV	Hepatopancreatic parvovirus
IHHNV	Infectious hypodermal and haematopoietic necrosis virus
IMNV	Infectious myonecrosis virus
ISO	International Organization for Standardization
L	Litre
LO	Lymphoid Organ
MBV	Monodon Baculovirus
μL	Microlitre
μM	Micromolar
mg	Milligrams
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MISA	Minimum Information required to support a Stimulant Assessment experiment
mL	Millilitre
MW	Molecular weight
NCBI	National Center for Biotechnology Information
NER	Ventral nerve cord
NPD	No pathogens detected
NT	Northern Territory
OIE	Office International des Epizooties, synonymous with WOA
PBS	Phosphate buffered saline
PCE	Pathogen challenge experiment
PCR	Polymerase chain reaction
PLEO	Pleopod
PstDV1	<i>Penaeus stylirostris</i> penstyldensovirus 1, synonymous with IHHNV

qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
s	Seconds
SE QLD	South-east Queensland
SNP	Single nucleotide polymorphism
SPERG	Shrimp PCE Reporting Guidelines
SPF	Specific pathogen free
SPR	Specific pathogen resistant
SPT	Specific pathogen tolerant
TEM	Transmission Electron Microscopy
TNA	Total nucleic acid
TSV	Taura syndrome virus
UV	Ultraviolet (light)
WOAH	World Organisation for Animal Health
WSSV	White spot syndrome virus
YHV-1	Yellow head virus genotype 1
YHV-2	Yellow head virus genotype 2, synonymous with GAV
YHV-7	Yellow head virus genotype 7

Chapter 1.

General Introduction

1.1 Aquaculture and food security

The average state of global marine fisheries is poor or declining, with many stocks predicted to experience further depletion under current management practices (Costello et al., 2020, 2016; Ritchie and Roser, 2021; Worm, 2016; Worm et al., 2009). Given this context, aquaculture has been the predominant contributor in meeting the rising demand for seafood generated by the doubling in global per capita fish consumption since 1960 (Mair et al., 2023; Stentiford et al., 2012). Aquaculture is defined by the Food and Agriculture Organization of the United Nations (FAO) as the farming of aquatic organisms including fish, molluscs, crustaceans, and aquatic plants, and currently is the fastest growing food production sector globally (FAO, 2022). Compared to land-based production of animal proteins, farming of aquatic animals is more resource efficient (Froehlich et al., 2018; Tsakiridis et al., 2020), with many aquatic species providing greater nutritional value at a lower environmental impact than terrestrial animal species (Bianchi et al., 2022). Therefore, aquaculture is uniquely positioned to significantly contribute to future food and nutrition security amidst global resource challenges (Costello et al., 2020). However, the realisation of this potential will depend on overcoming critical production challenges, including the impact of disease (Cain, 2022; Nguyen, 2024).

Infectious aquatic diseases are responsible for billions of dollars in losses per year across global aquaculture (Kumar et al., 2021b; Lafferty et al., 2015; Shinn et al., 2018; World Bank, 2014). The impacts of disease include losses from stock mortality, cost of disease control, loss of production or trade opportunity in the wake of disease outbreaks, and in yield-limiting effects such as growth disorders and deformities (Stentiford et al., 2017; Valappil et al., 2021). The emergence of diseases and their spread to naïve production regions has led to devastating collapses in aquaculture production with significant implications for regional economies, livelihoods, and the stability of global seafood trade (Asche et al., 2010; Shinn et al., 2018). Whilst the impacts of disease are felt across the diversity of aquaculture production industries, the magnitude of impacts are more pronounced in some species. For example, infectious diseases have particularly severe economic and social impacts for global shrimp production, where total losses may exceed 40% of global capacity (Stentiford et al., 2017), and where production has collapsed in several countries without recovering due to disease events (Asche et al., 2021). Therefore, addressing and overcoming the challenges of disease in aquaculture is pivotal to facilitate the sustainable growth of the sector.

1.2 Global shrimp aquaculture

Historically, shrimp have been among the most extensively traded seafood commodities, dominating the global production of crustaceans (FAO, 2022). Farming of marine shrimp began in the mid-20th century, with large-scale production increasing in the 1970's (Benzie, 2009; Stentiford et al., 2012). Early production relied on collection and on-growing of wild genetic resources and focused on a range of Penaeid species native to their global regions of production, including *Fenneropenaeus chinensis* (Osbeck, 1765), *Fenneropenaeus indicus* (Milne-Edwards, 1837), *Fenneropenaeus merguensis* (de Man, 1888), *Litopenaeus stylirostris* (Stimpson, 1874), *Marsupenaeus japonicus* (Bate, 1888), *Litopenaeus vannamei* (Boone, 1931), and *Penaeus monodon* (Fabricius, 1798). The developing industry rapidly grew with the advancement of culture practices in tandem with intensification and expansion of production area (Benzie, 2009; Kumar and Engle, 2016). However, beginning in the 1980s, the successive emergence and subsequent spread of diseases in shrimp aquaculture increasingly challenged production and prompted more focus on improving the industry's resilience to disease impacts (Walker and Mohan, 2009). Such advancements included improved biosecurity throughout production, movement away from wild genetic resources and towards domestication, and the development of breeding programs targeting the production of pathogen free and/or resistant stocks.

Initial efforts to domesticate shrimp proved successful with *L. vannamei*, given the species' adaptability to captive breeding, tolerance to higher intensity culture conditions and favourable rapid growth (Briggs et al., 2005; Lightner et al., 2012). Commercial genetic improvement programs with domesticated *L. vannamei* began in the USA during the late 1980s, with focus on the establishment of specific pathogen free (SPF) stocks. Pathogens targeted in SPF programs can be program-specific, but generally include at least those listed in the World Organisation for Animal Health (WOAH), formally Office International des Epizooties (OIE), Aquatic Animal Health Code. The WOAH listed diseases relevant to Penaeid production currently include acute hepatopancreatic necrosis disease (AHPND), and infections with decapod iridescent virus 1 (DIV-1), *Hepatobacter penaei*, infectious hypodermal and hematopoietic necrosis virus (IHHNV), infectious myonecrosis virus (IMNV), taura syndrome virus (TSV), white spot syndrome virus (WSSV) and yellow head virus genotype 1 (YHV-1) (WOAH, 2023a). The WOAH listed diseases comprise those which have had the most significant impact on global production. In parallel to the developing SPF programs, several

breeding programs were instigated across Latin America, with focus on establishing locally adapted, specific pathogen resistant (SPR) and tolerant (SPT) *L. vannamei* stocks (Alday-Sanz et al., 2018). The domestication and subsequent development of selectively bred SPF and SPR/SPT *L. vannamei* afforded significantly enhanced productivity (see section 1.4). As a result, many shrimp production systems converted farming of native species to domesticated *L. vannamei* (Alday-Sanz et al., 2018). Global aquaculture of *L. vannamei* has since grown from 155,000 tonne in 2000, to 5.8 million tonne in 2020, and now accounts for over 80% of global marine shrimp production (FAO, 2022).

As SPF *L. vannamei* production rapidly increased, capitalising on economies of scale, production of the previously higher farmed volume species, *P. monodon*, plateaued (Asmild et al., 2024). Between 2000 and 2020, the *P. monodon* industry only increased from 631,000 tonne to 717,000 tonne (FAO, 2022). Persistent challenges in successfully reproducing and rearing *P. monodon* under captive conditions has hampered domestication and industry development efforts for the species (Arnold et al., 2013; Coman et al., 2007b; Coman and Preston, 2008; Goodall, 2017). Currently, only a small number of *P. monodon* domestication programs have been established to supply commercial SPF seedstock, including those in Hawaii ([MOANA](#)), Madagascar ([UNIMA](#), formerly AQUALAMA) and Thailand ([CP Foods](#)), and the capacity of such suppliers remains limited. Therefore, many *P. monodon* production systems continue to heavily rely on wild-sourced broodstock. Nonetheless, advancements in domestication and selective breeding of *P. monodon*, coupled with rising market saturation and emerging disease issues (notably *Enterocytozoon hepatopenaei*; EHP) affecting *L. vannamei*, are fuelling considerable global interests to revive *P. monodon* production and domestication efforts (Fletcher, 2022; Lutz, 2023).

1.3 Disease in global shrimp aquaculture

Since its inception, the shrimp aquaculture industry has contended with, and evolved in response to, persistent challenges from a diverse array of pathogens including viruses, bacteria and parasites (Stentiford et al., 2012). Impacts of disease on shrimp production are considered among the most significant across the aquaculture sector (Asche et al., 2021). While advancements in production techniques and the availability of SPF/SPR/SPT seed supply has enabled substantive growth of global shrimp production, disease continues to have significant impact on the productivity and sustainability of the industry (Kumar and Engle,

2016; Shinn et al., 2018; Stentiford et al., 2012). Although aggregated global shrimp production values generally depict a steadily growing industry, annual production values from individual countries demonstrate significant fluctuations reflecting the episodic emergence and outbreak of disease (Kumar and Engle, 2016; Shinn et al., 2018). In some instances, annual yield metrics from individual producer countries document the collapse of production and subsequent failure to recover after significant disease outbreaks. Such collapses have manifested from intensification of existing infections and the emergence of previously unknown diseases, exemplified by the boom-and-bust cycle of Taiwan's *P. monodon*, and later *P. japonicus* farmed production (Asche et al., 2021; Kumar and Engle, 2016; Lo et al., 2003). Among the causative disease agents, viral pathogens are attributed to the most significant losses in shrimp production. Most notably, white spot syndrome virus (WSSV) is alone responsible for an estimated 10% loss in production output annually, and cumulative losses exceeding US\$15 billion since its emergence in 1992 (Lightner et al., 2012; Stentiford et al., 2012).

Impacts of disease on shrimp production have long been recognised as the result of a complex interplay of multiple biological, environmental and agrarian variables (Walker and Mohan, 2009). Contributing to this challenge is the unprecedented abundance of genetically diverse viruses that infect invertebrate arthropods, including shrimp (Li et al., 2015; Porter et al., 2019). Such viruses can have large host ranges, including insects, molluscs and crustacea, and can interact to exchange genetic information within their hosts (Pradeep et al., 2012; Yu et al., 2021). These characteristics promote the emergence and transmission of viral pathogens among a diversity of arthropod species (Li et al., 2015). Arthropods are the largest of the animal phyla and are abundant in almost all ecosystems, especially within marine and estuarine environments (Eisenhauer and Hines, 2021). With over 90% of shrimp aquaculture conducted in tropical coastal regions within marine and brackish-water earthen ponds (Boyd et al., 2022), shrimp farming is highly connected to the external environment including adjacent and/or direct connection to marine and estuary systems. Given this connectivity, pond-based shrimp farming is exposed to an abundance of arthropod species which inhabit the surrounding environment or water supply (Boyd et al., 2022; Krol et al., 2024; Nielsen et al., 2023; Stentiford et al., 2012). This exposure increases the probability of disease impacts in farmed populations, as susceptible species within the environment can act as pathogen reservoirs or vectors, facilitating transmission and recurrent infection in ponds (Raj et al., 2023; Small and Pagenkopp, 2011). Furthermore, the pond-based systems in tropical coastal regions are subject

to extreme equatorial weather conditions. Dramatic fluctuations, or sustained suboptimal physicochemical pond culture conditions, have significant impacts on the susceptibility of shrimp to disease, thus increasing the risk and severity of disease outbreaks (Millard et al., 2021). Shrimp farms are also commonly situated in close proximity to one another, with direct or indirect connectedness via common water sources, genetic resources or shared equipment (Patanasatienkul et al., 2023). This connectivity facilitates rapid transmission of pathogens between shrimp farms, amplifying the potential impact of disease on production. Regarding the transmission of shrimp viruses more broadly, increasing global trade of seafood and shrimp stocks with insufficient biosecurity regulation has facilitated transboundary distribution of pathogens, resulting in rippling globalised impacts to production and natural environments (Hulme, 2021; Stentiford et al., 2012).

1.4 Managing viral disease in shrimp aquaculture

The management of shrimp health and disease through appropriate farm design, the provision of adequate nutrition and management of optimal environmental conditions to reduce stressors are essential components of successful shrimp farming (Lightner, 2006; Millard et al., 2021). With this said, discussion herein will focus on the management of infectious diseases in shrimp aquaculture beyond the maintenance of ‘best-practice’ animal husbandry and farm management practices.

While prophylactic and therapeutic measures are common in modern aquaculture of vertebrate species, despite extensive research, there are no effective practical prophylaxis or treatments for viral disease in shrimp aquaculture. Vertebrates are equipped with both innate (i.e. physical, humoral and cellular defences) and adaptive clonotypical immunity (i.e. T-cells, B-cells and antibodies). Vertebrate adaptive immunity enables the development and maintenance of a long-term, pathogen-specific immune “memory” within the host from previous pathogen exposures. This mechanism facilitates more efficient immune responses upon subsequent exposures and is the basis of traditional vaccination approaches. Vaccines have been broadly successful for disease prevention in many vertebrate aquaculture species, such that modern finfish aquaculture is highly dependent on routine vaccination against a range of pathogens (Mondal and Thomas, 2022). Invertebrate species lack the molecular machinery characteristic of the adaptive immune systems found in vertebrates (Ottaviani, 2011). Therefore, traditional

vaccination approaches, such as those used in finfish production, are not viable for long-term protection against viral disease in shrimp production (Johnson et al., 2008).

There is developing evidence of the diverse and sophisticated capacities within invertebrate immunity which may afford specific and transgenerational immune ‘memory’ (Flegel, 2020; Norouzitallab et al., 2019; Roy et al., 2020; Taengchaiyaphum et al., 2021a). While theories regarding the mechanisms behind these immune capacities are evolving, the characterisation of invertebrate immune ‘memory’ systems is still nascent. Therefore, the development of practical disease prevention approaches targeting invertebrate immune ‘memory’ have not yet achieved consistent demonstrable success and commercial availability. Similarly, research and innovation in novel technologies such as immunostimulants, bioactive compounds and nanotechnologies are also providing initial promise (Seethalakshmi et al., 2021). However, significant further research and robust commercial validation is required before they will be capable of providing significant benefit to disease prevention and treatment in global shrimp aquaculture.

In the absence of vaccination or the availability of therapeutics, the shrimp production industry relies upon a number of alternative approaches to prevent and respond to shrimp disease, including the use of SPF and SPR shrimp, and the adoption of biosecurity practices throughout all phases of production (Asche et al., 2021; Moss et al., 2012; Nguyen, 2024). As previously described (see section 1.2), closure of the shrimp production cycle and development of SPF genetic improvement programs has facilitated sustained commercial success of *L. vannamei* production in the face of persistent disease challenges. Such programs enable improved disease resilience via numerous modes of action, including; i) captively breeding domesticated shrimp alleviates challenges related to the sporadic supply, inconsistent quality, and introduction of pathogens onto farms with wild-sourced stock, ii) use and dispersal of SPF stock reduces the likelihood of pathogen introduction onto receiving farms, iii) stocking SPF post-larvae affords an extended period free from exposure to pathogens throughout the growing cycle, iv) genetic improvement of domesticated stock can target heritable pathogen resistance or tolerance traits, in addition to production traits and, v) improving production traits, such as growth rate, reduces the length of the growing cycle, thereby, minimising the duration and risk of potential pathogen exposure during the grow-out phase. While the implementation of domestication and selective breeding programs have demonstrated success for improved pathogen resilience, these

approaches are technically complex and require significant time and financial investment (Moss et al., 2012).

In lieu of widespread established domestication and selective breeding programs, biosecurity regulations and practices serve as the foundation to shrimp disease management. These regulations and practices are applied across different scales. For example, at the national level, importation regulations implemented by relevant authorities aim to provide protection against incursion of exotic pathogens via imported goods, including certain live aquatic animals and seafood products (DAF, 2023a). Such mechanisms are particularly relevant to countries such as Australia where major shrimp diseases including YHV-1, EHP, DIV and AHPND are still considered exotic, and introduction of exotic pathogens to Australian shrimp populations via contaminated imported seafood is plausible (Scott-Orr et al., 2017) (also see section 1.5.1). At the enterprise level, on-farm biosecurity practices are adopted to mitigate pathogen introduction and spread within a farm (APFA, 2023; Diggles, 2022). Such practices include routine sanitation, zoning of production areas to specific staff and equipment, stock quarantine and treatment of intake water including filtration, ozonation and UV sterilisation.

The tenet ‘prevention is better than cure’ applies to the control of all infectious diseases and is particularly relevant to viral disease management in shrimp aquaculture, where no effective treatments are currently available. Domestication, use of SPF stocks, selective breeding, biosecurity policy and adoption of on-farm biosecurity practices all contribute to prevention. However, even the best management strategies do not eliminate the risk of pathogen exposure or guarantee protection from disease outbreak. Thus, effective disease mitigation requires reliable, accurate and cost-effective pathogen detection and diagnostics (see section 1.4.1). Early pathogen detection enables effective implementation of control strategies to minimise the transmission and impact of infection (Kumar et al., 2021b; Lightner, 2006; MacAulay et al., 2022).

1.4.1 A central pillar of biosecurity: Pathogen detection

Applications of biosecurity and disease management within shrimp aquaculture critically depend on the availability of accurate, reliable and cost-effective methods to determine the presence and quantity of infectious disease agents (pathogens) (Lightner, 2006). The presence of pathogens may be determined within a *surveillance* or *diagnostic* scope, depending on the

context of application. This context may include the detection methods applied, the type and source of the sample, the susceptibility and health status of the target population, and the epidemiological implications of detection (WOAH, 2023a). Understanding of this context is essential for appropriate interpretation of test results, as the detection of an infectious agent does not inherently equate to disease diagnosis. A brief clarification of surveillance and diagnostic scopes and their context for usage is provided below.

Pathogen screening methods are often selected to be highly sensitive, such that the tests correctly designate samples with the target pathogen present as being positive, producing very low false negative rates (i.e. target presence is rarely missed) (Maxim et al., 2014). The application of sensitive screening methods is therefore essential for surveillance and early detection of disease or risk factors for disease, including determining or monitoring pathogen presence in apparently healthy populations, in the absence of overt disease or clinical signs. In such cases, results may be used to inform proactive disease management strategies to reduce the potential transmission and impact of disease in the screened population, and populations which may be susceptible to the pathogen. For example, pathogen screening may be used for evaluating the pathogen burden of wild broodstock to inform their suitability for spawning, or for pathogen surveillance in wild crustacea to assess their potential as pathogen reservoirs or vectors into commercial production ponds. Surveillance efforts may be targeted to a specific disease or infection (e.g. for establishing freedom from a specific pathogen), or passive (e.g. general early pathogen detection including health observations) (WOAH, 2023a).

Diagnostic (*syn.* confirmatory) methods are required to definitively establish the cause of a disease following observation of clinical signs, or to confirm the presence of a specific pathogenic agent. Diagnostic methods are highly specific, providing correct designation of a sample without the target pathogen present as negative. Therefore, diagnostic methods have low rates of false positive detection and thus provide confidence to base further management strategies upon. Diagnostic methods are often applied to provide confirmation subsequent to positive detection from screening methods, especially for pathogens of high consequence where confirmed infection would trigger the induction of significant or costly management actions (WOAH, 2023a).

Ideally, the determination of pathogen presence should be based on methods that are both sensitive and specific, in addition to being timely and cost effective (MacAulay et al., 2022). Under such conditions, surveillance methods may be congruent with recommended diagnostic methods (WOAH, 2023a). Observation of clinical signs can be used to indicate the presence of sub-optimal conditions or infectious disease. However, many observable features of viral infection in shrimp (e.g. lethargy, abnormal increases or cessation of feeding, surface swimming, body discolouration, slow growth or mortality) are not specific to any single pathogen. Additionally, unlike monitoring of disease in terrestrial livestock, monitoring of aquatic stock is challenged by water quality factors, such as turbidity, impeding or reducing the sensitivity of visual inspection for observing clinical signs of disease (MacAulay et al., 2022). Moreover, visual clinical signs often present once significant infection or disease onset has already occurred, minimising the viability of this method for providing value to preventative disease management. Detection of shrimp pathogens can be more reliably achieved using laboratory-based detection methods (e.g. microscopy, histology, immunohistochemistry and, molecular detection) (Kumar et al., 2021b; MacAulay et al., 2022). Standardised protocols for the application of such methods for detection and diagnosis of listed aquatic pathogens are outlined within the WOAHA Manual of Diagnostic Tests for Aquatic Animals (WOAH, 2023b). These methods are evaluated based on key metrics, including; analytical sensitivity (AS_e; the smallest amount of analyte in a sample that can be detected by an assay with a specified level of certainty), analytical specificity (AS_p; the ability of an assay to differentiate the target analyte from other components in the sample matrix), diagnostic sensitivity (DS_e; the proportion of known infected reference animals correctly identified as positive by an assay), diagnostic specificity (DS_p; the proportion of known uninfected reference animals correctly identified as negative by an assay), availability, cost, timeliness, scalability of throughput and operability (WOAH, 2021a). The suitability of a test method, for a given application, based on these metrics is referred to as being “fit-for-purpose”. The array of pathogen detection technologies outlined within the WOAHA Aquatic Manual are diverse, with varying degrees of specificity, sensitivity, availability, cost, timeliness, sample throughput and operability. Against these qualifiers, application of real-time quantitative polymerase chain reaction (qPCR) for molecular detection of viral shrimp pathogens is regarded as highly fit-for-purpose. Detection by qPCR is specific, sensitive and quantitative, enabling reliable and accurate determination of pathogen target presence/absence and quantification, and can be readily scaled for high throughput, rapid turn-around analysis. Owing to these factors, suitable

application of qPCR extends across surveillance of apparently healthy animals for early detection purposes, presumptive diagnosis in clinically affected animals and for some confirmatory diagnostic purposes (WOAH, 2023b). Accordingly, in contemporary shrimp aquaculture practices, viral pathogen detection and quantification are primarily determined via qPCR.

1.5 Australian shrimp aquaculture

Australian shrimp aquaculture represents a small proportion of global production, derived from approximately 16 farms, less than half of which produce ~80% of annual production volume (DAF, 2024; Stephens, 2019). The Australian industry principally focusses on farming the giant black tiger *Penaeus monodon*, and banana *Fenneropenaeus merguensis* shrimp species. While these species are recognised as “prawns” within Australia (FRDC, 2019), to maintain consistency, the term “shrimp” is used to describe Penaeid species within an Australian and international context herein. Largely established within Queensland, shrimp production has seen rapid growth within the last 5 years, expanding from a 4,630 tonne industry valued at AUD\$26.8 million in 2018-19, to 9825.5 tonnes worth AUD\$212.9 million in 2022-23 (DAF, 2024, 2023b). In 2023, aquaculture of *P. monodon*, the primary farmed species, increased further to an estimated 10,500 tonnes (Jory, 2023a).

Australian national biosecurity regulations, aligned with the *Australia Environment Protection and Biodiversity Conservation Act 1999*, prohibit the importation of live shrimp into Australia, including stock achieving SPF status (Perez, 2018). Thus, the only genetic resources available to the industry include wild populations, or shrimp produced from Australian-based domestication programs developed from endemic animals (Coman and Preston, 2008). Research efforts within Australia to facilitate the development of *P. monodon* domestication programs have been substantial (e.g. Arnold et al., 2013; Coman et al., 2007b, 2007a; Dixon et al., 2008; Goodall, 2016; Guppy et al., 2020; Noble et al., 2020; Vu et al., 2020), including a significant multi-partner, industry-led domestication research project (Preston et al., 2007). This project was successful in producing domesticated lines and extending this research to industry participants. However, during the 2016-17 white spot disease (WSD) outbreak caused by WSSV (see section 1.5.1), all domesticated stock within the program were destroyed as part of the ensuing emergency response (Stephens, 2019). Recent large-scale investment within the industry, especially from [Seafarms Group Limited](#) and [Tassal Group Limited](#), has driven

developments towards establishing enterprise-specific *P. monodon* domestication programs (SFG, 2023, 2022, 2021; Tassal Group Limited, 2022). However, more broadly, and throughout the developmental phases of these programs, the industry continues to rely on wild-sourced *P. monodon* broodstock (Stephens, 2019).

1.5.1 Pathogens in Australian shrimp aquaculture

Australian wild and cultured stocks of *P. monodon* remain free from many of the significant pathogens which have caused collapse of shrimp farming industries abroad. Currently, Australia is considered free from YHV-1, TSV, EHP, AHPND and DIV-1. Australia has held an inconsistent status of freedom from WSSV, since 2000. In 2000, WSSV was detected in mud crabs *Scylla serrata* and *P. monodon* cultured within a Darwin aquaculture facility (East et al., 2004). Imported commodity shrimp used as feed were determined to be the source of the virus. Following an intensive biosecurity response, and a subsequent national survey for WSSV, Australia returned to WSSV freedom status at the time. However, in 2016, WSSV was detected in Australia again in shrimp farms located on the Logan River in Southeast Queensland (SE QLD) (WOAH notification ID [IN_10080](#)) (Moody et al., 2022). Similar to the Darwin incident, WSSV infected, imported commodity shrimp were suspected to also be the source of the 2016-17 outbreak (Scott-Orr et al., 2017). WSSV infection and eradication efforts subsequently devastated shrimp farms within the SE QLD region, which was accordingly classified as a [Movement Regulated Area](#) (MRA). A portion of the affected farms resumed production in late 2018, with varying success (Jory, 2023b). Since the initial outbreak, national surveillance efforts detected the same lineage of WSSV in wild populations of crustaceans in SE QLD, in the Moreton Bay area and at two farms on the Logan River during 2020 (WOAH notification ID [IN_17432](#)). The surveillance efforts, concluding in 2020, substantiated WSSV freedom in areas outside of the MRA (DAWE, 2020a). However, in August 2022, WSSV was detected at a biosecure shrimp hatchery in Yamba, Clarence Valley, New South Wales (NSW) (WOAH notification ID [IN_156716](#)), and again in February, March and May of 2023 from three shrimp farms along the Clarence River of NSW (WOAH notification IDs [FUR_159457](#), [FUR_159586](#), [FUR_160667](#), respectively). Genomic analysis of the WSSV detected from the affected Clarence River farms concluded that each were identical to each other, and similar to the detection from the biosecure hatchery in August 2022, suggesting the two events were linked. The origin of WSSV in these cases is yet to be determined, although the NSW cases are known to be genetically distinct from the 2016-17 and 2020 WSSV lineage detected in SE

QLD, representing a separate WSSV incursion event. While the infection within the biosecure hatchery was considered eradicated shortly following its identification, the affected Clarence River region remains under a [NSW biosecurity control order](#) and movement restriction zone until mid-2025 (Britton, 2023). The ensuing NSW WSSV surveillance program has most recently (Autumn of 2024) resulted in the detection of WSSV from wild-caught shrimp (*Metapenaeus macleayi*) from the inshore ocean area off Evans Head in NSW, approximately 40 km north of the 2022 and 2023 NSW detection sites (NSW DPI, 2024; WOA notification ID [IN_167297](#)). These detections are the first in NSW from wild shrimp, obtained directly from ocean waters and unrelated to a clinical WSD outbreak. A further [NSW biosecurity control order](#) and movement restriction zone has been placed on the Evans River region (Coombe, 2024), and genomic analysis is underway to determine its relation to the prior WSSV cases in NSW and SE QLD. For the Australian industry, similar future exotic pathogen incursion events or transmission of WSSV from wild hosts (i.e. within SE QLD and the northern NSW) to farmed populations represents an ominous and persistent danger.

Beyond the threat of exotic pathogens, Australian *P. monodon* stocks are also host to a range of enzootic viruses. Of the endemic viral pathogens commonly detected, several are associated with sub-optimal growth disorders of farmed shrimp (also referred to as runt deformity syndrome or monodon slow growth syndrome), including IHHNV, hepatopancreatic parvovirus (HPV) and Monodon baculovirus (MBV) (Valappil et al., 2021). Others, including gill-associated virus (GAV; genotype 2 of the yellowhead virus complex; YHV-2) and Mourilyan virus (MoV) have been associated with mortality syndromes (Oanh et al., 2011; Rajendran et al., 2006; Sellars et al., 2006; Spann et al., 1997). More recently, yellowhead virus genotype 7 (YHV-7) was discovered in Australian wild-sourced broodstock experiencing mortalities in 2012 (Mohr et al., 2015). Since its initial discovery in Australian stocks, YHV-7 has been associated with sporadic mass mortality events during pond-based grow out (Cowley et al., 2019). Similarly, bacterial outbreaks resulting in the production of the binary *Photobacterium* insect-related (Pir) toxins, PirA and PirB, have also been attributed to recent mass mortality events within hatchery (Condon et al., 2022) and grow-out (SFG, 2022) phases of Australian *P. monodon* production.

1.5.2 Pathogen management Australian shrimp aquaculture

With the range of endemic pathogens that impact production, and the ominous threat of exotic pathogen incursion, the Australian industry adopts a strongly precautionary approach to biosecurity. Propelled by the repeated WSSV events within Australia, focus on reviewing and strengthening Australian seafood import regulations has intensified to mitigate similar future exotic pathogen incursions (DAF, 2023a). Many Australian farms have also progressively increased adoption and stringency of practical on-farm biosecurity measures including staff and equipment zoning, treatment of intake water, reduction in water exchange, increased sanitation protocols and pathogen surveillance programs (Diggles, 2022, 2020).

Australia is a participant member of the World Trade Organization (WTO) and is obligated by the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS agreement) established in 1995. Within this framework, the WTO acknowledges WOA (formerly OIE) as the authoritative body for establishing international standards regarding sanitary measures for aquatic animals (WOA, 2023c). In meeting international obligations with respect to these measures, Australia maintains aquatic disease surveillance and reporting aligned with the standards published by WOA (DAWE, 2020b). Currently, legislated surveillance measures for domestic Australian shrimp production include confirmation of freedom of WSSV, YHV-1 and AHPND (PirAB toxin genes) by qPCR for translocation of wild or domesticated broodstock shrimp (DAF, 2018). Recognising the risks associated with epizootics, especially in light of recent endemic disease outbreaks (Condon et al., 2022; Mohr et al., 2015; SFG, 2022) some farms also opt to test stock for specific endemic pathogens, including YHV-7, IHHNV and GAV (APFA, 2023). Despite the potential for the collective testing being conducted across the industry to serve as a *de facto* large-scale disease survey, the data is not generally shared or aggregated amongst industry. Consequently, industry efforts and investment in pathogen testing is not leveraged to benefit understanding of the broader pathogen landscape to improve disease management strategy (e.g. for assessing spatiotemporal epidemiological patterns or evaluating and comparing wild populations for broodstock collection) (Stephens, 2019). Previous research has provided insight into the viral landscape of Australian wild stocks targeted for broodstock collection (summarised in Table 1.1). However, there is an acute need for more comprehensive data, collected within a contemporary timescale. This data would support the establishment of domestication programs founded by wild

resources targeting endemic and WOA listed pathogen freedom, or at minimum, enable strategic risk-averse collection of wild broodstock with lower viral burden.

Heightened biosecurity awareness arising from recent epizootics in Australia (Condon et al., 2022; Fensham et al., 2023; Mohr et al., 2015; SFG, 2022) has also increased the impetus for the Australian shrimp farming industry to integrate pathogen surveillance within the grow-out phase of production. Pathogen presence and prevalence in pond populations can be highly variable (Condon et al., 2020) and shrimp can tolerate single or multiple persistent infections in the absence of clinical signs (Arbon et al., 2022; Condon et al., 2020; Flegel, 2020), such that the presence of specific pathogens does not invariably result in disease outbreak. Given these complexities, understanding the interactions between the shrimp host, pathogen and culture environment, that drive disease manifestation from persistent or tolerated infections is critical for the effective prevention and management of disease (Arbon et al., 2024b). Currently, the lack of large-scale pathogen data throughout pond production represents a major limitation to elucidating these complexities. Beyond enabling early detection capability, the adoption of long-term farm-scale pathogen surveillance programs offers the opportunity to collect extensive pathogen detection and quantification datasets which can be aligned to farm productivity data. Alignment of these data sources, comprising detailed records of pathogen presence, prevalence and load, alongside key metrics of farm performance such as specific growth rates, feed conversion ratios, survival and harvest yield, and environmental parameters collected throughout the production period, yield integrated, farm-scale, pathogen-productivity datasets. Such datasets are a critical resource to understanding the sub-clinical impacts of infection(s) on production, trends in pathogen dynamics throughout production, and the system drivers of disease outbreak (Cahill et al., 2022). Farm pathogen surveillance should comprise routine sampling intervals across the entire system, employing a sensitive and reliable detection method. While select farms have begun integrating pathogen surveillance within production ponds and surrounding environments (Condon et al., 2020), the widespread adoption and maintenance of robust surveillance programs realising these characteristics largely remain cost prohibitive. Nonetheless, the potential losses incurred from disease outbreak due to absent or ineffective pathogen surveillance underscore the importance of both adopting and diligently maintaining effective early pathogen detection systems and understanding disease drivers throughout the production cycle. Thus, there is a critical need to ensure the accuracy and

reliability of surveillance methods, while simultaneously striving to achieve cost-efficiency for large-scale, routine farm implementation.

Modern advancements in molecular detection technologies, including nucleic acid extraction and qPCR, have yielded significant improvements in pathogen detection capabilities including detection sensitivity and reliability (Geraci-Yee et al., 2022; MacAulay et al., 2022). In this context, ‘reliability’ refers to the ability of a diagnostic test to consistently provide accurate detection results (i.e. true positives), within a specific sample matrix type. Accordingly, the adoption of modern pathogen detection technologies within both research and industry applications is increasingly widespread. However, in many instances, the rate of adoption of these technologies has outpaced the development of evidence to inform their optimal use. For example, despite the significantly improved detection sensitivity and reliability of modern molecular detection practices, evidence to determine appropriate target tissues to sample for such analysis are limited (Hou et al., 2023; Noble et al., 2018), or have not yet been established. In the absence of this supporting evidence, the efficacy of surveillance efforts may be reduced through sampling and analysis of suboptimal tissue types resulting in a loss of detection accuracy and reliability (Hou et al., 2023). Conversely, overlooking and non-usage of tissue types that were previously deemed unsuitable but are now recognised as appropriate for detecting a broader range of pathogens using modern methods (e.g. hepatopancreas for detection of IHHNV; Hou et al., 2023) may lead to the unnecessary analysis of multiple tissue samples, inflating the efforts and cost of surveillance. The paucity of supporting evidence to guide the application of modern detection tools is especially apparent for Australian endemic shrimp pathogens which have not received global research attention, or emerging pathogens which are not yet well characterised. Nevertheless, contemporary evidence, including descriptions of tissue-specific pathogen presence and quantification in *P. monodon*, is required to ensure surveillance programs are effective and can provide meaningful datasets to advance our understanding of the host, pathogen and environment dynamics.

Table 1.1. Summary of endemic viral target detections applying (q)PCR from previous studies assessing wild-sourced Australian *Penaeus monodon* collected for use as commercial broodstock. NEQ = North Eastern Queensland, GoC = Gulf of Carpentaria, NWA = North Western Australia, JBG = Joseph Bonaparte Gulf, NT = Northern Territory, IHHNV = infectious hypodermal and hematopoietic necrosis virus, HPV = hepatopancreatic parvovirus, MBV = Monodon baculovirus, GAV = gill-associated virus, YHV-7 = yellow head virus genotype 7, MoV = Mourilyan virus, When-2 = Wenzhou shrimp virus 2

Reference	Year collected	Origin	IHHNV	HPV ¹	MBV ¹	GAV	YHV-7	MoV	When-2
(Cowley et al., 2000)	1997-1998	NEQ				98% (57/58)			
		NEQ				97% (144/148)			
(Walker et al., 2001)	1997-1999	GoC				0% (0/4)			
		NWA				0% (0/12)			
		NEQ	7% (10/151)	25% (38/151)	1% (2/151)	72% (111/155)	2% (3/155)	43% (65/153)	
(Cowley et al., 2015)	2013-2014	GoC	10% (5/50)	14% (7/50)	2% (1/50)	16% (11/67)	1% (1/67)	3% (2/67)	
		JBG	3% (4/136)	5% (7/143)	0% (0/143)	27% (73/271)	9% (25/271)	3% (4/150)	
		NT	34% (162/475)	16% (78/475)		83% (393/475)	12% (57/475)		18% (84/475)
(Condon et al., 2020)	2018	NEQ	60% (293/489)	23% (115/489)		96% (467/489)	11% (52/489)		46% (226/489)

¹ Non-target tissue used for analysis

While improving the reliability and accuracy of pathogen detection from host tissue sampling approaches is critical to disease diagnosis, the cost-effectiveness of tissue sampling remains limiting for large-scale surveillance efforts. For example, to conduct a farm-scale pathogen monitoring program, which may aim to detect a target occurring at a prevalence of 10% with 95% confidence within large pond populations (e.g. 100,000 to 600,000 shrimp; Eid et al., 2020; Mena-Herrera et al., 2006; Zeng et al., 2020), at least 39 shrimp per pond must be sampled (<https://epitools.ausvet.com.au/ppfreedom>; assuming a reasonable test sensitivity of 0.8 for pooled tissue samples of 3 individuals). The sampling effort and cost of analysis, scaled for the number of ponds on the farm and repeated at regular intervals for the monitoring program to provide early detection capability and valuable temporal insights, rapidly becomes prohibitive for farms to implement (Brunner, 2020). Therefore, a more efficient, alternative sampling approach is required to facilitate large-scale pathogen surveillance and monitoring programs.

Environmental sampling approaches are gaining increased popularity for this purpose, as was demonstrated during the recent global COVID-19 pandemic for tracing community infections through sampling and analysis of human wastewater (Rafiee et al., 2021; Schang et al., 2021; Sikorski and Levine, 2020). Environmental samples, involving the collection of DNA and RNA (collectively eNA) shed into the water, soil or sediment, can be analysed in a similar manner to host tissue samples to provide evidence of pathogen presence within a system. Detections from eNA samples can act as a proxy for infection of host organisms within the system, following appropriate validation (Bass et al., 2023). Sampling methods utilising eNA can be 2 to 10 times more cost-efficient than traditional sampling, and can achieve high levels of detection sensitivity (MacAulay et al., 2022; Smart et al., 2016). Therefore, eNA sampling approaches have the potential to significantly enhance the capacity and scope for early detection and monitoring of pathogens in aquatic environments. Recent studies have successfully demonstrated the application of eNA for early detection and monitoring of viral pathogens and investigating host-pathogen dynamics in aquatic systems. Exemplar studies include those which have analysed infection and outbreak dynamics of ranavirus in wood frogs (*Rana sylvatica*) (Hall et al., 2018) and pertinently, iridovirus in red sea bream (*Pagrus major*) within aquaculture systems (Kawato et al., 2021). Shrimp aquaculture stands to benefit

significantly from the application and validation of analogous methodologies, including substantial advancements from expanding surveillance scope and cost effectiveness.

The success of contemporary disease management strategies in shrimp aquaculture is predicated on robust knowledge of the conditions and interactions between the host, pathogen and environment that promote disease (i.e. ‘systems-based’) (Flegel, 2019). Advancements in the effectiveness of pathogen detection, through the optimised application of modern molecular tools and adoption of emerging strategies such as environmental sampling, will improve the capacity for early pathogen detection and understanding of host-pathogen dynamics and drivers of disease outbreaks within production systems. Studying the outcomes of viral disease *in situ* is relevant to understanding the impact of disease under the complex influence and interactions of factors within the whole system (Noble et al., 2020b). However, in some cases, the complexity of disease as a manifestation of interactions within the system can limit the confidence and robustness of *in situ* studies. As a consequence, correlation-causal relationships between pathogen presence and the observed impacts on productivity or shrimp health cannot be justified (Munro et al., 2011; NACA, 2019; Valappil et al., 2021). Viral pathogens endemic to Australian *P. monodon*, including GAV and IHNV, have been inconsistently associated with productivity issues *in situ* (Montgomery-Brock et al., 2007; Sellars et al., 2019; Withyachumnarnkul et al., 2006). To date conclusive evidence to demonstrate such infections as the cause of sub-optimal growth or mortality in *P. monodon* is yet to be established. To facilitate the development of robust disease mitigation and management strategies, elucidation of these associations is required (Valappil et al., 2021).

Pathogen challenge experiments (PCEs) offer a powerful mechanism for elucidating such associations robustly. PCEs have been applied extensively with this aim for many important shrimp pathogens (Arbon et al., 2024b). However, in many instances, key studies have neglected to either consider, or accurately report, experimental details which would support the generation of robust and comparable experimental conclusions (e.g. analysis to eliminate other possible, causative pathogens within the experimental cohort or challenge inoculum). To address similar concerns, lateral fields have developed ‘best-practice’ guidelines to support quality consideration and reporting of experimental details for challenge trials, including within the fields of human medicine (Consolidated Standards of Reporting Trials; Moher et al., 2001) and livestock veterinary studies (Reporting Guidelines for Randomised Controlled Trials for

Livestock and Food Safety; O'Connor et al., 2010; Sargeant et al., 2010). Currently, no such guidelines are available for shrimp which has limited the capacity for previous research to make progressive advancements towards elucidating the impact of Australian enzootic shrimp viruses on *P. monodon* and contribute meaningfully to contemporary shrimp disease management strategies.

When considered together, the imperative for effective biosecurity systems to mitigate the impacts of disease on shrimp aquaculture and facilitate its continued and sustainable growth is clear. Global trends in pathogen spread and increasing disease pressures (Hulme, 2021; Samsing and Barnes, 2024; Valappil et al., 2021) underscore the necessity for increasingly robust, sensitive and cost-effective modern diagnostic and surveillance systems to effectively support the management of shrimp health and disease. Similarly, understanding of host-pathogen-environment dynamics and the drivers of disease outbreaks within production systems represents a critical requisite for successful contemporary disease management strategies targeting a 'systems-based' approach. The threats posed by significant endemic pathogens and the possibility for future exotic pathogen incursions foster significant impetus to address these needs within Australia. The strong commitment of the Australian shrimp (prawn) farming industry to not only adopt, but also continue to bolster and advance its biosecurity systems in response to this impetus is clearly reflected in the current industry research and development investment priorities ([2020-2025 APFA RD&E Priorities](#)). The research comprising this thesis aims to address key objectives to support advancements in pathogen detection, biosecurity, and disease management within the Australian *P. monodon* farming industry, with broader relevance to shrimp farming globally.

1.6 Thesis aims and structure

The overarching aim of this thesis was to advance biosecurity in shrimp aquaculture, with particular focus on the Australian farmed *P. monodon* industry, through improved application of molecular pathogen detection (qPCR). Initially, the research aimed to characterise the contemporary landscape of viral pathogens in Australian wild-sourced *P. monodon* broodstock. Using this information, research reported in subsequent chapters aimed to develop knowledge to support improved application of molecular detection technologies for reliable and cost-effective detection of endemic viral pathogens. Additionally, the research sought to devise and validate a novel environmental sampling approach to improve sampling efficiency and broaden

the scope of pathogen surveillance for large-scale industry application. Finally, a review and synthesis of prior research were conducted on the factors influencing viral disease outcomes in commercially important Penaeid shrimp species. Leveraging the findings of this review, evidence-based guidelines for shrimp disease research were developed to facilitate the translation of future PCE research findings into actionable strategies for advancing disease management within shrimp aquaculture.

Chapter 2 reports the largest audit of commercially important viral pathogens in wild-sourced Australian *P. monodon* broodstock conducted to date. The study defined the contemporary viral landscape of the two major wild stocks that are the primary sources of broodstock for the Australian shrimp farming industry using qPCR. Defining the prevalence and loading of pathogens within commercially relevant wild stocks was significant to inform improvements in stock collection strategy and support industry endeavours to establish domesticated endemic *P. monodon* breeding lines targeting specific pathogen freedom. The study highlighted the critical need for strong entry-level biosecurity, and robust, reliable pathogen detection to inform broodstock management decisions. The viral pathogens that were detected at the highest prevalence (IHHNV and GAV), and an emerging pathogen of concern to the Australian industry detected within the audit (YHV-7) were earmarked for further analysis in Chapter 3 (IHHNV, GAV and YHV-7), Chapter 4 (IHHNV and GAV) and, Chapter 5 (IHHNV and GAV). Chapter 2 was published in the peer reviewed journal *Aquaculture* (Arbon et al., 2022) and is presented within this thesis with only minor modifications to improve the readability of the thesis as a whole.

Chapter 3 investigated the tissue specific distribution (tropism) of IHHNV, GAV and YHV-7 in naturally infected *P. monodon* using qPCR. The study evaluated quantitative viral detection across six tissue types, including gill, pleopod, hepatopancreas, hindgut, ventral nerve cord and muscle. Beyond establishing contemporary evidence of viral distribution within shrimp tissues to improve understanding of host-viral interactions, understanding the tropism of shrimp viruses directly supports appropriate target tissue selection for reliable pathogen detection. Ensuring shrimp tissue sampling yields both reliable pathogen detection and is practical, is a critical measure to improve the adoption and maintenance of strong pathogen surveillance programs. The findings of this study indicated that pleopod tissue was practical to sample and generally as suitable as other tissues for the detection of IHHNV, GAV and YHV-7. Given

sampling of pleopod for pathogen screening is currently industry standard practice, further investigation into the reliability of pathogen detection from pleopod tissue was conducted in Chapter 4. Chapter 3 was published in the peer reviewed journal *Aquaculture* (Arbon et al., 2024a) and is presented within this thesis with only minor modifications.

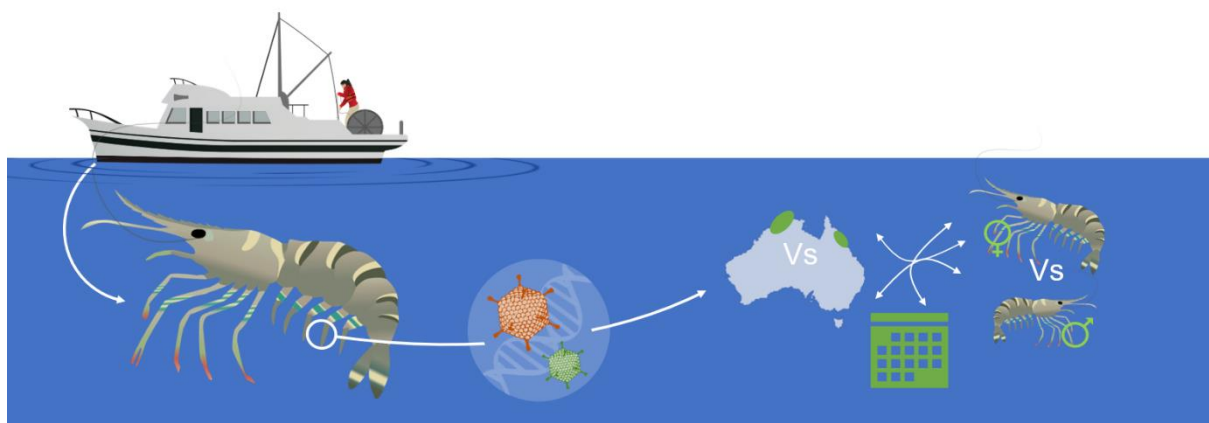
Chapter 4 examined the reliability of IHHNV and GAV detection from pleopods of naturally infected *P. monodon*. Determining the reliability of detection from pleopod tissue was critical to optimise sampling strategies and ensure appropriate interpretation of presence/absence and quantitative detection results. Variability in pathogen detection between pleopods of individual shrimp, and the representativeness of detection from pleopods to detection from other target tissues, including hepatopancreas and gill, were defined. A suite of pleopod tissue sampling and testing scenarios were statistically simulated for the experimental cohort of naturally infected *P. monodon*. The simulations underscored the trade-offs between detection reliability, and the commensurate increases in required sampling effort. Acknowledging the latter factor of this trade-off, the requirement of an alternative, more cost effective and accurate sampling approach suitable to large-scale pathogen surveillance applications was earmarked for further investigation in Chapter 5. The findings of Chapter 4 provide evidence to support improved interpretation of IHHNV and GAV detection results from pleopods.

Chapter 5 comprised the application and validation of a novel environmental sampling approach for shrimp pathogen detection and monitoring in commercial production ponds. The novel sampling approach involved incubation of simple filter units in shrimp aquaculture ponds to passively collect genetic materials (eNA) within the water. When compared to traditional tissue sampling approaches, passively collected eNA provided highly correlated pond detection results suitable for proxy-quantification of pathogen loading in the shrimp hosts. These results validated the utility of the novel approach. In addition, the study determined the effectiveness of the passive eNA sampling with various incubation times, which demonstrated that indicative detection could be achieved after only 1 h of immersion. The findings of this chapter established the value of passively collected environmental samples as a sensitive surveillance tool and proxy for indicating viral shrimp infection. The approach offers significant advantages through greatly improved cost-efficiency, increased sampling scope, suitability to farm-scale deployment and reduced handling stress on host animals. Application of the environmental

sampling approach developed will enhance the capacity for data-driven farm biosecurity management and future systems-level host-pathogen-environment research.

Chapter 6 comprehensively reviewed and synthesised prior research on viral disease in *P. monodon* and other commercially important shrimp species to identify key factors that influence the outcomes of disease. The findings of the review highlighted the complexity of interactions between the shrimp host, viral pathogen, and environmental conditions, which impact the manifestation of disease, supporting the multifactorial systems-based approach to disease management strategies and research. The review also revealed a paucity of experimental detail reported in many published shrimp PCEs. To address this significant gap, a guideline was formulated to describe ‘best-practice’ development and reporting in publication of shrimp viral PCEs. The guidelines, in the form of a checklist, were borne from the host pathogen and environmental components identified to have significant influence on disease outcomes within the broader review. Ultimately, the guidelines were developed to enhance the robustness, transparency and standardisation of experimental design and reporting, and facilitate the translation of future PCE findings to directly inform improvements in contemporary shrimp disease management strategies. Chapter 6 was published in the peer reviewed journal *Reviews in Aquaculture* (Arbon et al., 2024b) and is presented within this thesis with only minor modifications.

In total, the research comprising this thesis offers significant advancement in understanding the detection and dynamics of viral pathogens in shrimp aquaculture systems and proposes how best to apply this understanding to advance biosecurity within the Australian *P. monodon* farming industry, and global shrimp aquaculture more broadly.



Chapter 2.

Molecular detection of six viral pathogens from Australian wild sourced giant black tiger shrimp (*Penaeus monodon*) broodstock

PUBLISHED - (Arbon et al., 2022)

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Foreword

Chapter 2 provides a comprehensive analysis detailing the presence and prevalence of endemic viral pathogens in wild-sourced Australian *P. monodon* broodstock, collected from the two primary fisheries that support the Australian shrimp aquaculture industry. The findings highlighted the viral pathogens of highest prevalence and offer fundamental context on the extent of viral pathogen exposure of the Australian shrimp aquaculture industry via wild sourcing of stock. Moreover, the results affirm the critical requirement for further research to ensure biosecurity practices, especially pathogen detection capacity and methodology, are optimised to support improved disease mitigation and management.

The de-identified data within Chapter 2 is presented with permissions from participant industry collaborators. The authors sincerely acknowledge each industry participant and the value of this collaboration.

2.1 Abstract

Disease presents a considerable challenge to the sustainability and development of global shrimp aquaculture. The Australian black tiger shrimp (*Penaeus monodon*) aquaculture industry is heavily reliant on wild-sourced broodstock for seedstock production, representing a large and under evaluated biosecurity risk. Currently, there is a paucity of quantitative, large-scale data detailing the extent of pathogenic agents in wild-sourced *P. monodon* broodstock. This study presents a comprehensive investigation of the presence and level of detection of endemic pathogen targets in wild-sourced *P. monodon* broodstock collected from the two primary sources of supply for the Australian shrimp aquaculture industry using quantitative polymerase chain reaction (qPCR). Broodstock pleopod samples (N = 7472) were analysed by TaqMan qPCR for the detection of six viral pathogens. In total, 44.9% of wild-sourced broodstock were positive for the detection of at least one pathogen target. White spot syndrome virus and yellow head virus-1 were not detected in any sample. Infectious hypodermal haematopoietic necrosis virus (30%) and gill-associated virus (28.1%) were the most prevalent pathogen targets detected. Whenzhou (*syn.* Wenzhou) shrimp virus-2 and yellow head virus-7 were each cumulatively detected in less than 3% of broodstock samples. Geographic source, sex and year of collection of broodstock significantly influenced prevalence of detection. The current study will be valuable to the Australian shrimp aquaculture industry to improve understanding of the presence of pathogens in wild-sourced broodstock, along with informing management decisions related to wild broodstock collection and associated biosecurity practices.

Keywords: *Penaeus monodon*, TaqMan qPCR, viral pathogens, broodstock, shrimp aquaculture

2.2 Introduction

Disease presents a considerable and pervasive challenge to the sustainability and development of global shrimp aquaculture (Flegel, 2019; Norouzitallab et al., 2019). An estimated 40% of tropical shrimp production is lost due to disease, predominantly due to viral pathogens (Stentiford et al., 2012). To reduce the impact of disease outbreaks on shrimp aquaculture, domestication and genetic selection approaches have been adopted in the culture of *Litopenaeus vannamei* (Alday-Sanz et al., 2018; Briggs et al., 2005; Tan-Wong and Subramaniam, 2009; Wyban, 2019). One domestication approach has involved the development of breeding lines that are tested and certified free of specific pathogens (Specific Pathogen Free - SPF). Pathogens targeted in SPF programs include those listed in the OIE Aquatic Manual, which includes acute hepatopancreatic necrosis disease (AHPND), and infections with *Hepatobacter penaei*, infectious hypodermal haematopoietic necrosis virus (IHHNV), infectious myonecrosis virus (IMNV), Taura syndrome virus (TSV), white spot syndrome virus (WSSV), yellow head virus-1 (YHV-1), spherical baculovirus (MBV) and tetrahedral baculovirus (BP) (OIE, 2020a). Alternative approaches that select animals that are refractory/ genetically resistant to infection, or disease, after challenge with a specific pathogen (Specific Pathogen Resistant - SPR) have also been adopted (Alday-Sanz et al., 2018). A third approach, termed Specific Pathogen Tolerant (SPT), selects for genetic lines that are not refractory to infection, but rather do not develop signs of disease when infected with specific pathogens (Alday-Sanz et al., 2018). The advantages in productivity derived from the domestication and development of SPF, SPR and SPT lines of *L. vannamei* has led to the conversion of many global shrimp production systems from native species of *P. stylirostris*, *P. chinensis*, *P. indicus* and *P. monodon*, to domesticated *L. vannamei*.

Although domesticated *P. monodon* stocks have been commercially developed in Hawaii (MOANA), Madagascar (AQUALAMA) and Thailand (CP Foods), national biosecurity regulations prevent the importation of such lines into certain countries, including Australia. Countries that have retained *P. monodon* production and attempted domestication programs using endemic lines have reported variable levels of success with domestication (Coman and Preston, 2008; Tan-Wong and Subramaniam, 2009; Towers, 2012). Many unsuccessful domestication programs have failed to incorporate, or consider pathogen detection, and programs have faltered when low-level viral infections have progressed to disease when shrimp are reared under suboptimal conditions (Coman and Preston, 2008). Given the ongoing

aspiration for commercial establishment of domesticated endemic *P. monodon* lines, there is a requirement to understand the pathobiome of wild-sourced candidate founding broodstock. Generally, there is a paucity of large-scale data that describes the prevalence and intensity of infection of the range of pathogens present in wild-sourced *P. monodon* broodstock. The development of such datasets could inform strategic pursuit of domesticated, SPF, SPR or SPT lines, or at a minimum, could better inform hatchery managers of the risks associated with the introduction of wild-sourced broodstock for seedstock production.

The aim of this study was to apply TaqMan qPCR analysis to investigate the presence of pathogen targets in wild-sourced *P. monodon* targeted as candidate broodstock for use in commercial shrimp production. The analysed targets included pathogens that can be detected from non-destructive sampling of pleopod tissues and have been associated with mortality events, or are present as emerging or significant threats to shrimp production in Australia. Specifically, the pathogen targets included white spot syndrome virus (WSSV), yellow head virus-1 (YHV-1), gill-associated virus/yellow head virus-2 (GAV/YHV-2), infectious hypodermal haematopoietic necrosis virus (IHHNV, *syn* *Penaeus stylirostris penstyldensovirus* 1; PstDV1), yellow head virus-7 (YHV-7) and, a potential pathogen of unknown significance, Whenzhou (*syn*. Wenzhou) shrimp virus-2 (When-2). Analysis to detect a number of pathogens that are considered exotic to Australia, including *Hepatobacter penaei*, TSV, IMNV, and EHP was not conducted.

This study is the most comprehensive assessment of pathogens in wild-sourced Australian *P. monodon* to date. The findings will improve understanding of the presence of known pathogens within wild-sourced *P. monodon* broodstock. Improved understanding in this area will aid in the synthesis of well-founded, evidence-based management decisions regarding broodstock biosecurity practices and, inform the strategic pursuit of domesticated lines, SPF, SPR or SPT programs from wild endemic stocks.

2.3 Materials and methods

2.3.1 Sample collection and handling

P. monodon examined in this study ($N = 7472$) originated from the Northern Territory (NT; $n = 5091$) and the East Coast (EC QLD; $n = 2381$) of Australia (Figure 2.1) and were collected by commercial fishing trawlers specifically tasked for broodstock collection to supply the Australian shrimp aquaculture industry. Broodstock samples were collected between July 2018 and October 2020. The number of broodstock samples analysed from each single collection location, and within each temporal (year) or sex (male or female) subset, exceeded the minimum number of samples required to detect a 5% prevalence in an unknown population size with 95% confidence using an assay with Type 1 and Type 2 error rate less than 5% (<https://epitools.ausvet.com.au/prevalence>).

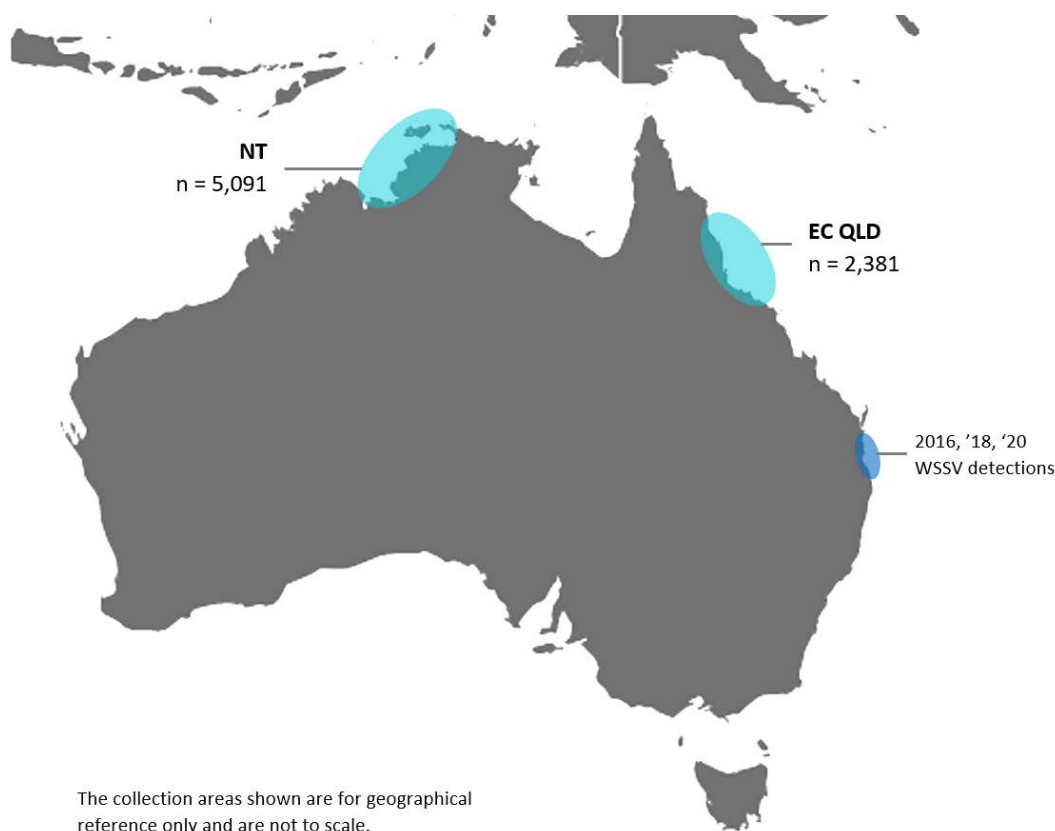


Figure 2.1. Penaeus monodon broodstock collection locations including the Northern Territory (NT) and East Coast of Queensland (EC QLD) and the number of samples collected from each location. Also shown is the region of WSSV detections in south-eastern Queensland in 2016, 2018 and 2020 (not sampled as part of this study).

The live broodstock shrimp were received onto commercial farms, where tissue samples from one pleopod per individual broodstock were taken ($\sim 5 \text{ mm}^2$) and placed immediately into 2 mL

Lysing Matrix D tubes (Cat. 6913050, MP Biomedicals™, OH, USA) containing 350 µL of MagMAx™ CORE Lysis Solution (Cat. A32702 Ref. 100043807, Applied Biosystems™, VIC, Australia). Samples were transported and stored at room temperature in the lysis tubes for < 48 h prior to nucleic acid extraction. Pleopod tissue was used for analysis in this study as sampling from broodstock shrimp required non-destructive and individually traceable sample collection.

2.3.2 Nucleic acid extraction

For total nucleic acid extraction from tissue samples, 5 µL of 20 mg mL⁻¹ MagMAx™ CORE Proteinase K (Cat. A32702 Ref. 100043773, Applied Biosystems™, VIC, Australia) was added to each 2 mL Lysing Matrix D tube (Cat. 6913050, MP Biomedicals™, OH, USA) containing the sample and 350 µL of MagMAx™ CORE Lysis Solution (Cat. A32702 Ref. 100043807, Applied Biosystems™, VIC, Australia). The sample was homogenized for 30 s at 5000 rpm using a Precellys® 24 tissue homogenizer (Cat. P000669-PR240-A, Bertin Technologies, France). The homogenised samples were incubated at ~72 °C for 60 min. Total nucleic acid (TNA) extraction from the homogenised and digested samples was conducted using the MagMAx™ CORE Nucleic Acid Purification Kit (Cat. A32702, Applied Biosystems™, VIC, Australia) with a KingFisher™ Flex 96 Deep-Well Magnetic Particle Processor (Cat. A31508, ThermoFisher Scientific™, VIC, Australia) as per the manufacturer's instructions. Total nucleic acid was eluted into 100 µL of MagMAx™ CORE Elution Buffer (Cat. A32702 Ref. 100043817, Applied Biosystems™, VIC, Australia) and stored at -20 °C until required.

2.3.3 Quantitative real-time PCR (qPCR) target detection

Quantitative polymerase chain reaction (qPCR) analysis was used for the detection of six pathogen targets; two viruses presumed exotic to the collection sites, namely white spot syndrome virus (WSSV) and yellow head virus-1 (YHV-1), and four endemic pathogen targets, including infectious hypodermal haematopoietic necrosis virus (IHHNV, *syn* *Penaeus stylirostris penstyldensovirus* 1; PstDV1), yellow head virus-7 (YHV-7), gill-associated virus/yellow head virus-2 (GAV/YHV-2) and, a potential pathogen of unknown significance, Whenzhou (*syn*. Wenzhou) shrimp virus-2 (When-2). Additionally, the *P. monodon* Dicer-1 gene (Dicer-1) was targeted for detection using RT-qPCR as a protocol integrity control (Su et al., 2008).

Target DNA and RNA in each TNA extract were quantified using TaqMan Real-Time quantitative PCR (qPCR). For the detection DNA targets, the SensiFAST™ Probe Lo-ROx Kit (Cat. BIO-84050, Meridian Bioscience™, NSW, Australia) was used. Alternatively, for the detection of RNA targets, the reverse transcriptase (RT) SensiFAST™ Probe Lo-ROx One-Step Kit (Cat. BIO-78005, Meridian Bioscience™, NSW, Australia) was used. A total of 1.25 µL of sample template was added to 8.75 µL of the SensiFAST™ Kit master-mix to yield a 10 µL reaction; inclusive of 0.1 µL of the respective target primers (0.2 µM final concentration) and 0.025 µL of target probe (0.05 µM final concentration). Primer and probe sequences are listed in Table 2.1. Analysis was conducted using a QuantStudio™ 5 Real-Time PCR System, 384-well (Cat. A28140, Applied Biosystems™, VIC, Australia). Cycle conditions were standardised for both DNA and RNA targeting assays, consisting of a reverse-transcription (RT) step of 45 °C for 10 min, initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing and extension at 60 °C for 30 s. Raw data were processed using QuantStudio™ Design & Analysis Software v1.5.2 (ThermoFisher Scientific™, www.thermofisher.com), using the ΔR_n method with a relative threshold of 0.05, to produce cycle threshold (Ct) values of positive detections. Samples which did not amplify beyond the relative threshold within 40 cycles were categorized as undetermined. A positive target control and non-template control were included in each analysis.

Table 2.1. Quantitative polymerase chain reaction (qPCR) primer and probe details used in the present study.

Pathogen target	Status	Nucleic target	Primer/ Probe	Sequence (5'-3')	Length [bp]	Product length [bp]	Reference
White spot syndrome virus (WSSV)	exotic ²	DNA	qF	CCGACGCCAAGGGAAC	17	77	CSIRO (Callinan & Barlow, 2013)
			qR	TTCAGATTCGTTACCGTTTCCA	22		
			qProbe	CGCTTCAGCCATGCCAGCCG	20		
Yellowhead virus 1 (YHV-1)	exotic	RNA	qF	AGTCTACAGTGCTCTGATCT	20	150	CSIRO (Cowley et al., 2015)
			qR	GATTCTTGAAGCGCATGAGT	20		
			qProbe	TCTCATGTGTCATGATATTCTCAAGCGAGT	30		
Whenzhou (<i>syn.</i> Wenzhou) shrimp virus 2 (When-2)	endemic	RNA	qF	GGCTCTTTAGCCTGACTTTATCT	23	114	This chapter (Arbon et al., 2022)
			qR	GCAGAGGACAGGAAGTGATTA	21		
			qProbe	ACCTCACTGTCTGAGTTCTGCACA	24		
Infectious hypodermal and haematopoietic necrosis virus (IHHNV)	endemic	DNA	qF	CCTAAAGAAAACAGTGCAGAATAT	24	98	(Cowley et al. 2018)
			qR	TCATCGTCAAGTTTATTGACAAGTTC	26		
			qProbe	CTCCAACACTTAGTCAAA	18		
Hepatopancreatic parvovirus (HPV)	endemic	DNA	qF	CTACTCCAATGGAACTTCTGAGC	24	140	(La Fauce et al., 2007)
			qR	GGCACTTCCTGTATTTTCCCG	22		
			qProbe	TACCGCCGCACCGCAGCAGC	20		
Yellowhead virus 7 (YHV-7)	endemic	RNA	qF	CATCCAACCTATCGCCTACA	20	79	(Cowley et al. 2015)
			qR	TGTGAAGTCCATGTGAACGA	20		
			qProbe	CAACGACAGACACCTCATCCGTGA	24		
Gill-associated virus (GAV)	endemic	RNA	qF	GGGATCCTAACATCGTCAACGT	22	81	(De La Vega et al., 2004)
			qR	AGTATGGATTACCCTGGTGCAT	22		
			qProbe	TCAGCCGCTTCCGCTTCCAATG	22		
<i>Penaeus monodon</i> Dicer-1 gene (Dicer-1)	internal control	RNA	qF	TGGTACCAAAGTCACCCATTAG	22	91	(Su et al., 2008)
			qR	ACCTTCCCATCAACAAGACGTT	22		
			qProbe	AACCAGAAACAGCCAAAT	18		

²*Contained within movement regulated areas (MRA).

2.3.4 Statistical analysis

Statistical analysis was conducted using R (R Core Team, 2021). Descriptive statistical parameters, including total number of samples tested, total number of positive detections, rate of positive detection (prevalence, %) and the average (mean), standard deviation (SD) and range (Ct range) of positive detection Ct values were calculated for each target and across subsets of broodstock geographic source, year and sex. Significant differences in positive detections (Ct values) between broodstock subsets were assessed using Permutation testing (R package: *lmPerm*; $\alpha = 0.05$; Wheeler and Torchiano, 2016). Significant differences in prevalence (positive detection rate) between broodstock subsets was assessed using generalised linear modelling with binomial response (R package: *stats*; $\alpha = 0.05$; R Core Team, 2021). Post-hoc analysis was conducted with pairwise comparisons using Z-tests, corrected with Holm's sequential Bonferroni procedure (R package: *multcomp*; Hothorn et al., 2008) (R package: *emmeans*; $\alpha = 0.05$; Lenth, 2021). Non-parametric tests were applied as the normality and homoscedasticity assumptions for the parametric test alternatives were unable to be fulfilled, even after data transformation.

2.4 Results

Pathogen prevalence and intensity of infection are important parameters to consider within biosecurity management protocols and in the preferential selection of founding broodstock for domestication programs pursuing SPF, SPR and SPT lines. The results from the current study indicated that various pathogen targets were highly prevalent in the broodstock sampled. Furthermore, the findings indicated significant variation in the prevalence and Ct values of detection of pathogen targets associated with broodstock sex, temporal sampling and geographic source.

2.4.1 Summary of pathogen detection

A considerable proportion of *P. monodon* broodstock (44.9%; 3354/ 7472) were positive for the detection of at least one pathogen target (Figure 2.2). IHHNV (30%; 2238/7472) and GAV (28.1%; 2100/7472) were the most prevalent targets detected, followed by When-2 (2.9%; 220/ 7472). Detected in only three samples, YHV-7 (0.04%; 3/7472) was the least prevalent endemic pathogen (Table 2.2). WSSV and YHV-1 were not detected in any of the broodstock tested. All samples were positive for the detection of the *P. monodon* Dicer-1 target (Table 2.2), confirming the procedural integrity of the qPCR analysis.

The Ct values were highly variable between the different pathogen targets (Table 2.2), with the Ct value of positive detections for each pathogen target ranging from ~15 to 39 (Table 2.2). When-2 was detected at the lowest average Ct value of all the pathogen targets (30.36 ± 6.04 Ct; $Z = -25.02$, $p < 1e-04$); however, it was also the most variable (Table 2.2). YHV-7 was detected at the highest average Ct value, although this was not significant (35.92 ± 1.12 Ct; $Z = 0.62$, $p = 0.96$) (Table 2.2). Low Ct values were observed for three out of the four endemic targets, as indicated by minimum Ct values of IHHNV (15.44 Ct), GAV (21.92 Ct) and When-2 (20.61 Ct) (Table 2.2).

Table 2.2. Descriptive statistics for target detections by qPCR of *Penaeus monodon* broodstock. Mean $\pm$ SD and Ct range are expressions of Ct values for positive detections. NPD = No pathogen detected, EC QLD = East Coast Queensland, NT = Northern Territory. Data is subset to broodstock geographic location and sex. For each target, significantly higher ($p < 0.05$) pathogen prevalence and significantly lower Ct values ($p < 0.05$) between subset groups of sex is denoted by an asterisk, while differences between subset groups of location is denoted by a diamond.

	WSSV	YHV-1	GAV	IHHNV	When-2	YHV-7	Dicer-1	NPD
All samples (N = 7472)								
Positive detections (%)	(0%)	(0%)	2100 (28.1%)	2238 (30%)	220 (2.9%)	3 (0.04%)	7472 (100%)	4118 (55.1%)
Mean Ct $\pm$ SD	0 $\pm$ 0	0 $\pm$ 0	35.06 $\pm$ 2.15	34.57 $\pm$ 3.63	30.36 $\pm$ 6.04	35.92 $\pm$ 1.12	28.56 $\pm$ 1.68	-
Ct range	0 - 0	0 - 0	21.92 - 39.97	15.45 - 39.94	20.61 - 39.56	34.63 - 36.6	20.05 - 36.39	-
EC QLD (N = 2381)								
Positive detections (%)	(0%)	(0%)	1316 (55.3%) [◇]	1385 (58.2%) [◇]	199 (8.4%) [◇]	(0%)	2381 (100%)	515 (21.6%)
Mean Ct $\pm$ SD	0 $\pm$ 0	0 $\pm$ 0	35.12 $\pm$ 2.06	33.98 $\pm$ 3.79 [◇]	29.61 $\pm$ 5.86 [◇]	0 $\pm$ 0	28.82 $\pm$ 1.51	-
Ct range	0 - 0	0 - 0	22.6 - 39.97	15.45 - 39.58	20.61 - 38.96	0 - 0	24.38 - 35.74	-
NT (N = 5091)								
Positive detections (%)	(0%)	(0%)	784 (15.4%)	853 (16.8%)	21 (0.4%)	3 (0.1%)	5091 (100%)	3603 (70.8%) *
Mean Ct $\pm$ SD	0 $\pm$ 0	0 $\pm$ 0	34.97 $\pm$ 2.3	35.53 $\pm$ 3.15	37.47 $\pm$ 1.18	35.92 $\pm$ 1.12	28.44 $\pm$ 1.75 *	-
Ct range	0 - 0	0 - 0	21.92 - 39.93	27.08 - 39.94	35.3 - 39.56	34.63 - 36.6	20.05 - 36.39	-
Male (N = 3563)								
Positive detections (%)	(0%)	(0%)	949 (26.6%)	852 (23.9%)	91 (2.6%)	2 (0.1%)	3563 (100%)	2169 (60.9%) *
Mean Ct $\pm$ SD	0 $\pm$ 0	0 $\pm$ 0	34.92 $\pm$ 2.27 *	34.57 $\pm$ 3.25	30.79 $\pm$ 5.98	35.58 $\pm$ 1.34	28.6 $\pm$ 1.74	-
Ct range	0 - 0	0 - 0	22.27 - 39.97	17.25 - 39.82	20.91 - 39.41	34.63 - 36.52	21.18 - 35.55	-
Female (N = 3909)								
Positive detections (%)	(0%)	(0%)	1151 (29.4%)	1386 (35.5%) *	129 (3.3%)	1 (0%)	3909 (100%)	1949 (49.9%)
Mean Ct $\pm$ SD	0 $\pm$ 0	0 $\pm$ 0	35.18 $\pm$ 2.04	34.58 $\pm$ 3.85	30.05 $\pm$ 6.09	36.6	28.54 $\pm$ 1.63	-
Ct range	0 - 0	0 - 0	21.92 - 39.93	15.45 - 39.94	20.61 - 39.56	36.6 - 36.6	20.05 - 36.39	-

Table 2.2 Continued.

	WSSV	YHV-1	GAV	IHHNV	When-2	YHV-7	Dicer-1	NPD
EC QLD - Male (n = 961)								
Positive detections (%)	(0%)	(0%)	549 (57.1%) [◇]	555 (57.8%) [◇]	79 (8.2%) [◇]	(0%)	961 (100%)	208 (21.6%)
Mean Ct ± SD	0 ± 0	0 ± 0	35.06 ± 2.21	34.37 ± 3.24	29.85 ± 5.85 [◇]	0 ± 0	28.82 ± 1.54	-
Ct range	0 - 0	0 - 0	22.6 - 39.97	17.25 - 39.58	20.91 - 38.43	0 - 0	24.56 - 35.55	-
EC QLD - Female (n = 1420)								
Positive detections (%)	(0%)	(0%)	767 (54%) [◇]	830 (58.5%) [◇]	120 (8.5%) [◇]	(0%)	1420 (100%)	307 (21.6%)
Mean Ct ± SD	0 ± 0	0 ± 0	35.16 ± 1.95	33.73 ± 4.09 ^{*◇}	29.45 ± 5.88 [◇]	0 ± 0	28.82 ± 1.48	-
Ct range	0 - 0	0 - 0	25.75 - 39.83	15.45 - 39.53	20.61 - 38.96	0 - 0	24.38 - 35.74	-
NT - Male (n = 2602)								
Positive detections (%)	(0%)	(0%)	400 (15.4%)	297 (11.4%)	12 (0.5%)	2 (0.1%)	2602 (100%)	1961 (75.4%) ^{*◇}
Mean Ct ± SD	0 ± 0	0 ± 0	34.72 ± 2.34 [*]	34.94 ± 3.24 [*]	37.01 ± 1.14	35.58 ± 1.34	28.51 ± 1.81	-
Ct range	0 - 0	0 - 0	22.27 - 39.9	27.13 - 39.82	35.3 - 39.41	34.63 - 36.52	21.18 - 34.96	-
NT - Female (n = 2489)								
Positive detections (%)	(0%)	(0%)	384 (15.4%)	556 (22.3%) [*]	9 (0.4%)	1 (0.04%)	2489 (100%)	1642 (66%) [◇]
Mean Ct ± SD	0 ± 0	0 ± 0	35.23 ± 2.22	35.85 ± 3.05	38.07 ± 0.98	36.6	28.37 ± 1.68	-
Ct range	0 - 0	0 - 0	21.92 - 39.93	27.08 - 39.94	36.82 - 39.56	36.6 - 36.6	20.05 - 36.39	-

2.4.2 Multiple pathogen detections

Near half (44.9%; 3354/7472) of all sampled broodstock tested positive for the detection of at least one pathogen target (Figure 2.2). IHHNV (15.9%; 1187/7472) and GAV (14.0%; 1045/7472) were the most common targets detected in single pathogen detections (Figure 2.2). Broodstock with single pathogen detections of When-2 or YHV-7 were very uncommon, cumulatively accounting for less than 0.5% (34/7472) of all samples tested (Figure 2.2). Of the broodstock with pathogen detections, 32.4% (1088/3354) were positive for the detection of multiple pathogens. Co-detection of GAV and IHHNV was the most prevalent combination observed, occurring in 12.0% (899/7472) of all broodstock (Figure 2.2). There were no multiple detections that did not include either GAV or IHHNV. Triple pathogen detection was only observed for the combination of IHHNV, GAV and When-2, and accounted for 1.6% (119/7472) of all samples (Figure 2.2).

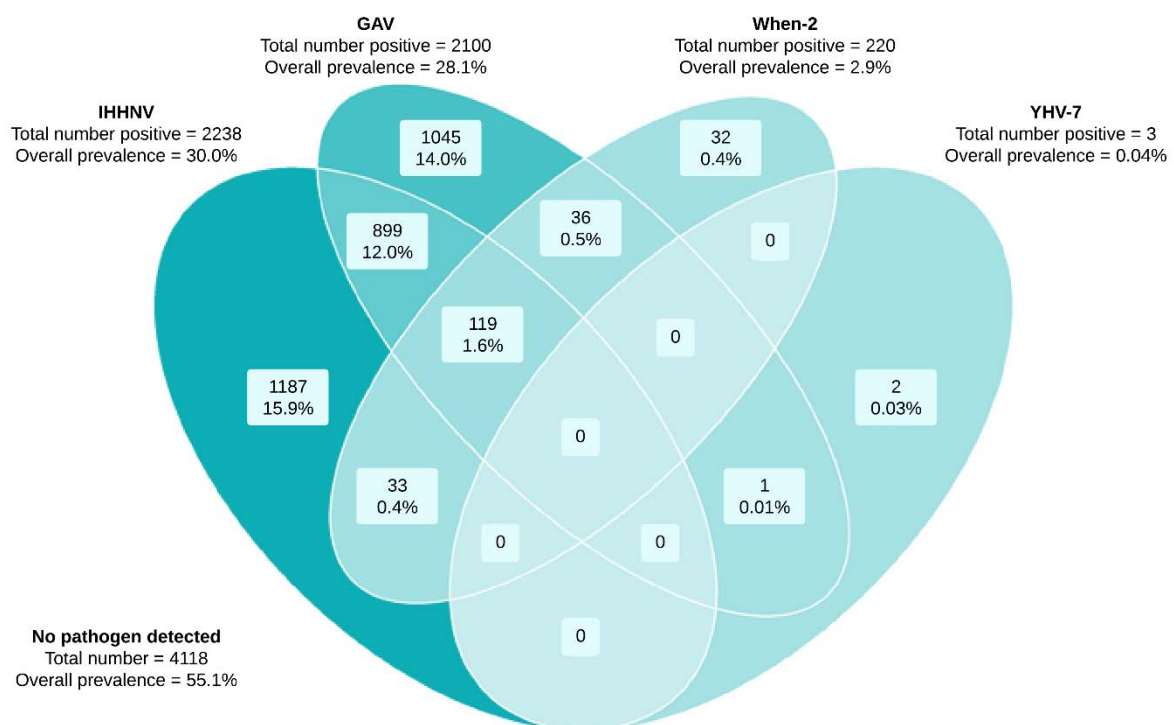


Figure 2.2. Multiple pathogen detections by qPCR of *P. monodon* broodstock. Percent values are an expression of overall prevalence (from $N = 7472$ broodstock).

2.4.3 Influence of broodstock sex and geographic source on pathogen detection

The frequency of pathogen target detection varied significantly between the sexes for IHHNV ($Z = -10.83, p < 2e-16$), whereby females (35.5%; 1386/3909) were positive significantly more often than males (23.9%; 852/3563) (Table 2.2). The positive detection rate of GAV in female broodstock (29.4%; 1151/3909) was also higher compared to male broodstock (26.6%; 949/3563) ($Z = -2.70, p = 0.007$). The difference in When-2 positive detection rate was not significant between the sexes ($Z = -1.9, p = 0.057$) (Table 2.2). Overall, male broodstock (60.9%; 2169/3563) were negative for the detection of all pathogen targets more often, compared to the female broodstock (49.9%; 1949/3909) ($Z = 9.54, p < 2e-16$) (Table 2.2). This trend in pathogen detection by sex continued for the multiple pathogen detections in the male and female broodstock, with higher overall proportions of female broodstock observed with co-pathogen detections compared to males ($Z = -3.99, p = 6.5e-05$) (Table 2.3). Differences in the Ct value of positive detections between male and female broodstock were only significant for GAV ($Z = -2.8, p = 0.005$), with male broodstock having a lower average Ct of GAV (34.92 ± 2.27) compared to female broodstock (35.18 ± 2.04) (Table 2.2).

Table 2.3. The number of shrimp with multiple pathogen detections collected from East Coast Queensland (EC QLD) and Northern Territory (NT) of Australia. Detections are subset into sex (Male: M and Female: F) for each location. Percentages are calculated from the totals of each subset. For each number of pathogens detected, significantly higher ($p < 0.05$) prevalence between sexes within location groups is denoted by an asterisk (), and significantly higher prevalence between location groups for all samples is denoted by a diamond (◇).*

		No. pathogens detected			
		0	1	2	3
EC QLD	F	307 (22%)	578 (41%)	466 (33%)	69 (5%)
	M	208 (22%)	369 (38%)	338 (35%)	46 (5%)
	ALL	515 (22%)	947 (40%) ◇	804 (34%) ◇	115 (5%) ◇
NT	F	1642 (66%)	746 (30%)*	99 (4%)*	2 (0.1%)
	M	1961 (75%)*	573 (22%)	66 (3%)	2 (0.1%)
	ALL	3603 (71%) ◇	1319 (26%)	165 (3%)	4 (0.1%)
TOTAL	F	1949 (50%)	1324 (34%)*	565 (14%)*	71 (2%)
	M	2169 (61%)*	942 (26%)	404 (11%)	48 (1%)
	ALL	4118 (55%)	2266 (30%)	969 (13%)	119 (2%)

Differences were evident in the prevalence of detection and Ct values of positive detection between shrimp sourced from the NT and EC QLD. NT-sourced broodstock had significantly lower prevalence of IHHNV (16.8%; 853/5091; $Z = -34.52$, $p < 2e-16$), GAV (15.4%; 784/5091; $Z = -33.82$, $p < 2e-16$) and When-2 detections (0.04%; 21/5091; $Z = -13.39$, $p < 2e-16$), compared to EC QLD-sourced samples (58.2%; 1385/2381, 55.3%; 1316/2381 and 8.4%; 199/2381, respectively) (Table 2.2). Overall, 70.8% (3603/5091) of NT-sourced broodstock were negative for the detection of all pathogen targets. In contrast, only 21.6% (515/2381) of EC QLD-sourced broodstock were negative for the detection of all pathogen targets ($Z = 37.1$, $p < 2e-16$; Table 2.2). Similarly, multiple pathogen detections from samples were less common in NT-sourced broodstock (all: $Z < -8.19$, $p < 2e-16$) (Table 2.3). The average Ct values of both IHHNV and When-2 detections were significantly lower in EC QLD-sourced samples (33.98 ± 3.79 Ct and 29.61 ± 5.86 Ct, respectively), compared to NT-sourced samples (35.53 ± 3.15 Ct and 37.47 ± 1.18 Ct, respectively) (both: $Z > 6.12$, $p < 9.13e-10$; Table 2.2).

The interactions of geographic source and sex of broodstock significantly influenced detection patterns. The interaction of geographical source and sex was significant for both IHHNV prevalence (χ^2 ($df = 1$, $N = 7472$) = 45.66, $p < 1.4e-11$) and the prevalence of shrimp with no pathogen detections (NPD; χ^2 ($df = 1$, $N = 7472$) = 14.73, $p = 0.0001$). While IHHNV prevalence was generally higher in EC QLD than NT, a higher prevalence of IHHNV in female broodstock was observed for the NT-sourced broodstock (~11% difference; $Z = 10.27$, $p < 1e-05$), compared to their male counterparts, than that observed in the EC QLD broodstock (~1% difference; $Z = 0.34$, $p = 0.99$; Table 2.2). With respect to shrimp with no pathogen detected (NPD), EC QLD had a lower overall prevalence and there was no difference in prevalence between the sexes ($Z = -0.014$, $p = 1$). Conversely, the difference in NPD prevalence between the sexes of the NT-sourced shrimp was considerable (~9.4% difference; $Z = -7.34$, $p < 1e-10$), with males having a higher prevalence compared to their female counterparts. The interaction of geographical source and sex also influenced the Ct value of positive detection of IHHNV ($F = 41.65$, $p < 2e-16$). IHHNV Ct values were generally lower in EC QLD shrimp compared to NT shrimp. However, for EC QLD shrimp, female broodstock had a lower average Ct value, compared to male broodstock ($Z = 3.29$, $p = 0.0057$); this pattern was inverse for broodstock sourced from the NT ($Z = -3.58$, $p = 0.0021$; Table 2.2), with male broodstock having lower average Ct values, compared to their female counterparts.

2.4.4 Influence of year on pathogen detection

Despite the variation in overall prevalence of pathogen detection between the two geographic locations (Table 2.2), the changes in pathogen prevalence between years followed similar trends throughout the period assessed in this study. Compared to 2018, IHHNV prevalence peaked in 2019, at 86.9% (1103/1269) for EC QLD ($Z = -11.66$, $p < 1e-06$) and 28.1% (583/2076) for NT ($Z = -11.18$, $p \leq 1e-06$) (Table 2.4). Following the peak in 2019, IHHNV prevalence in 2020 reduced in EC QLD sourced broodstock to 18.4% (162/881) ($Z = 28.11$, $p \leq 1e-06$), well below the 2018 level of 51.9% (120/231) ($Z = 9.94$, $p \leq 1e-06$). Meanwhile in NT shrimp, IHHNV detections in 2020 (9.1%; 190/2090) also declined from their 2019 peak ($Z = 15.06$, $p \leq 1e-06$), returning to similar levels as observed in 2018 (8.6%; 80/925) ($Z = -0.392$, $p = 0.99$) (Table 2.4). In an expectedly inverse manner, the prevalence of shrimp with no pathogens detected (NPD), was lowest in 2019 for NT (55.3%; 1149/3345) ($Z = 3.48$, $p = 0.006$) and EC QLD (5.0%; 63/1269) ($Z = 6.56$, $p < 0.001$), and dramatically increased in 2020 for both NT (89.9%; 1879/2090) ($Z = -23.21$, $p < 0.001$) and EC QLD (46.7%; 411/ 881) ($Z = -19.33$, $p < 0.001$). For both collection locations, GAV detections steadily declined from 2018 to 2020, with the most recent NT levels reaching only 1% (21/2090) compared to 31.9% (295/925) in 2018 ($Z = 16.63$, $p < 0.001$). A steady decline in When-2 prevalence was also observed for EC QLD broodstock from 2018 to 2020 ($Z = 9.65$, $p < 0.001$). Given the low prevalence of When-2 in NT shrimp it is difficult to discern any distinct pattern in detection through time, however, prevalence was highest in 2018 at 2.2% (20/925; Table 2.4).

Table 2.4. Prevalence of pathogen detections through time (years) between collection locations (East Coast Queensland: EC QLD and Northern Territory: NT). NPD = no pathogens detected. Significant differences between years of the same subset are indicated by superscript letters. Significantly higher prevalence in the same year, between location subsets are indicated by an asterisk.

		No. tested	GAV	IHHNV	When-2	YHV-7	NPD
EC QLD	2018	231 (3.1%)	171 (74%) ^{a, *}	120 (51.9%) ^{a, *}	56 (24.2%) ^{a, *}	(0%)	41 (17.7%) ^a
	2019	1269 (17%)	792 (62.4%) ^{b, *}	1103 (86.9%) ^{b, *}	126 (9.9%) ^b	(0%)	63 (5%) ^b
	2020	881 (11.8%)	353 (40.1%) ^{c, *}	162 (18.4%) ^{c, *}	17 (1.9%) ^{c, *}	(0%)	411 (46.7%) ^c
NT	2018	925 (12.4%)	295 (31.9%) ^a	80 (8.6%) ^a	20 (2.2%) ^a	2 (0.2%)	575 (62.2%) ^{a, *}
	2019	2076 (27.8%)	468 (22.5%) ^b	583 (28.1%) ^b	(0%)	1 (<1%)	1149 (55.3%) ^{b, *}
	2020	2090 (28%)	21 (1%) ^c	190 (9.1%) ^a	1 (<1%) ^b	(0%)	1879 (89.9%) ^{c, *}
TOTAL		7472 (100%)	2100 (28.1%)	2238 (30%)	220 (2.9%)	3 (0%)	4118 (55.1%)

2.5 Discussion

Domestication of an aquaculture species offers many growth and risk mitigation benefits, yielding superiority over production systems that rely on the collection of wild sourced broodstock (Wyban, 2019). Maintaining broodstock within hatcheries throughout their entire life cycle facilitates increased nutritional-induced maturation, scheduling of spawning events, genetic selection for favoured phenotypes and the opportunity to reduce the risk of pathogen introduction to the culture system. However, domesticated and established endemic SPF/SPR/SPT lines are not yet commercially available for a large sector of global *P. monodon* aquaculture. The majority of farmed *P. monodon* production relies on wild-sourced broodstock. Sectors relying on wild-sourced broodstock are exposed to considerable biosecurity risks and are unable to realize the economic benefits that can result from domestication and subsequent genetic selection. Improved understanding of the presence and infection intensities of pathogens in wild-sourced broodstock would provide immediate advantages to support data-driven biosecurity management decisions. This knowledge may also aid in the estimation of broodstock availabilities and resource requirements for the development of domestication programs adopting an SPF, or SPR/SPT approach. The current study has established a comprehensive knowledge base detailing the detection of six pathogen targets important to Australian shrimp aquaculture. The targets were selected from those that can be detected from non-destructive sampling of broodstock pleopod tissues and are currently considered of commercial relevance due to association with mortality, or are present as emerging and/or significant threats to production. The results of this study are discussed in the context of the risk to production posed by the targeted pathogens and the preferential selection of founding broodstock for domestication programs pursuing SPF, SPR and SPT lines.

2.5.1 IHHNV

IHHNV is included in the OIE Aquatic Manual, OIE listed pathogens and in SPF listings (OIE, 2020a, 2019a). Despite being considered a significant pathogen, there is ambiguity in both the detection of IHHNV and the economic significance of IHHNV in *P. monodon* production. There are four genotypes documented for IHHNV, of which only two, genotype 1 (America and Eastern Asia) and genotype 2 (South-East Asia), are considered infectious (Rai et al., 2012). Two additional variants, genotypes 3 (Type A: Madagascar, India, Mauritius and Australia) and genotype 4 (Type B: East Africa, Mozambique and Tanzania) are not infectious and considered integrated into the *P. monodon* genome (Rai et al., 2012).

The accurate detection of IHHNV in Australian *P. monodon* stocks has been elusive. Australian, African, Thailand and Indian stocks of *P. monodon* contain partial sequences of the IHHNV (Genotypes 3 or 4) genome integrated into the *P. monodon* genome (Rai et al., 2012; Saksmerprome et al., 2011; Tang and Lightner, 2006) and can also be infected with genotype 1 or 2. Despite the presence of IHHNV in both farmed and wild Australian shrimp stocks, there has been scarce research focused on genome sequencing analysis to describe the infectious genotypes. All four IHHNV genotypes are positively detected with the OIE IHHNV TaqMan PCR and the IQ 2000 IHHNV assay (1608F/ 1688R; Tang and Lightner, 2001), including the non-infectious types 3 and 4. As such, investigations that have studied presence/absence of clinical signs of disease in *P. monodon* associated with the detection of IHHNV using the OIE qPCR or the Intelligene IQ 2000 assay are not able to reliably generate or base conclusions specific to infectious genotypes (Chayaburakul et al., 2005; Withyachumnarnkul et al., 2006). A PCR (309F/R) assay that differentially detects infectious (genotype 1 & 2) IHHNV from genotypes 3 or 4 has since been published (Tang et al., 2007). Following this, Cowley et al. (2018) published a TaqMan qPCR (IHHNV-q309) that targets the same region as the 309 nt amplicon of the 309F/R “IHHNV” PCR (Tang et al., 2007).

Applying the IHHNV-q309 assay, IHHNV was the most frequently detected pathogen target in broodstock in the present study (30%). Previously, analysis of wild-sourced Australian *P. monodon* stocks, using the IHHNV 309F/R PCR (Tang et al., 2007) detected IHHNV in Australian broodstock at a prevalence of ~7% (10/151) from wild EC QLD-sourced shrimp and ~ 10% (5/50) from NT-sourced shrimp (Cowley et al., 2015). The results of the present study suggest considerably higher prevalence levels of IHHNV from both geographic sources, in addition to a significant prevalence differential between the two sources (NT: 16.8%, $n = 853/5091$ and EC QLD 58.2%, $n = 1385/2381$; $Z = -34.52$, $p < 2e-16$).

Beyond the ambiguity and pervasiveness of accurate detection of IHHNV, there also exists limited and conflicting published information regarding the impact of IHHNV on productivity in *P. monodon*. Although numerous publications note an association in the detection of IHHNV with reduced growth and deformities (Montgomery-Brock et al., 2007; Sellars et al., 2019), other researchers report no impact of the virus on production (Chayaburakul et al., 2005; Withyachumnarnkul et al., 2006). Unlike experimental challenges of *L. vannamei* and *P.*

stylirostris (Bell and Lightner, 1984), there have been no reported experimental challenges of *P. monodon* with IHHNV. Furthermore, since the historical reports of an association between IHHNV and slow growth in *P. monodon* (Quinitio and Primavera, 2000), other pathogens including MBV, *Enterocytozoon hepatopenaei* (EHP), Hepandensovirus (HDV/HPV) and Laem Singh Virus (LSVN) have also been associated with reduced growth in *P. monodon* (Janakiram et al., 2013; Poornima et al., 2012; Valappil et al., 2021). Despite this, screening for the range of pathogens that are associated with slow growth has not ensued in many of the recent reports that describe reduced productivity in association with IHHNV (Sellars et al., 2019). In the absence of the fulfillment of Koch's postulates with IHHNV in *P. monodon*, caution should be exercised when assessing the apparent impact and significance of IHHNV infection in *P. monodon*. Considering the significant costs associated with pathogen screening for the purpose of removing IHHNV positive animals and the prevalence of IHHNV detected in this study, further research should be conducted to elucidate the significance of IHHNV genotypes and strains on the health and productivity of Australian *P. monodon*.

2.5.2 GAV

GAV, *syn.* YHV-2, is a member of the yellow head virus (YHV) complex. GAV is not an OIE listed pathogen (OIE, 2020a), but is included in SPF listing (Brock, 2013). In the present study, GAV was the second most frequently detected pathogen target in broodstock (28.1%). Congruently, high prevalence of subclinical GAV infections have been reported for both farmed and wild *P. monodon*, especially from EC QLD stocks (De La Vega et al., 2006). The NCBI database contains sequences listed as GAV collected from Australia, Indonesia, Malaysia, Thailand and Taiwan (<https://www.ncbi.nlm.nih.gov/>, accessed 9/08/21), however, disease events in *P. monodon* production have only been reported from Australian sourced samples (Spann et al., 1997). GAV was associated with mortality events termed Mid-Crop Mortality syndrome (MCMS) that occurred in Australian *P. monodon* pond systems during 1996/97 (Anderson and Owens, 2001). GAV particles in viral extracts from these disease events were observed under TEM (Spann et al., 1997). Although experimental challenges have been conducted for GAV in *P. monodon* (Noble et al., 2020a, 2017; Spann et al., 1997), as with many historical reports, the challenge inoculum was not a purified viral extract and comprehensive screening for other pathogens in the viral inoculum is not widely reported (reviewed in Arbon et al., 2024b). The findings of the present study suggest that the majority of wild-sourced shrimp positive for the detection of GAV have co-detections of one or more

other pathogen targets (50.2%) including IHNV, YHV-7 and When-2. As with IHNV, experimental challenge trials with a purified and sequenced viral inoculum are required to unequivocally determine the significance of GAV strain/s on the health and productivity of Australian *P. monodon*.

2.5.3 When-2

Wenzhou (*syn.* Wenzhou) shrimp virus-2 (When-2) is not an OIE listed pathogen and also is not included in SPF pathogen listings (OIE, 2020a). The viral genome was initially identified in RNA deep sequencing of *P. monodon* originating from Wenzhou in Zhejiang province, China (Li et al., 2015). This uncharacterized viral genome was also recently identified in Australian *P. monodon* stocks (Huerlimann et al., 2018) and the extent of its presence in Australian *P. monodon* stocks has not yet been described. The present study detected When-2 at an overall prevalence of 2.9%, with the majority of positive detections originating from EC QLD (199/220 positive). The results of this study provide an early indication into the presence of this potential pathogen within Australian stocks. However, the significance of these detections is unknown as the impact of this virus on the health and productivity of *P. monodon* is yet to be demonstrated. If the virus is demonstrated to have an impact on production, and When-2 were to be added to the pathogens in an SPF listing, this study suggests NT-sourced broodstock would be preferential over EC QLD-sourced broodstock due to the significantly lower prevalence of When-2 in NT.

2.5.4 YHV-7

YHV-7 is a member of the YHV complex of viruses that includes YHV- 1, GAV (*syn.* YHV-2) and eight other YHV strains (YHV-3 to YHV-10) (Mohr et al., 2015). YHV-7 is not included in the OIE Aquatic Manual, OIE listed pathogens or SPF listings (OIE, 2020, 2019). The present study detected YHV-7 from a small number (0.1%, $n = 3/5091$) of NT-sourced broodstock. In 2012, YHV-7 was identified from wild NT *P. monodon* broodstock stocked into a commercial EC QLD hatchery, resulting in cumulative stock mortality of 81.4% (Mohr et al., 2015). Following this initial incident, YHV-7 has been detected in wild NT-sourced and EC QLD-sourced *P. monodon* at relatively low prevalence (1–9%) (Cowley et al., 2015). The pathogenicity of YHV-7 in *P. monodon* was demonstrated by inoculation, yielding mortality of ~60–70% by day 28 post inoculation (Moody and Crane, 2015). RT-qPCR analysis of the inoculated shrimp revealed 100% positive detection at Ct values ranging between 12.9 and 22.6 (Moody and Crane, 2015). The YHV-7 inoculum was negative for the detection of YHV-1,

AHPND, TSV, WSSV, MBV, IHHNV, IMNV, and Mourilyan virus (syn. Wenzhou shrimp virus-1), but was positive for the detection of HPV (Moody and Crane, 2015). This study of Moody and Crane (2015) is one of few experimental infection challenges in shrimp that report analysis to determine the absence of other pathogens in the experimental inoculum. In 2017, a disease event on an EC QLD shrimp farm was associated with YHV-7 (Cowley et al., 2019). All samples tested were positive for the detection of YHV-7, at Ct values ranging from 14.6 to 24.2. The Ct values reported by Cowley et al. (2019) and Moody and Crane (2015) relating to disease events and mortality are considerably lower than those detected in the present study (min Ct = 34.63). Nonetheless, the demonstrated pathogenicity of YHV-7 justifies inclusion of this pathogen in screening Australian broodstock for commercial production. The presence and potential for introduction of YHV-7 into the hatchery environment via infected broodstock remains a significant risk, as increased stressors related to the handling of broodstock can result in rapid increases of viral infection (De La Vega et al., 2004).

2.5.5 WSSV & YHV-1

WSSV and YHV-1 are included in the OIE Aquatic Manual, are OIE listed pathogens and are in SPF listings (OIE, 2020, 2019). Both viruses lead to mass mortality and induce severe economic consequences in shrimp production systems (OIE, 2019). WSSV and YHV-1 were not detected from any broodstock in this study. This result in relation to YHV-1 continues the historic absence of this pathogen's detection in Australia. WSSV was also not detected from the 7472 wild-sourced *P. monodon* broodstock sourced from Northern Australia. Prior to this study, in mid-2000, a national survey designed to detect a within-site prevalence of WSSV greater than 10% with 95% confidence, demonstrated Australia to be free from WSSV (East et al., 2005). However, in 2016, WSSV was recorded for the first time from shrimp farms in South-eastern Queensland (Figure 2.1). Subsequent surveys of other crustacean species in the wild resulted in several positive detections of WSSV in 2017, 2018 and 2020 within 100 km of the originally affected shrimp farms (Oakey et al., 2019; OIE, 2020b). The results of the present study indicates the two major commercial broodstock collection locations in Australia can be considered free of WSSV, noting that these locations are geographically distanced (>1600 km) from areas where WSSV has been detected in Australia.

2.5.6 Influence of broodstock sex and geographic source on pathogen detection

Anecdotal evidence of superior growth and survival, attributed to lower prevalence and intensity of infection with endemic viruses, influenced the Australian shrimp farming industry towards increasingly sourcing broodstock from the NT, rather than EC QLD (Cowley et al., 2015). The findings of the present study demonstrate significantly lower prevalence of IHHNV, GAV and WNV targets in NT-sourced broodstock, compared to the EC-sourced broodstock, with 70.8% of NT-sourced broodstock negative for the detection of all pathogen targets, compared to only 21.6% of EC QLD- sourced broodstock. These findings maintain the recommendation of Cowley et al. (2015) to transition stock collection from EC QLD to NT stocks. Speculative influences, including variation in environmental conditions e.g. water temperature (Millard et al., 2021; Montgomery-Brock et al., 2007), genetic divergence of shrimp populations (Vu et al., 2020; Waqairatu et al., 2012), anthropogenic effects e.g. agricultural runoff (Hook et al., 2018; Smith et al., 2012) and recreational fisheries as a pathway for pathogen introduction (Karunasagar and Ababouch, 2012; McColl et al., 2004) may contribute to the differences observed in pathogen presence, prevalence and load between locations. However, the mechanisms resulting in this apparent variation are unknown and more robust and structured investigation is required.

Overall, 49.9% (1949/3909) of the female broodstock, were negative for the detection of all the targeted pathogens, compared to 60.9% (2169/3563) of male broodstock. The prevalence of IHHNV was significantly higher in the female broodstock assessed in this study. Similarly, Noble et al. (2020b) found that GAV was significantly more prevalent in commercially reared *P. monodon* females (50.2%), compared to males (41.8%), and that on average the females were heavier (+ 0.5 g) than the males within the cohort. This variation may be associated with a shift in resource allocation in females due to sexually dimorphic growth patterns (Powell et al., 2018), or during sexual maturation and vitellogenesis (Chan et al., 2014; Long and Ka, 2008; Saetan and Chotigeat, 2019; Wonglapsuwan et al., 2009) resulting in a reduction in immune response capacity and thus increased susceptibility to pathogens, compared to their male counterparts. However, as with the influence of geographic source, the actual mechanisms resulting in this apparent sex dependent variation are unknown.

Notwithstanding the unknown mechanisms driving this variation, the implications of such marked differences in pathogen prevalence and detection intensity between geographic sources and sexes are considerable. The collection of stock from EC QLD presents an increased risk of pathogen introduction into farm hatcheries; however, collection of broodstock predominately, or solely from NT stocks, may reduce the genetic resilience potential of foundation broodstock, and may have implications for the future development of breeding programs (Houston et al., 2020).

2.5.7 Influence of year on pathogen detection

Temporal fluctuation in pathogen prevalence in shrimp has been widely observed in relation to seasonal conditions, such as monsoons (Chakrabarty et al., 2014; Mallik et al., 2016; Remany et al., 2012; Withyachumnarnkul et al., 2003). More broadly, temporal variation may align to shifts in climate phenomena including El Nino Southern Oscillation (ENSO), or anthropogenic drivers such as policy change or shifts in importation trends (Knibb et al., 2018). Although interesting, interpretation of the temporal variation captured within this study is limited, and further investigation capturing data over an extended duration is required to elucidate the potential temporal drivers for the observed changes. Nonetheless, temporal variation in pathogen prevalence of wild-sourced broodstock should be recognized as a dynamic risk to be considered in the development and maintenance of breeding programs targeting high health or, pathogen free lines.

2.5.8 Considerations for Australian *P. monodon* domestication programs

Considering the prevalence of the targeted pathogens demonstrated in this study, there is a high likelihood of introduction of viral pathogens in hatchery production systems by wild-sourced broodstock. These findings substantiate the pursuit of domesticated endemic stock to avoid the continued exposure of industry to the annual introduction of pathogens from wild broodstock. Although domestication of *P. monodon* has been attempted in many countries, there has been limited sustained success in establishing large numbers of domesticated families in Australian *P. monodon* programs. Failure to establish domesticated lines has been primarily attributed to biological reproductive difficulties in producing fertile broodstock and commercially sustainable larval output from breeders (Arnold et al., 2013). However, consideration of the pathobiome has not been included in many domestication programs, or other programs that have attempted to improve reproductive performance in domesticated *P. monodon* (Goodall, 2017).

Previous analyses (East et al., 2005), coupled with the results of the present study, suggest the Australian industry currently has access to wild shrimp stocks that are free of the vast majority of pathogens listed in the OIE and SPF shrimp pathogen listings and other emerging pathogens, such as Decapod Iridovirus-1 (DIV-1) (DAF, 2019). However, the recent WSSV disease outbreaks in the SE QLD region in Australia and subsequent detection of WSSV in wild stocks within a 100 km zone in SE QLD in 2017, 2018 and 2020 (Oakey et al., 2019; OIE, 2020b) (also see section 1.5.1) suggests the timeline for the Australian industry to access WSSV-free wild-sourced broodstock may be declining. Consequently, the Australian shrimp aquaculture industry is facing increased pressure to more actively pursue broodstock domestication programs, with focus on realizing high health lines.

Advancements towards domestication and genetic selection for disease resilience (SPR, SPT, SPF or otherwise), in *P. monodon* will be contingent on the realization of key milestones and development of fundamental understandings. Specifically, (i) overcoming lagging developments in domestication which currently constitutes a barrier to implementing advanced selection technologies; (ii) establishing robust evidence surrounding the significance and impact of pathogens currently associated with disease and productivity losses to inform ongoing management and selection strategy, and; (iii) establishing understanding of the genetic architecture for disease resistance and high health traits to determine their viability for inclusion in selection programs. Further work is required promptly to resolve these limiting factors and enable the targeted development of domesticated high health Australian *P. monodon*.

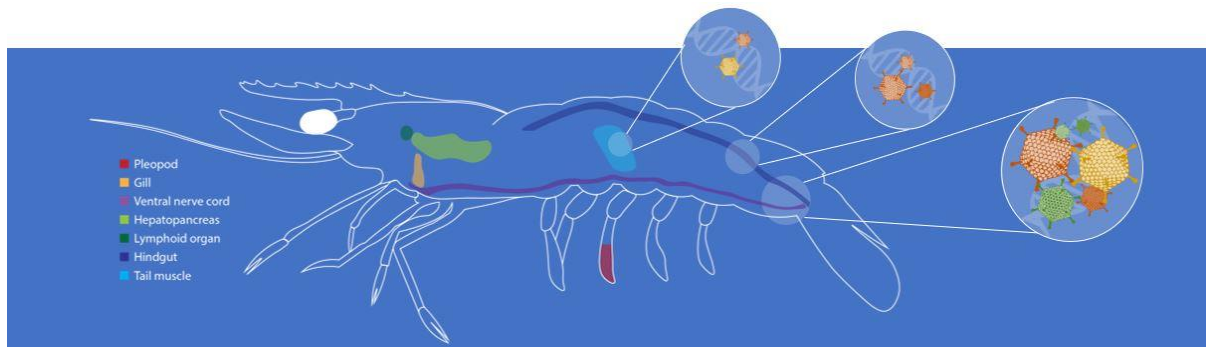
2.5.9 Data applicability

In the present study, TaqMan qPCR analysis was conducted to detect the presence of six viral pathogen targets. Although qPCR is the assay recommended in the OIE Manual of Diagnostic Tests for Aquatic Animals as fit for purpose for the surveillance of viral pathogens in crustaceans, the authors acknowledge positive detection by qPCR does not support diagnosis of viable viral infection. Ideally, analysis by qPCR should be supported by an additional assay such as histology, however, to align with commercial pursuit of optimal spawning performance of the study animals, sampling of additional tissues for histology was not possible within the scope of the current investigation.

Single pleopod samples were collected from individual broodstock as soon as possible after receipt onto farms. As suggested by Cowley et al. (2015) sampling was conducted in this way to ensure the samples were as representative of their wild complements as possible. However, any time delay between stock collection and tissue sampling, or potential elevation in stock stress due to handling and increase in stock density during transport and holding, may have resulted in increased viral transmission between stock, or amplification of infections within stock (De La Vega et al., 2004; Raja et al., 2015; Walker and Mohan, 2009). Additionally, the use of single tissue samples (pleopods or gill filaments) for the detection of pathogen targets by qPCR can yield underestimates of infection severity given the variability of infection loads among samples of the same individual (Arbon et al., 2024a; Noble et al., 2018). Considering the aforementioned factors, the data yielded from this study does not attempt to directly assess the prevalence and intensity of pathogen detections in wild *P. monodon* populations and may underestimate pathogen target loadings of the analysed broodstock. Accordingly, the results of this study should be considered with direct relevance to the aquaculture industry framework, as the collection and sampling protocol used in this study is consistent with current Australian industry practice.

2.5.10 Conclusions

The present study provides a comprehensive insight into the detection of pathogens in wild-sourced *P. monodon* broodstock. Both prevalence and intensity of pathogen detection varied significantly with geographic source, year of collection and sex of the broodstock. The high prevalence of pathogens detected in this study suggests industry pursuits to establish domesticated endemic *P. monodon* lines targeting high health should be prioritised to avoid continued exposure to pathogen introduction via wild-sourced *P. monodon* broodstock. Further research to robustly elucidate the impact and significance of viral and bacterial pathogens in *P. monodon* production will be requisite to the realisation of selective breeding programs targeting high health lines.



Chapter 3.

Determining patterns of tissue tropism for IHHNV, GAV and YHV-7 infection in giant black tiger shrimp (*Penaeus monodon*) using real-time RT-qPCR

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Foreword

Chapter 2 highlighted the viral pathogens that were detected at the highest prevalence in wild-sourced Australian *P. monodon* broodstock (IHHNV and GAV), and an emerging pathogen of high concern to the Australian industry (YHV-7). These findings underscored the critical need for robust pathogen detection systems to support disease mitigation and management, and the commonality of this need throughout all phases of shrimp aquaculture. However, owing to the rapid evolution of pathogen detection technologies, in many cases, evidence to support their optimised and robust application has not yet been generated. The absence of this evidence can reduce the sensitivity and efficiency of pathogen detection efforts. Leveraging the findings of Chapter 2, Chapter 3 sought to develop such evidence for the detection of key viral pathogens of concern to the Australian industry, namely of IHHNV, GAV and YHV-7, from naturally infected *P. monodon*. Specifically, the analysis assessed tissue-specific viral distribution patterns using qPCR to identify appropriate target tissues for sampling and detection.

3.1 Abstract

Disease management in shrimp aquaculture is heavily dependent on accurate and rapid pathogen detection and diagnosis. Understanding the presence and distribution of pathogens within various tissues of the shrimp host is critical to ensure detection efforts are targeted for optimal sensitivity and reliability. Modern advancements in molecular technologies, including nucleic acid extraction and RT-qPCR, have yielded significant improvements in pathogen detection capabilities. Despite these advancements and their widespread adoption within both research and industry applications, evidence to inform their optimised use, such as revisions to tissue-specific viral detection to support selection of appropriate target tissues, has not been established. To address these gaps, this study aimed to establish contemporary evidence of viral tissue tropism for three major shrimp pathogens. TaqMan qPCR analysis was used to determine tissue-specific loading of viral pathogens in naturally infected *Penaeus monodon* pleopod, gill, hepatopancreas, lymphoid organ, abdominal muscle tissue, hindgut and ventral nerve cord. The viral targets analysed included infectious hypodermal haematopoietic necrosis virus (IHHNV, *syn Penaeus stylirostris* penstyldensovirus 1; PstDV1) and, two genotypes of the yellow head virus complex, including genotype 2 known as gill associated virus (YHV-2/ GAV), and genotype 7 (YHV-7). qPCR analyses demonstrated the highest level of genetic IHHNV detection from gill tissue, followed sequentially by hindgut, pleopod, hepatopancreas, lymphoid organ, ventral nerve cord and abdominal muscle. For the yellow head viruses (GAV and YHV-7), no significant differences in genetic viral detection were demonstrated, although a non-significant advantage was observed for lymphoid organ and hindgut tissue. Beyond establishing contemporary evidence of viral distribution to improve understanding of host-viral interactions, these findings offer supporting evidence for revisions to appropriate target tissue recommendations, within the World Organisation for Animal Health (WOAH, founded as OIE) Manual of Diagnostic Tests for Aquatic Animals, for optimised modern shrimp pathogen detection.

Keywords: aquaculture, pathogen detection, TaqMan, viral distribution, sampling

3.2 Introduction

Rapid expansion and intensification of global aquaculture has coincided with the rise of aquatic diseases (Kennedy et al., 2016; Pulkkinen et al., 2010; Walker and Winton, 2010). Consequently, effective disease management presents a central challenge for both sustaining current and increasing future aquaculture production (Stentiford et al., 2017, 2012). Reliable and rapid pathogen detection and disease diagnosis is fundamental to effective disease management in aquaculture (Kumar et al., 2021b; Lightner and Redman, 1998; WOA, 2023b).

While diseases are of major concern across the diversity of primary aquaculture species, shrimp aquaculture is one of the most severely impacted (Asche et al., 2021; Leung and Bates, 2013). Global shrimp production endures sustained impacts from disease that range from reduced productivity via disorders of fecundity and growth, to outbreaks of diseases resulting in mass mortality events (Shinn et al., 2018; Valappil et al., 2021). Until recently, diagnosis of shrimp diseases has remained heavily reliant on classical disease diagnostic techniques including microscopy, histology and histochemistry, and has suffered severe limitations in the development and availability of diagnostic tools given the absence of crustacean cell culture methods (Kumar et al., 2021b; Lightner and Redman, 1998; WOA, 2023b). The adoption of advancements in molecular technologies, derived from the fields of human and veterinary medicine, has underpinned the development of contemporary shrimp disease surveillance and diagnostic strategy (Lightner, 2006). Currently, shrimp pathogen detection for surveillance and diagnostics relies primarily on real-time quantitative PCR (qPCR) analysis due to its availability, utility, diagnostic specificity, sensitivity, and scalability of throughput (WOA, 2023b).

Application of technologies, including PCR and qPCR, for the detection and diagnosis of aquatic disease is guided by the World Organisation for Animal Health (WOA, founded as OIE). The WOA Manual of Diagnostic Tests for Aquatic Animals (herein referred to as the Aquatic Manual), provides information to support a standardised and validated approach for the detection and diagnosis of diseases listed in the WOA Aquatic Animal Health Code (herein referred to as the Aquatic Code) (WOA.org). Since the first edition in 1995, successive editions of the Aquatic Code and Aquatic Manual have expanded in response to the emergence of new pathogens and improved diagnostic technologies. Expansions have

incorporated new chapters and updates to recommended practices in line with the latest developments in knowledge. However, some sections have evolved more rapidly, outpacing revisions to supporting sections. For example, where the definition of suitable target tissues for diagnosis and surveillance has remained unchanged, despite the significantly improved detection sensitivity and reliability of the updated detection practices. Definition and recommendation of appropriate target tissues is crucial to support sensitive pathogen detection and efficient sampling practices. Inconsistent updates to these sections may result in the sampling and analysis of suboptimal tissue types, resulting in a loss of efficiency and detection accuracy (Hou et al., 2023). Conversely, the non-inclusion of tissue types that may be suitable for detection of a broad range of pathogen targets may result in multiple tissue analysis that needlessly increases costs of surveillance.

Accordingly, this study aimed to generate evidence supporting appropriate target tissue selection for viral pathogen detection using contemporary techniques. The study investigated tissue-specific qPCR detection of infectious hypodermal haematopoietic necrosis virus (IHHNV, *syn Penaeus stylirostris penstyldensovirus* 1; PstDV1) and two genotypes of the yellow head virus complex, including genotype 2 known as gill associated virus (YHV-2/GAV), and genotype 7 (YHV-7), in naturally infected giant black tiger shrimp, *Penaeus monodon*.

3.3 Material and methods

3.3.1 Experimental animals

Two cohorts of shrimp were used for analysis in this study. Cohort 1 ($n = 10$, 35.99 ± 6.63 g) originated from a commercial shrimp farm in Queensland, Australia and were known to be naturally infected with both IHHNV and GAV based on previous qPCR and reverse transcriptase (RT) qPCR pathogen screening conducted throughout the production cycle. Cohort 2 ($n = 10$, 37.46 ± 9.17 g) originated from a commercial shrimp farm in Queensland, Australia and were known to be naturally infected with YHV-7 based on previous RT-qPCR pathogen screening conducted throughout the production cycle.

3.3.2 Sample collection

Seven tissues including pleopod (PLEO), gill (GILL), hepatopancreas (HP), lymphoid organ (LO), hindgut (HG), ventral nerve cord (NER) and, abdominal tail muscle (TAIL), were sampled from each individual shrimp (Figure 3.1). Each tissue sample was weighed and placed immediately into 2 mL Lysing Matrix D tubes (Cat. 6913050, MP Biomedicals™, OH, USA) containing 350 μ L of MagMAX™ CORE Lysis Solution (Cat. A32702 Ref. 100043807, Applied Biosystems™, VIC, Australia). Samples were stored at ambient temperature (~ 25 °C) in lysis solution for 48 h prior to nucleic acid extraction.

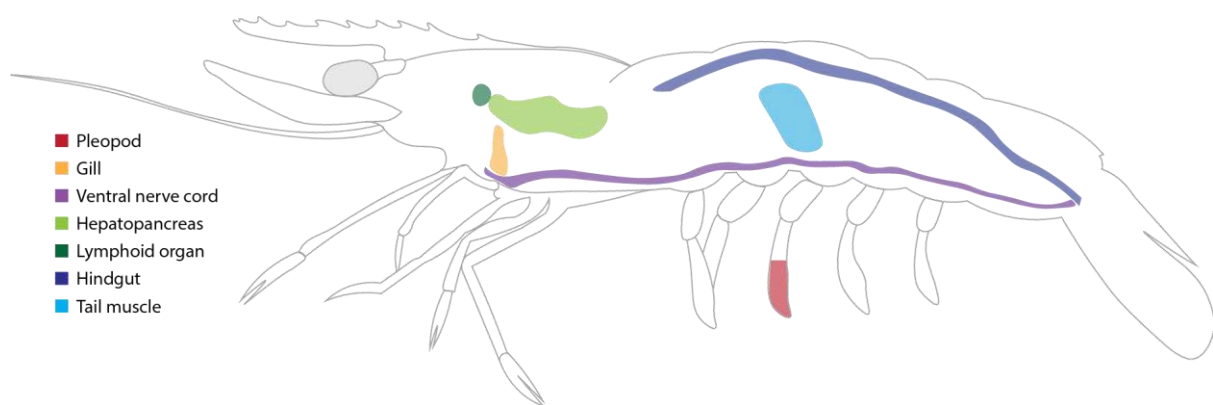


Figure 3.1. Tissue specific sample collection diagram.

3.3.3 Nucleic acid extraction

Nucleic acid extraction was conducted as per Arbon et al. (2022) with minor adaption. Briefly, 5 μ L of 20 mg mL⁻¹ MagMAX™ CORE Proteinase K (Cat. A32702 Ref. 100043773, Applied Biosystems™, VIC, Australia) was added to each 2 mL Lysing Matrix D tube (Cat. 6913050,

MP Biomedicals™, OH, USA) containing the sample and 350 µL of MagMAX™ CORE Lysis Solution (Cat. A32702 Ref. 100043807, Applied Biosystems™, VIC, Australia). The samples were homogenised for 30 s at 5000 rpm using a Precellys® 24 tissue homogeniser (Cat. P000669-PR240-A, Bertin Technologies, France). The homogenised samples were incubated at ~72 °C for 60 min. Total nucleic acid (TNA) extraction from the homogenised and digested samples was conducted using the MagMAX™ CORE Nucleic Acid Purification Kit (Cat. A32702, Applied Biosystems™, VIC, Australia) with a KingFisher™ Flex 96 Deep-Well Magnetic Particle Processor (Cat. A31508, ThermoFisher Scientific™, VIC, Australia) as per the manufacturer's instructions. All tissue samples belonging to each experimental cohort were extracted on 96 well plates together using the same reagent batches to minimise inter-run variation on nucleic acid extraction. TNA was eluted into 100 µL of MagMAX™ CORE Elution Buffer (Cat. A32702 Ref. 100043817, Applied Biosystems™, VIC, Australia). Template was stored at -20 °C until required.

3.3.4 Quantitative real-time PCR (qPCR)

RT-qPCR and qPCR analysis was conducted as per Arbon et al., (2022) with minor modification for the detection of GAV and IHHNV from cohort 1, and YHV-7 from cohort 2. Additionally, the *Penaeus monodon* Dicer-1 gene (Dicer-1) (Su et al., 2008) was targeted for detection using RT-qPCR as a protocol integrity control. Target DNA and RNA in each TNA extract were quantified using TaqMan Real-Time quantitative PCR (qPCR). For the detection of IHHNV (DNA target), the SensiFAST™ Probe Lo-ROx Kit (Cat. BIO-84050, Meridian Bioscience™, NSW, Australia) was used. Alternatively, for the detection of GAV (YHV-2), YHV-7 and Dicer-1 (RNA targets), the reverse transcriptase (RT) SensiFAST™ Probe Lo-ROx One-Step Kit (Cat. BIO-78005, Meridian Bioscience™, NSW, Australia) was used. A total of 1.25 µL of sample template was added to 8.75 µL of the SensiFAST™ Kit master-mix to yield a 10 µL reaction; inclusive of 0.1 µL of the respective target primers (0.2 µM final concentration) and 0.025 µL of target probe (0.05 µM final concentration). Primer and probe sequences are listed in Table 2.1. Analysis was conducted using a QuantStudio™ 5 Real-Time PCR System, 384-well (Cat. A28140, Applied Biosystems™, VIC, Australia). Cycle conditions were standardised for both DNA and RNA targeting assays, consisting of a reverse-transcription (RT) step of 45 °C for 10 min, initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 10 s and annealing and extension at 60 °C for 30 s. Raw data were processed using QuantStudio™ Design & Analysis Software v1.5.2

(ThermoFisher Scientific™), using the ΔR_n method with a relative threshold of 0.05, to produce cycle threshold (Ct) values of positive detections. Samples which did not amplify beyond the relative threshold within 45 cycles were categorised as undetected.

To minimise non-biological variation introduced during qPCR analysis, three 1.25 μ L aliquots of each TNA extract were tested as technical replicates for each target. Technical replicates of TNA extracts that were extracted on the same 96-well plate were tested on the same 384-well plate using the same reagent batches to minimise inter-run variation. Within each analysis run, a non-template control and positive target control for each target were included in triplicate. The positive target controls comprised nucleic acid extracts from *P. monodon* samples, derived using the methods described in section 3.3.3, which were previously confirmed positive for each target using the assays described in Table 2.1 (data not shown). The Ct values of each positive control were monitored across runs and verified to fall within an established, acceptable range to confirm consistency across all analyses (Geraci-Yee et al., 2022).

3.3.5 Conversion of Ct value to target copy number (μ L⁻¹)

Serial 10-fold dilutions of gBlock™ Gene Fragments (Integrated DNA Technologies™, NSW, Australia) were used as synthetic linear dsDNA template for IHHNV, GAV and YHV-7 (Table 3.1). The gBlock™ Gene Fragments were designed to comprise the IHHNV, GAV or YHV-7 genomic sequence targeted by the qPCR or RT-qPCR assays described in Table 2.1, including flanking sequence, derived from sequences available on the National Centre for Biotechnology Information (NCBI) database. Each point on the 10-fold dilution series was analysed in triplicate by qPCR. Each dilution series were used to generate standard curves, from which the conversion of Ct value to target copy number (μ L⁻¹) could be estimated in future analyses (Equation 3.1) of IHHNV (Figure 3.2, Equation 3.2), GAV (Figure 3.3, Equation 3.3) and YHV-7 (Figure 3.4, Equation 3.4). Samples categorised as undetected were assumed to have a viral copy number of zero.

Table 3.1. Synthetic linear dsDNA gBlock™ Gene Fragments (Integrated DNA Technologies™, NSW, Australia) details, as used in the present study. Sequence targeted by the respective IHHNV, GAV or YHV-7 qPCR assays within the gBlock™ oligonucleotides are presented in bold, with the flanking primer binding sequence underlined.

Pathogen target	Nucleic target	Sequence (5'-3')	Length [bp]	GC [%]	MW [Daltons]
Infectious hypodermal and haematopoietic necrosis virus <i>syn. Penaeus stylirostris</i> penstyldensovirus 1 (PstDV1)	DNA	CCGAGCTGAAGAAAAA CCTAAAGAAAACAGTGCAGAATATGA CTACCTCCAACACTTAGTCAAAACCAAATCAGCAAGAACAGT TCAAGAACTTGTCAATAAACTTGAC CGATGAAGAATACAAACAA CTATGGACCCGTACCAGAGGACAATATAAAGACAAACTCAGAGG AATACTGACATACTACAACAACAAGAAAAAGTCAAACCAAAGC CAACTGTCACTCATTACAAATCTTCAAAATATATCAAAGAGGAA ACCAGACTACGACAATATGCAGTGGATAAAATATATGTTAGCCA ACAACGACATCCGTGTACCAGAAATCTTAGCTTGGATAATCAT	345	37.1%	213007
Gill-associated virus <i>syn. Yellowhead virus 2</i> (YHV-2)	DNA	TTCACAAGGGATCCTAACATCGTCAACGTAGCTGTCAGCCGC TTCCGCTTCCAATGTATCTGCATGCACCAGGGTAATCCATAC TACTCTAAACTTCCTTACTACAACACAGCACAGATTTACTTCGAG AAATCAACCACAGTCATCGCTTATAACGGCCCCAACAACAAACT CTCAAACATGTACACTGATAATCTCAAACCATTTCCCATACCATAC CCTTGAGAACCGTTACCAGAGCGAGAAGGCTAAGTATCTCGGTA AGAAATTAATCTTGCATAACAATCCGTTTCGAGACACTTAAGGAA GCCAAGAAGGTGTTACACCGTGAAGACAACAAGATATGGGCTAA AGTCTCCGCTGAAGTCATGACCCGTCTGTTGTTTCGAGAAATTCAA CAATCCTGAACCTCTCTAAACACCTAATT	423	43.3%	261219
Yellowhead virus 7 (YHV-7) ³	DNA	GGTTCAAGAATCCTCTCTCCAAC ATCCAACCTATCGCCTACACA GCTATACACAACGACAGACACCTCATCCGTGATTTCGTCTCGT TCACAT TGGACTTCACATCGACCACCTCGACCTAACACCATAC	128	49.2%	78966

³ YHV-7 gBlock™ design was constrained by limited genetic sequence data available on NCBI for the region surrounding the qPCR primer and probe target.

Equation 3.1. Copy number (μL^{-1}) calculation from whole concentration of Gblock oligonucleotide ($\text{ng } \mu\text{L}^{-1}$)

$$\text{Copy number } (\mu\text{L}^{-1}) = \frac{\chi \text{ ng } \mu\text{L}^{-1} * 6.0221 \times 10^{23} \frac{\text{molecules}}{\text{mole}}}{\text{MW} * 1 \times 10^9 \frac{\text{ng}}{\text{g}}}$$

Where χ is the concentration of the gBlock™ oligonucleotide dilution in $\text{ng } \mu\text{L}^{-1}$ and, MW is the molecular weight of the gBlock™ oligonucleotide (in Daltons or g/mole) as provided by the manufacturer specifications.

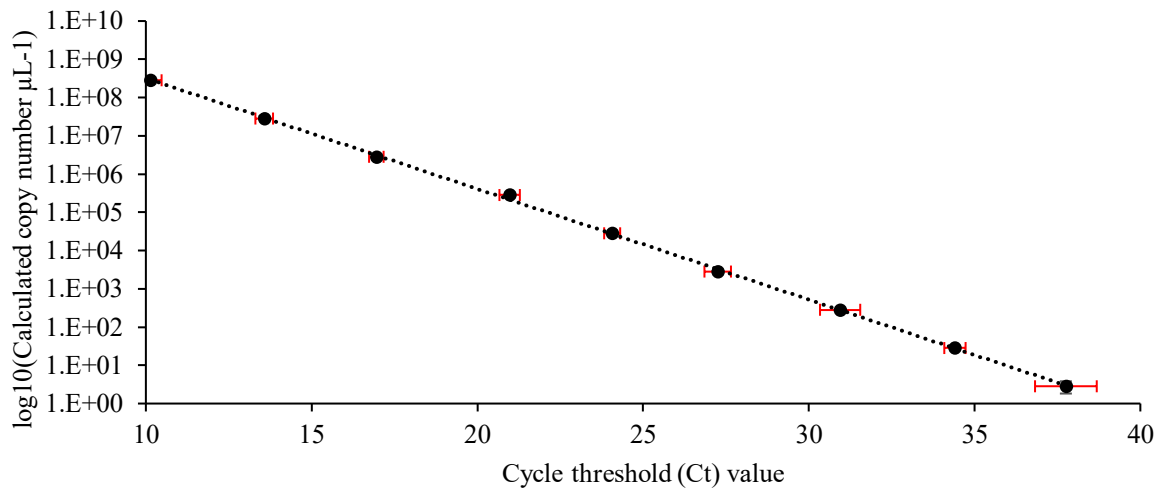


Figure 3.2. Standard qPCR curve for conversion of cycle threshold (Ct) value to calculated copy number (μL^{-1}) on a logarithmic scale for infectious hypodermal and haematopoietic necrosis virus (IHHNV), generated using a 10-fold serial dilution of gBlock™ Gene Fragments. Standard deviation of Ct value across the three technical replicates for each dilution are shown in red. The 10th dilution was not detected in any of the triplicate qPCR tests.

Equation 3.2. Infectious hypodermal and haematopoietic necrosis virus (IHHNV) cycle threshold (Ct) value to copy number (μL^{-1}) standard curve equation

$$\text{IHHNV copy number } (\mu\text{L}^{-1}) = 251,426,615,616.215 \times e^{-0.666505 * \chi}$$

Where χ is the IHHNV cycle threshold (Ct) value determined using the ΔRn method from QuantStudio™ Design & Analysis Software v1.5.2 (ThermoFisher Scientific™, www.thermofisher.com)

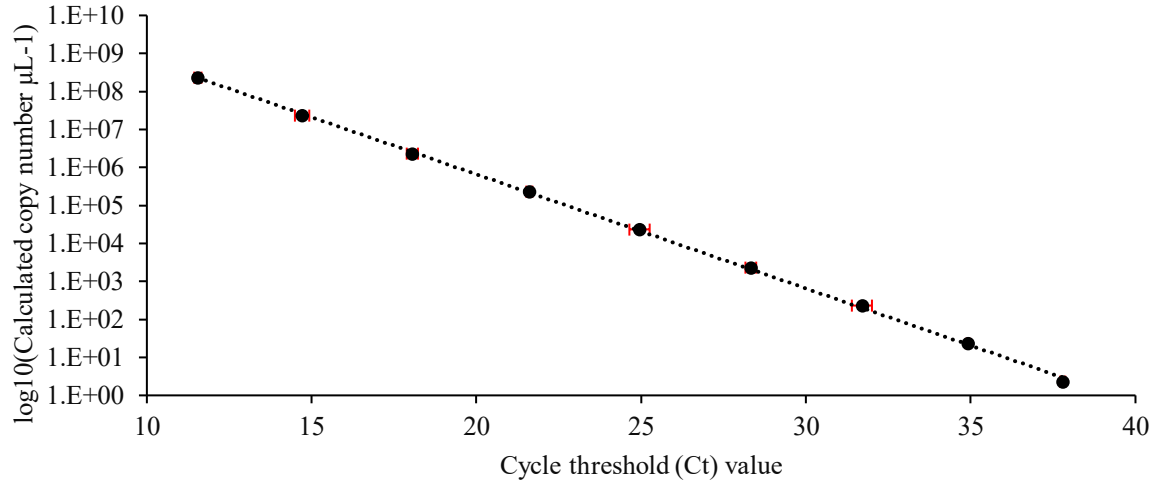


Figure 3.3. Standard qPCR curve for conversion of cycle threshold (Ct) value to calculated copy number (μL^{-1}) on a logarithmic scale for gill-associated virus (GAV), generated using a 10-fold serial dilution of gBlock™ Gene Fragments. Standard deviation of Ct value across the three technical replicates for each dilution are shown in red. The 10th dilution was not detected in any of the triplicate qPCR tests.

Equation 3.3. Gill associated virus (GAV) cycle threshold (Ct) value to copy number (μL^{-1}) standard curve equation

$$\text{GAV copy number } (\mu\text{L}^{-1}) = 673,260,590,458.418 \times e^{-0.691881 \times \chi}$$

Where χ is the GAV cycle threshold (Ct) value determined using the ΔRn method from QuantStudio™ Design & Analysis Software v1.5.2 (ThermoFisher Scientific™, www.thermofisher.com)

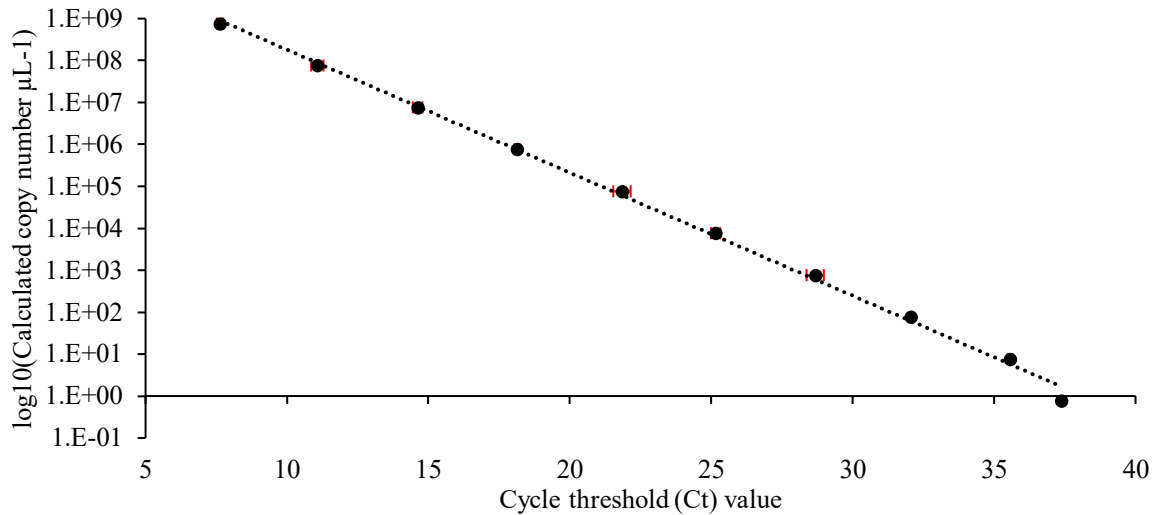


Figure 3.4. Standard qPCR curve for conversion of cycle threshold (Ct) value to calculated copy number (μL^{-1}) on a logarithmic scale for yellowhead virus 7 (YHV-7), generated using a 10-fold serial dilution of gBlock™ Gene Fragments. Standard deviation of Ct value across the three technical replicates for each dilution are shown in red. The 10th dilution was only detected in two of the triplicate qPCR tests.

Equation 3.4. Yellowhead virus 7 (YHV-7) cycle threshold (Ct) value to copy number (μL^{-1}) standard curve equation

$$\text{YHV7 copy number } (\mu\text{L}^{-1}) = 155,481,715,224.916 \times e^{-0.67542 \times \chi}$$

Where χ is the YHV-7 cycle threshold (Ct) value determined using the ΔR_n method from QuantStudio™ Design & Analysis Software v1.5.2 (ThermoFisher Scientific™, www.thermofisher.com)

Correlation of the Ct values derived from the gBlock™ serial dilutions and the log₁₀(calculated copy number μL^{-1}) values yielded linear relationships described by the equations within Table 3.2, with the form $y = mx + c$, where y defines any given Ct value detected for the a given gBlock™ calculated copy number (x), m defines the slope of the linear relationship and c is the y intercept. Using these parameters, calculation of the qPCR efficiency was conducted as described by Equation 3.5. Efficiency values for qPCR should range between 90-100% (thermofisher.com). The linear relationships describing each of the standard curves, and their associated calculated efficiency are presented in Table 3.2.

Equation 3.5. qPCR efficiency calculation

$$\text{Efficiency} = 10^{\left(\frac{-1}{\text{slope}}\right)} - 1$$

Table 3.2. Linear standard curve equations describing the correlation of Ct values derived from 10-fold gBlock™ serial dilutions and the $\log_{10}$ (calculated copy number μL^{-1}) for GAV, IHNV and YHV-7 assays. For each standard curve, the correlation coefficient (R^2) and qPCR efficiency (Equation 3.5.) were calculated.

Target	Linear standard curve	R^2	qPCR efficiency
IHNV	$y = -3.4535x + 39.38$	0.9996	95%
GAV	$y = -3.314x + 39.478$	0.9992	100%
YHV-7	$y = -3.4295x + 38.304$	0.9997	96%

3.3.6 Statistical analysis

Statistical analysis of qPCR detection data was conducted using R (R Core Team, 2022). Calculated target copy number μL^{-1} TNA extract was standardised to target copy number mg^{-1} tissue for each sample for further analyses. Descriptive statistical parameters were calculated for each target and between tissue types. For individual shrimp, tissues were ranked from 1 to 7, with a rank of 1 representing the tissue yielding the highest copy number mg^{-1} for that specific shrimp, and 7 representing the lowest. The mean and modal rank of each tissue type was calculated for shrimp within each cohort, for each pathogen target. Further statistical analysis to evaluate the differences in copy number and correlation of detection between tissues were conducted using $\log_{10}$ transformed copy number mg^{-1} tissue, after zero values were adjusted to one. Significant differences in IHNV and YHV-7 detection between the various tissue types were determined using an ANOVA with Tukey HSD post-hoc test. Differences in GAV detections were determined using Kruskal-Wallis analysis with Dunn test post-hoc analysis (Ogle et al., 2022) due to significant deviation from homogeneous variances, as determined via a Levene's test (Fox and Weisberg, 2019). The statistical relationship of target detections between the various tissue types for individual shrimp was determined using Pearson's correlation analysis (Harrell, 2023).

3.4 Results

3.4.1 Overall detection of pathogens

Detection of IHNV across all tissue types ranged between 1.2×10^4 and 1.58×10^8 copies mg^{-1} tissue, yielding the highest average copy number of the three targets analysed (mean $\pm$ SD; $2.2 \times 10^7 \pm 3.15 \times 10^7$ copies mg^{-1} tissue). YHV-7 detection across all tissue types ranged between 1.3 and 9.66×10^6 copies mg^{-1} tissue, yielding an average detected copy number of $4.6 \times 10^5 \pm 1.49 \times 10^6$ copies mg^{-1} tissue (mean $\pm$ SD). GAV was detected at the lowest average copy number across all tissue types of the three viral targets analysed. GAV detections ranged from no detection to 3.52×10^6 copies mg^{-1} tissue (mean $\pm$ SD; $7.4 \times 10^4 \pm 4.28 \times 10^5$ copies mg^{-1} tissue). Tissue-specific detection levels for the shrimp analysed in this study are detailed in Table 3.3.

3.4.2 IHNV

Detection of IHNV was the highest and most consistent across tissue types for all pathogen targets analysed, ranging between $\sim 10^4$ and 10^8 copies mg^{-1} tissue (Table 3.3), with a maximum $\log_{10}$ fold difference in copies mg^{-1} tissue of 1.5 between tissues of the same shrimp (TAIL: 5.06×10^6 , LO: 1.53×10^8). Overall, per mg of tissue, average IHNV copy number was highest in GILL, followed sequentially by HG, LO, PLEO, HP, NER, and TAIL (Figure 3.5A). Mean $\log_{10}$ IHNV copy number mg^{-1} tissue varied significantly between tissue types ($f_6 = 4.39$, $p = 0.000915$). On average, GILL yielded significantly higher detection of IHNV than both NER ($p = 0.013$) and TAIL ($p = 0.003$). HG yielded significantly higher detection than TAIL ($p = 0.022$). When tissues were ranked to represent highest to lowest copy number detected within individual shrimp, on average, GILL ranked as the best tissue, followed by HG, PLEO, HP, LO, NER and TAIL. Analysing tissue ranking for individual shrimp, as opposed to observing the average IHNV copy number for each tissue across the whole cohort, HP and PLEO were revealed to be more consistently advantageous for the detection of IHNV than LO, despite yielding lower average copy number across the cohort. IHNV copy number mg^{-1} were significantly linearly correlated between all tissue types within individual shrimp ($r \geq 0.65$, $p \leq 0.05$; Figure 3.5B).

Table 3.3. IHNV, GAV and YHV-7 detection in different tissues of naturally infected *Penaeus monodon* (GILL = gill, HG = hindgut, HP = hepatopancreas, LO = lymphoid organ, NER = ventral nerve cord, PLEO = pleopod, TAIL = tail muscle). Tissue quantity (mg) used in TNA extraction for each shrimp is given as mean $\pm$ SD for each tissue type. Mean and modal tissue rank (1 = highest detection to 7 = lowest detection) from individual shrimp was calculated for each pathogen across all shrimp, within each relevant cohort (IHNV and GAV in cohort 1, YHV-7 in cohort 2).

Tissue		IHNV copy mg ⁻¹ tissue			GAV copy mg ⁻¹ tissue			YHV-7 copy mg ⁻¹ tissue		
Type	Weight (mg) mean $\pm$ SD	Rank mean (mode)	Mean $\pm$ SD	Min - max	Rank mean (mode)	Mean $\pm$ SD	Min - max	Rank mean (mode)	Mean $\pm$ SD	Min - max
GILL	40.76 $\pm$ 15.82	1.6 (1)	5.0 $\times 10^7 \pm$ 4.15 $\times 10^7$	1.4 $\times 10^6$ - 1.05 $\times 10^8$	4.2 (6)	2.3 $\times 10^2 \pm$ 3.49 $\times 10^2$	1.8 $\times 10^1$ - 1.17 $\times 10^3$	3.8 (3)	1.3 $\times 10^5 \pm$ 1.98 $\times 10^5$	3.3 $\times 10^1$ - 6.35 $\times 10^5$
HG	90.13 $\pm$ 33.88	2.5 (2)	2.9 $\times 10^7 \pm$ 2.52 $\times 10^7$	1.9 $\times 10^5$ - 6.79 $\times 10^7$	2 (2)	7.6 $\times 10^4 \pm$ 1.15 $\times 10^5$	9.2 $\times 10^0$ - 3.32 $\times 10^5$	2.1 (2)	3.3 $\times 10^5 \pm$ 4.45 $\times 10^5$	1.2 $\times 10^1$ - 1.44 $\times 10^6$
HP	144.62 $\pm$ 58.31	3.6 (4)	2.1 $\times 10^7 \pm$ 1.81 $\times 10^7$	3.5 $\times 10^5$ - 4.66 $\times 10^7$	4.1 (5)	2.5 $\times 10^3 \pm$ 4.33 $\times 10^3$	6.7 $\times 10^{-1}$ - 1.09 $\times 10^4$	4.8 (5)	3.1 $\times 10^4 \pm$ 3.28 $\times 10^4$	1.5 $\times 10^0$ - 9.90 $\times 10^4$
LO	23.75 $\pm$ 10.24	4 (5)	2.4 $\times 10^7 \pm$ 4.80 $\times 10^7$	1.7 $\times 10^5$ - 1.58 $\times 10^8$	2.2 (1)	4.4 $\times 10^5 \pm$ 1.10 $\times 10^6$	undetected - 3.52 $\times 10^6$	1.8 (1)	2.6 $\times 10^6 \pm$ 3.27 $\times 10^6$	9.4 $\times 10^0$ - 9.66 $\times 10^6$
NER	80.47 $\pm$ 15.78	6.2 (6)	5.2 $\times 10^6 \pm$ 6.92 $\times 10^6$	1.2 $\times 10^4$ - 2.09 $\times 10^7$	5.6 (7)	2.2 $\times 10^2 \pm$ 3.64 $\times 10^2$	undetected - 1.15 $\times 10^3$	6.6 (7)	5.8 $\times 10^3 \pm$ 8.66 $\times 10^3$	1.3 $\times 10^0$ - 2.74 $\times 10^4$
PLEO	41.93 $\pm$ 15.93	3.5 (4)	2.2 $\times 10^7 \pm$ 2.96 $\times 10^7$	6.8 $\times 10^5$ - 8.95 $\times 10^7$	4.9 (7)	8.9 $\times 10^1 \pm$ 1.13 $\times 10^2$	6.4 $\times 10^0$ - 3.83 $\times 10^2$	3.8 (4)	8.8 $\times 10^4 \pm$ 1.04 $\times 10^5$	3.0 $\times 10^1$ - 2.42 $\times 10^5$
TAIL	9.36 $\pm$ 4.54	6.6 (7)	2.1 $\times 10^6 \pm$ 2.01 $\times 10^6$	1.4 $\times 10^4$ - 5.06 $\times 10^6$	4.6 (5)	4.6 $\times 10^2 \pm$ 7.77 $\times 10^2$	undetected - 2.56 $\times 10^3$	5.1 (6)	1.2 $\times 10^4 \pm$ 1.05 $\times 10^4$	3.2 $\times 10^1$ - 2.52 $\times 10^4$

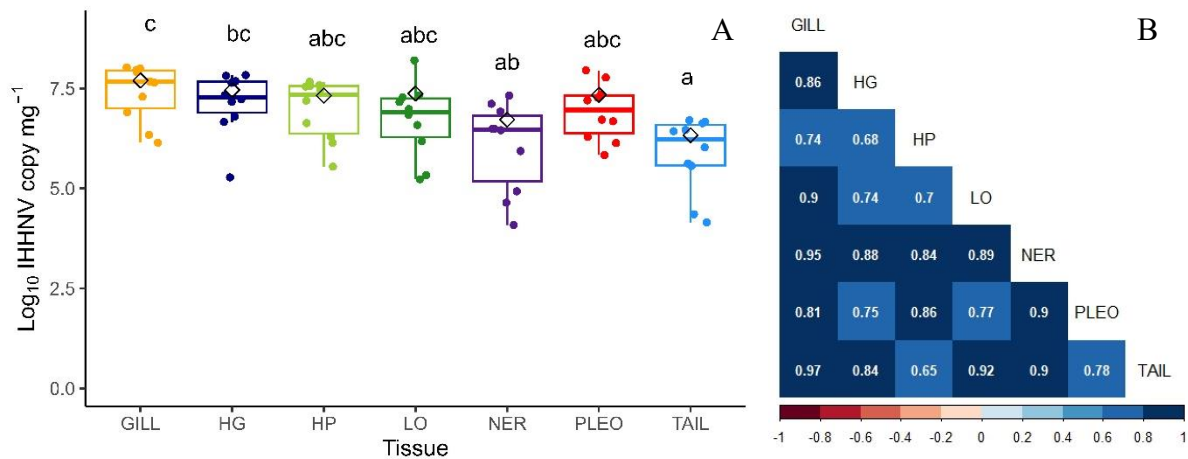


Figure 3.5. (A) IHHNV detection in different tissues of naturally infected *Penaeus monodon*. Each point represents the $\log_{10}(\text{IHHNV copy number mg}^{-1} \text{ tissue})$ calculated from the average cycle threshold value of technical triplicate RT-qPCR. Mean $\log_{10}(\text{IHHNV copy number mg}^{-1} \text{ tissue})$ for each tissue are indicated by a black diamond. Letters indicate significant difference between means ($\alpha = 0.05$). (B) Pearson's correlation coefficient matrix of $\log_{10}(\text{IHHNV copy number mg}^{-1} \text{ tissue})$ between tissue types. (GILL = gill, HG = hindgut, HP = hepatopancreas, LO = lymphoid organ, NER = ventral nerve cord, PLEO = pleopod, TAIL = tail muscle).

3.4.3 GAV

Detected GAV was lower than the detected IHHNV copies mg^{-1} tissue from cohort 1 (GAV mean copy number mg^{-1} tissue $\sim 10^4$; Table 3.3). Detection across tissue types was also less consistent for GAV, ranging from undetected to 10^6 copies mg^{-1} tissue, with a maximum $\log_{10}$ fold difference of 4.4 between tissues of the same shrimp (HP: 1.33×10^2 , LO 3.52×10^6). Per mg of tissue, average GAV copy number was highest in LO, followed sequentially by HG, HP, TAIL, GILL, NER and PLEO (Figure 3.6A). Despite the apparent inconsistency of GAV detection levels between tissue types, mean $\log_{10}$ GAV copy mg^{-1} tissue did not significantly vary. When tissues were ranked to represent highest to lowest copy number detected within individual shrimp, on average, HG ranked as the best tissue, followed by LO, HP, GILL, TAIL, PLEO and NER. Analysing tissue ranking for individual shrimp compared to observing the average GAV copy number for each tissue across the cohort, HG was revealed to be more consistently advantageous for the detection of GAV than LO. This is likely strongly influenced by the single shrimp from which GAV was undetected in the LO. Similarly, while the cohort averaged GAV copy number detected from TAIL was higher than detection from GILL, ranking tissues in individual shrimp revealed GILL to be more consistently advantageous, likely due to the two shrimp from which GAV was undetected in TAIL. With the same rationale, PLEO ranked better than NER, despite having lower average copy number across the

cohort. Except for PLEO and GILL, detection levels in the various tissues of the experimental shrimp were significantly linearly correlated with all other tissue types ($r \geq 0.70$, $p \leq 0.05$; Figure 3.6B). While copy number mg^{-1} tissue in PLEO and GILL correlated significantly with one another ($r = 0.66$, $p = 0.04$), GAV load in PLEO did not correlate significantly with any other tissue types. Detection in GILL significantly correlated with tissues excluding HG ($r = 0.29$) and HP ($r = 0.47$).

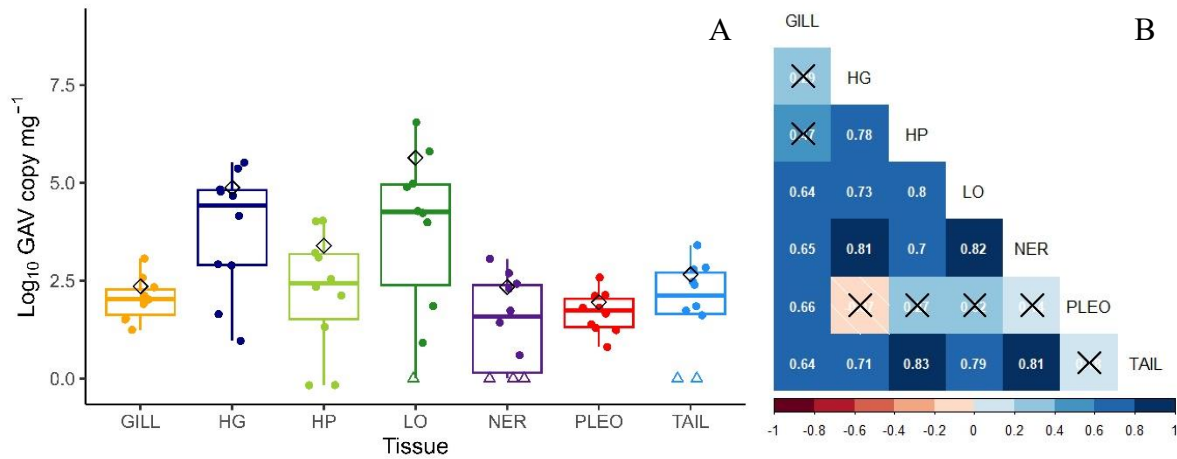


Figure 3.6. (A) GAV detection in different tissues of naturally infected *Penaeus monodon*. Each point represents the $\log_{10}(\text{GAV copy number } \text{mg}^{-1} \text{ tissue})$ calculated from the average cycle threshold value of technical triplicate RT-qPCR. Tissue samples where GAV was undetected (assigned zero value copy number) are indicated by a triangle. Mean $\log_{10}(\text{GAV copy number } \text{mg}^{-1} \text{ tissue})$ for each tissue are indicated by a black diamond. No significant difference between means ($\alpha = 0.05$) were found. (B) Pearson's correlation coefficient matrix of $\log_{10}(\text{GAV copy number } \text{mg}^{-1} \text{ tissue})$ between tissue types. Non-significant correlations ($\alpha = 0.05$) are marked by a cross. (GILL = gill, HG = hindgut, HP = hepatopancreas, LO = lymphoid organ, NER = ventral nerve cord, PLEO = pleopod, TAIL = tail muscle).

3.4.4 YHV-7

Detection of YHV-7 across tissue types ranged between <10 and 10^6 copies mg^{-1} tissue (Table 3.3), with a maximum $\log_{10}$ fold difference of 2.6 between tissues of the same shrimp (GILL: 2.13×10^4 , LO: 9.66×10^6). Per mg of tissue, average YHV-7 copy number was highest in LO, followed sequentially by HG, GILL, PLEO, HP, TAIL and NER (Figure 3.7A). No significant differences were observed for mean $\log_{10}$ YHV-7 copy number mg^{-1} tissue between tissue types. When tissues were ranked to represent highest to lowest copy number detected within individual shrimp, rankings reflected the order of tissues defined by the cohort average YHV-7 copy number mg^{-1} tissue. YHV-7 detection between all tissue types were highly significantly correlated ($r \geq 0.86$, $p \leq 0.001$; Figure 3.7B).

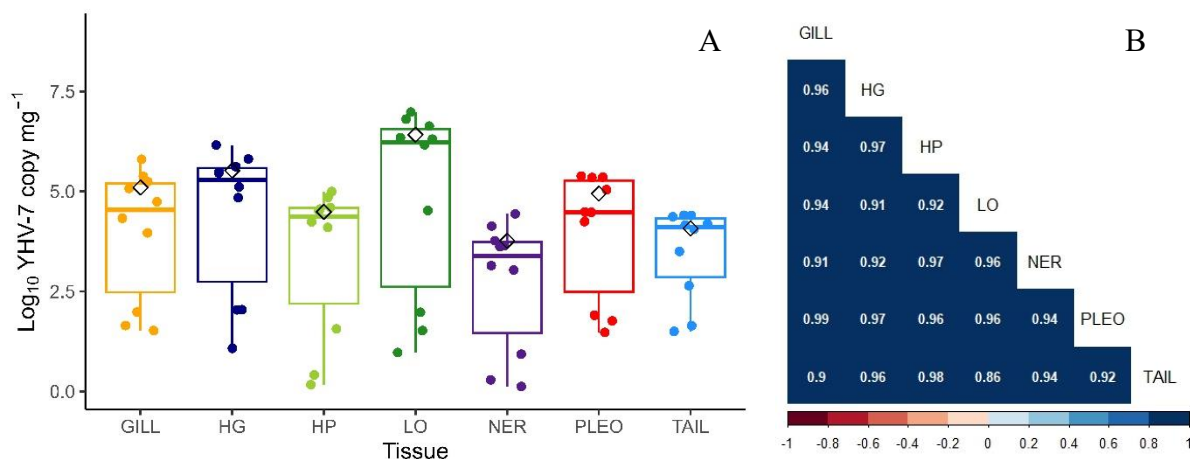


Figure 3.7. (A) YHV-7 detection in different tissues of naturally infected *Penaeus monodon*. Each point represents the $\log_{10}(\text{YHV-7 copy number } \text{mg}^{-1} \text{ tissue})$ calculated from the average cycle threshold value of technical triplicate RT-qPCR. No significant difference between means ($\alpha = 0.05$) were found. (B) Pearson's correlation coefficient matrix of $\log_{10}(\text{YHV-7 copy number } \text{mg}^{-1} \text{ tissue})$ between tissue types. (GILL = gill, HG = hindgut, HP = hepatopancreas, LO = lymphoid organ, NER = ventral nerve cord, PLEO = pleopod, TAIL = tail muscle).

3.4.5 Dicer-1

Dicer-1 was detected consistently from all tissues analysed. No negative correlations between the quantity of tissue used for TNA extraction and the level of Dicer-1 detected were observed within any of the tissue types analysed, suggesting that there was no significant inhibition of DNA extraction or PCR from co-precipitated specimen matrix inhibitors.

3.5 Discussion

This study aimed to generate contemporary evidence of viral distribution patterns of IHHNV, GAV and YHV-7 using qPCR detection, in naturally infected *P. monodon*. The findings of this study provide an enhanced, modern understanding of infection patterns to improve detection for surveillance and disease diagnosis purposes. This work will also be valuable to support ongoing maintenance and modernisation of the Aquatic Manual, in alignment with the 2021 WOAHS Strategy on Aquatic Animal Health.

3.5.1 IHHNV

IHHNV was listed in the first edition of the Aquatic Code and is the only WOAHS listed pathogen that is frequently detected in the Australian shrimp aquaculture industry (Arbon et al., 2022; OIE, 1995; WOAHS, 2023b). IHHNV is generally described as a systemic virus, infecting tissues of ectodermal and mesodermal origin, and as such, does not replicate in endodermal or enteric tissues. Accordingly, the Aquatic Manual specifically defines hepatopancreas and midgut tissues as unsuitable for surveillance and diagnosis of IHHNV (WOAHS, 2023b). The historical studies cited to support this exclusion assert that such tissues (endoderm-derived hepatopancreas, midgut and midgut caeca) show no histological signs of infection with IHHNV and are usually negative by *in-situ* hybridisation (ISH) (Lightner, 1993; Lightner et al., 1983b, 1983a; Lightner and Redman, 1998; OIE, 2019a; WOAHS, 2023b). Histology is often used to detect IHHNV-specific Cowdry type A inclusion bodies, which are considered pathognomonic for IHHNV infection. However, recent studies have since confirmed that tissues naturally and experimentally infected with IHHNV frequently lack corroborative observation of Cowdry type A inclusion bodies and positive reaction by ISH, especially in *P. monodon* and *P. stylirostris*, or when infection loads are low (Aranguren Caro et al., 2022; Chayaburakul et al., 2005). As such, determining IHHNV infection status using histopathological observation of Cowdry type A inclusions or ISH may potentially yield false negative results. Therefore, molecular detection of IHHNV represents a more reliable means for determining IHHNV infection status.

There is growing evidence to substantiate the suitability of hepatopancreatic tissue as a target tissue for detection of IHHNV DNA using molecular techniques. For example, in naturally infected *P. monodon* similar infection levels were detected by PCR between hepatopancreas, heart, haematopoietic tissue, gill, subcutaneous tissue, pleopod, abdominal muscle, ventral

nerve cord, lymphoid organ and haemolymph (Chayaburakul et al., 2005). Severe IHHNV infection of the hepatopancreas was detected by PCR in naturally infected *Macrobrachium rosenbergii*, with corroborating histology and ISH analysis (Hsieh et al., 2006). IHHNV DNA was also detected by PCR from hepatopancreas tissue in frozen commodity *Cherax quadricarinatus* (Lee et al., 2021). Most recently IHHNV was detected in hepatopancreas of experimentally challenged *Penaeus vannamei* by qPCR (Hou et al., 2023). In these studies, IHHNV was detected more reliably in hepatopancreas tissue, compared to other target tissues including gill (Lee et al., 2021), pleopod, and muscle tissue (Hou et al., 2023; Hsieh et al., 2006). In our study, detection of IHHNV from hepatopancreas yielded high copy number mg^{-1} tissue, with significant linear correlation to detection in other target tissues including the gill, lymphoid organ and pleopod. Our findings enhance the existing body of evidence, confirming that PCR-based detection of IHHNV from the hepatopancreas is possible and reliable. This evidence supports consideration for revision of current recommendations within the Aquatic Manual. Specifically, that hepatopancreas be considered as a suitable tissue type for the detection of IHHNV.

Detection of IHHNV in the hindgut also yielded high IHHNV copy number mg^{-1} tissue, with significant linear correlation to detection in other target organs. This finding contrasts the Aquatic Manual's classification of enteric tissues as unsuitable for IHHNV surveillance and diagnostics. Nevertheless, our results suggest the potential suitability of this tissue, under the examined and specific conditions of this study. However, it is important to caution that commercial shrimp feeds frequently contain detectable template of shrimp pathogens (reviewed in Tacon, 2017). The potential for feed present in the gut of shrimp, containing non-viable pathogen template, to confound pathogen detection from hindgut samples should be considered. This study recognises the possible impact of this occurrence, especially since the timing between shrimp sampling and preceding feeding instances was not controlled. While this limitation is acknowledged, for all pathogen targets analysed, detection in the hindgut was significantly correlated with detection in other examined target organs. This strong correlation implies that any residual feed within the hindgut had minimal to no impact on the accurate detection of IHHNV, GAV, or YHV-7. Nonetheless, the potential for non-viable viral template in feed to erode the reliability of pathogen detection from the hindgut must be considered in result interpretation from this tissue.

The present study found IHHNV in the ventral nerve cord and tail muscle tissue to be significantly lower than other target organs, including gills. Chayaburakul et al. (2005) also found that the ventral nerve cord and muscle tissue of *P. monodon* were among the lowest ranking tissues for the detection of IHHNV, compared to eight other tissue types. Similarly, IHHNV detected in the tail muscle of *P. vannamei* in the study by Hou et al. (2023), yielded the lowest average copy number mg^{-1} tissue of all tissue types analysed. These findings together confer that muscle tissue and ventral nerve cord may be less suitable for reliable diagnostics and surveillance of IHHNV at lower viral loading, or early in infection progression. The ventral nerve cord and ganglia are currently recommended as suitable target tissue for molecular detection of IHHNV within the Aquatic Manual (WOAH, 2023b). Based on our analysis, and the additional works referenced, we suggest that sampling of nerve tissue is not advantageous for detection of IHHNV compared to other, more easily sampled tissue types such as gill or pleopod.

When considering non-destructive tissue sampling, gill and pleopod tissues are recommended for the detection of IHHNV (WOAH, 2023b). Similar detection of IHHNV from pleopod and gill tissues was observed in a comparable study by Hou et al. (2023) in *P. vannamei*. In the present study, both gill and pleopod yielded high IHHNV copy number mg^{-1} tissue, were ranked among the best tissues, and detection between the two tissue types was highly correlated. This evidence suggests that gill or pleopod tissue would provide similar detection sensitivity for surveillance and diagnosis of IHHNV infection. Given the susceptibility of gill filaments to further damage during sampling (Mitchell et al., 2023), pleopod is likely preferential for non-lethal tissue sampling for IHHNV detection, despite gill tissue yielding negligibly increased copy number mg^{-1} .

3.5.2 YHV (Genotype 2 and 7)

Yellow head virus (YHV-1) has long been considered a significant pathogen of global shrimp aquaculture and was accordingly listed in the first edition of the Aquatic Code (OIE, 1995). While YHV-1 has never been reported from Australia, local variants of the yellow head complex including yellow head virus genotype 7 (YHV-7) and yellow head virus genotype 2, *syn* gill associated virus (GAV), are considered endemic within Australia.

Based on sequence analysis of ORF1b, the seventh distinct YHV genotype (YHV-7) shares highest similarity to YHV-1 (Mohr et al., 2015). YHV-7 was discovered in association with elevated mortalities among broodstock *P. monodon* originating from Northern Australia in 2012 (Mohr et al., 2015), and has since been detected in *P. monodon* sampled from various regions across Northern Australia between 2013 and 2019 (Arbon et al., 2022; Cowley et al., 2015, 2019). Pathogenicity of YHV-7 was demonstrated in *P. monodon* by experimental inoculation, resulting in cumulative mortality of approximately 60% at 28 days post-infection (Moody and Crane, 2016). YHV-7 outbreaks have also been associated with sporadic disease events in pond reared *P. monodon* (Cowley et al., 2019). As yet, YHV-7 has not been reported outside of Australia. Given the relatively modern discovery and its current containment to Australia, there are no known studies which investigate the tissue tropism of YHV-7. From the few studies published reporting the detection of YHV-7, detection has only been reported from pleopod, gill and epidermis tissues (Arbon et al., 2022; Cowley et al., 2019; Mohr, 2020; Mohr et al., 2015). Due to its apparent pathogenicity as indicated by both its association with mass mortality events on farm, and the experimental challenge conducted, current Australian shrimp aquaculture industry practice prioritises removal of broodstock with positive YHV-7 detection by RT-qPCR. As such, it is important to elucidate its tissue tropism for optimised detection by RT-qPCR.

YHV-7 and GAV are not listed in the Aquatic Code or Manual, therefore, no guidelines describing the selection of target tissues for their detection are available. However, given their relatedness to YHV-1 and current taxonomic classification within the same viral complex, the recommendations established for YHV-1 will be used as a proxy for YHV-7 and GAV (Mohr et al., 2015; Munro and Owens, 2007; Wijegoonawardane et al., 2009). These pathogens will herein be collectively referred to as yellow head viruses (YHV).

YHV is known to target tissues of the ectoderm and mesoderm (De La Vega et al., 2004; Munro and Owens, 2007; WOA, 2023b). While lymphoid organ is determined to be the primary target organ of YHV replication, the presence of YHV has been historically confirmed by bioassay, TCID₅₀ assay, Monoclonal Antibody, PCR, RT-qPCR, and classical histopathology in shrimp tissues including gill, gut, muscle, heart, nerve cord, hepatopancreas, haematopoietic tissue and haemocytes, connective tissue, eyestalk and gonads, and hence YHV is considered a systemic virus (Chantanachookin et al., 1993; Cowley et al., 2002b, 2001; Lu et al., 1995;

Soowannayan et al., 2002). To this body of evidence, we add the supporting results of the present study, which demonstrate non-significant differences in viral loading for both YHV genotypes (2 and 7) across pleopod, gill, lymphoid organ, hepatopancreas, hindgut, ventral nerve cord and abdominal muscle tissue. While no significant differences in average copy number mg^{-1} tissue were detected, a clear advantage of lymphoid organ was observed for detection of YHV-7 and GAV, yielding the highest average copy number mg^{-1} tissue of the seven tissues analysed. Accordingly, this study supports the WOAHA recommendation of lymphoid organ for detection of YHV (WOAHA, 2023b).

Given the systemic nature of YHV, currently, specific tissues that are unsuitable for the detection of YHV are not determined (WOAHA, 2023b). While detection of both YHV genotypes was statistically consistent among tissue types in the present study, there were clear disadvantages associated with sampling and analysis of ventral nerve cord and muscle tissues. This was especially evident for detection of GAV, where the overall range of detected GAV was lower, possibly reflective of sub-acute, chronic, or tolerated infection within the cohort. Based on GAV detection under these conditions, the results of the present study indicate that ventral nerve cord and tail muscle are least suitable for YHV detection as they may yield false negative results.

Gill and haemolymph are recommended as the most suitable samples for detection of YHV using non-destructive methods (WOAHA, 2023b). While detection of YHV genotypes 2 and 7 from haemolymph was not analysed in the present study, previous studies have strongly established its utility for reliable detection of YHV using RT-PCR and RT-qPCR (De La Vega et al., 2004; Kiatpathomchai et al., 2004; Ma et al., 2008). Detection of YHV genotypes 2 and 7 from gill tissue was analysed in the present study. Under the conditions of our analysis, consistent detection of GAV and YHV-7 between gill and pleopod tissues was observed, with strong correlation of detection within individual shrimps. Similar observations were made by Noble et al. (2018), where gill and pleopod tissues were demonstrated to yield similar GAV loading and variability from naturally infected *P. monodon*. As was apparent for the detection of IHHNV, this evidence suggests that gill or pleopod tissue would provide comparable detection sensitivity for surveillance and diagnosis of YHV infection.

3.5.3 Industry practices and implications

Based on the conditions analysed in the present study, lymphoid organ and hindgut consistently ranked among the best target tissues for IHHNV and YHV genotype 2 and 7. Targeting of lymphoid organ is recommended for the detection of IHHNV and YHV under surveillance conditions, when shrimp appear grossly normal (WOAH, 2023b). Our findings support this strategy for the detection of YHV-7, YHV-2 (GAV), and IHHNV, when destructive sampling is permissible. The observed advantage of the lymphoid organ is likely related to its function in clearance of foreign material from the haemolymph (Rusaini and Owens, 2010). The involvement of the lymphoid organ in shrimp antiviral and antibacterial immunity potentiates it as a sensitive target tissue for detecting a range of pathogens, or their degraded products, in broader non-targeted screening and surveillance applications.

However, the lymphoid organ in Penaeid shrimp is relatively small (1-4 mm in diameter), and its size and position within the shrimp cephalothorax can vary with shrimp species, sex, maturation stage, size, and health status (reviewed in Rusaini and Owens, 2010). Although there are advantages of sampling the lymphoid organ for detection sensitivity, sampling of the lymphoid organ can be challenging due to the small size and inconsistencies in its location within any given shrimp. Reliable collection of the lymphoid organ can become especially challenging when sampling is required from shrimp of a small size, shrimp which have been freeze-thawed, or when sampling is being conducted by untrained staff. Furthermore, for large-scale sampling, targeting such an organ becomes labour intensive and prone to error.

The findings of this study indicate that sampling of hindgut may permit a similar level of detection sensitivity to lymphoid organ for IHHNV and YHV. Given the difficulties of sampling the lymphoid organ, hindgut tissue may be a more practical target tissue for sampling. However, detection of any target from a hindgut sample must consider the presence of non-viable viral template in remnant feed within the hindgut (Tacon, 2017). Further work is required to elucidate the utility of the hindgut with respect to these factors, and throughout infection progression.

Detection from gills yielded the highest viral copy number of the non-destructive sample types assessed for all pathogen targets analysed. The highly correlated and comparable level of detection from pleopods and gills indicates that detection from pleopods will likely provide a similar level of detection sensitivity as gill tissue (Noble et al., 2018). Pleopods are already

currently sampled from shrimp broadly across the shrimp aquaculture industry for routine pathogen screening (Arbon et al., 2022). Furthermore, sampling of gill tissue is more labour and skill intensive, and can frequently result in extensive damage to the sensitive gill structures (Mitchell et al., 2023). Continued use of pleopods for detection of IHNV, as aligned with WOA recommendations, and the addition of pleopods as a recommended target tissue for detection of YHV is expected to achieve comparable detection sensitivity while reducing the sampling burden on industry and potential damage to stock during sampling.

In addition to improving the detection of pathogens for surveillance and disease diagnosis, having an accurate understanding of virus distribution in shrimp tissues is an important consideration when devising risk management protocols to prevent the global transfer of shrimp pathogens within uncooked crustacean product (Lee et al., 2021). For example, Australia currently permits importation of uncooked shrimp product, given compliance of the imported product to specific conditions. The conditions permitting the import of uncooked shrimp product dictate the shrimp must be frozen, have head and shell removed, be deveined, and if not from a disease-free source, test free from WSSV and YHV-1 (DAF, 2023a). While batch testing to confirm freedom of WSSV and YHV-1 provides adequate protection against importation of high risk material in most instances, this protection can be undermined by non-compliance with testing conditions by importers, weak border security assessment and inspection procedures, and variation in laboratory testing procedures and interpretation (Scott-Orr et al., 2017). In these cases, where testing cannot achieve reliable or complete protection, and for other pathogens beyond just WSSV and YHV-1, removal of the head, shell and hindgut from frozen shrimp is expected to reduce biosecurity risk to an appropriate level. In the present study, detection of the yellow head viruses was statistically similar from muscle tissue to other target tissues which are considered higher risk, including lymphoid organ, hepatopancreas and hindgut. These results indicate that current measures to de-risk importation of uncooked crustacean product into Australia, by removal of the head, shell, and deveining, may be inadequate to reduce exposure to yellow head virus, and other potential pathogens of consequence beyond those currently tested for (YHV-1 and WSSV) to confirm freedom. Further research is warranted to extend this evidence to pathogens of concern to national border security.

3.5.4 Considerations of experimental design

Biological and technical variability can produce differences in viral detection using qPCR across distinct tissue types. Biological variability relates to divergent virion concentration in different tissues due to complex host-pathogen interactions (referred to as tissue tropism). Technical variability stems from potential errors arising during sampling and analysis, such as procedural or human error, as well as the technical capabilities and limitations of the technologies, including nucleic acid extraction platforms and protocols. To ensure the fidelity of this study's findings in reflecting biological variation of pathogen loading among tissues, various measures were taken to minimise the impact of potential technical variations on the analysis. Specifically, all sampling and analysis was conducted in compliance with international standard ISO/IEC 17025:2017 (ISO, 2017), and nucleic acid extraction and testing plans were designed to minimise potential inter-run or inter-batch reagent variation on subsequent tissue specific pathogen quantitation. Additionally, the potential for non-biological variability of viral quantification arising from the presence of PCR inhibitors within the different tissue samples was evaluated and considered.

Shrimp tissues including the compound eye and hepatopancreas are known to contain PCR inhibitors such as melanin-type compounds and polyphenols (Lin et al., 2022; Lo et al., 1997; Schrader et al., 2012; Wang et al., 1996), which may be co-extracted with target nucleic acid. Such inhibitors interfere with PCRs, preventing target amplification via interactions with the template, polymerase, or other reaction components (Buckwalter et al., 2014; Opel et al., 2010). Many modern commercial nucleic acid extraction and purification kits/protocols, including those employed in the present study, are increasingly robust against co-extraction of inhibitors and incorporate multiple measures to achieve this (e.g. use of solid-phase magnetic bead extraction (Bordelon et al., 2013), addition of a chaotropic agent such as guanidinium thiocyanate (Nelson and Krawetz, 1992), digestion with proteinase K (Rezadoost et al., 2016), and multiple staged wash phases (Jue et al., 2020)). The presence of PCR inhibitory compounds in shrimp tissues, including the hepatopancreas, has historically impacted the reliability of PCR analysis, and as such, these tissues are precluded from recommended diagnostic and surveillance samples types for certain WOA listed diseases (Moody and Mohr, 2022; Richards, 1999; WOA, 2023b). However, for enteric diseases such as acute hepatopancreatic necrosis disease (AHPND) and infection with *Hepatobacter penaeii*, the Aquatic Manual recommends the targeted use of hepatopancreas for surveillance and diagnosis using qPCR

(WOAH, 2023b). While modern extraction chemistry and protocols are increasingly robust to the presence and impact of inhibitors, in these instances where recommended target tissues may contain inhibitors, the inclusion of an internal process control (e.g. qPCR assay targeting a shrimp house-keeping gene) is required to demonstrate that the nucleic acid extracted from these tissues is free from inhibitors (Moody and Mohr, 2022; Schrader et al., 2012). In the present study, the *P. monodon* Dicer-1 gene (*syn* Pm Dcr1) was used as an internal control. Dicer-1, involved in the crustacean RNA interference pathway, was selected due to its systemic and independent expression from viral genetic loads in shrimp naturally infected with GAV (Su et al., 2008). Within all examined tissue types, no significant, or notable non-significant negative correlations were observed between the detection levels of the Dicer-1 and the quantity of tissue used in nucleic acid extraction and purification. This measure, indicative of the potential presence of co-precipitated inhibitors, demonstrates either the absence of inhibitors in the purified template or the robustness of subsequent qPCR analysis to their influence. In either case, the absence of a discernible effect supports the robustness of the results and attribution of the variability observed in pathogen detection between tissue types to biological causes including viral tissue tropism and host-pathogen interactions.

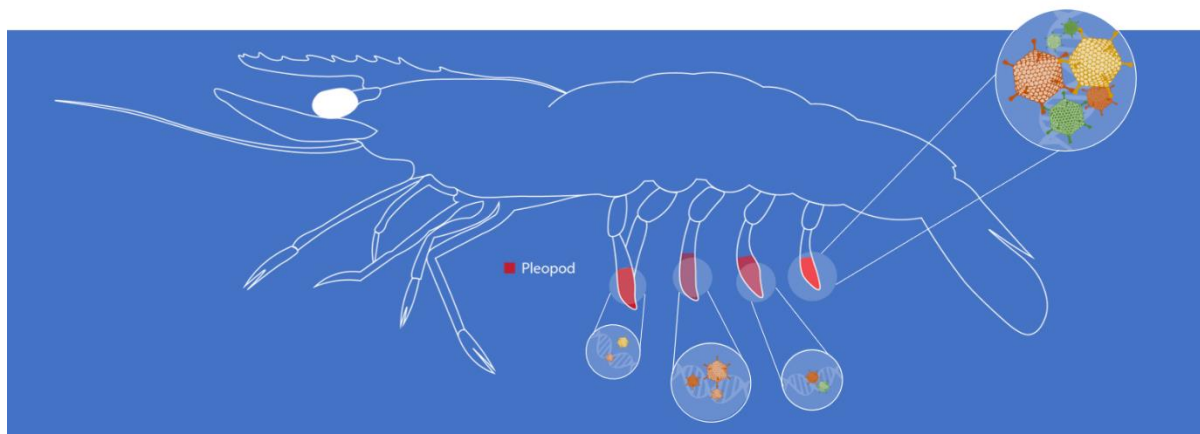
While multiple measures were incorporated to ensure the fidelity of its results with respect to the detection of real biological variation of viral loading between tissues, there are two notable limitations of our study. Firstly, while the detections of each pathogen target were diverse (IHHNV: $\sim 10^4 - 10^8$, YHV-7: $<10 - 10^6$, GAV: undetectable – 10^6 copies mg^{-1} tissue), all shrimp analysed were naturally infected, and were collected from a single production pond at a single timepoint. Consequently, infection progression was not controlled for, or able to be measured. Diagnostic sensitivity from specific tissues may be reduced at lower infection severity, or early in the progress of infection (Chayaburakul et al., 2005; Kim et al., 2023). As such, further analysis may be required to elucidate the qPCR detection patterns observed in the present study at different stages of infection.

The multiple measures conducted to ensure the fidelity of the results, could also be considered as a limiting factor for this study. While the nucleic acid extraction platform (MagMAX™ CORE Nucleic Acid Purification Kit with KingFisher™ Flex 96 Deep-Well Magnetic Particle Processor), qPCR assays (Cowley et al., 2018, 2019; De La Vega et al., 2004; Su et al., 2008), qPCR master mix (SensiFAST™ Probe Lo-ROx Kit and SensiFAST™ Probe Lo-ROx One-

Step Kit) and instrument (QuantStudio™ 5 Real-Time PCR System, 384-well) utilised in this study were selected to yield highly reliable detection, robust to the presence of PCR inhibitors, we recognise that these conditions are not widely attainable. Consequently, it is acknowledged that factors including the presence of inhibitory compounds in shrimp tissues presents ongoing challenges for diagnosis and surveillance of shrimp pathogens when such conditions have not yet been adopted or are not readily available. Extension of these studies to incorporate analysis of detection results from tissues extracted and tested using other platforms and commercially available kits is needed (Buckwalter et al., 2014). Irrespective of the availability of such evidence, it remains essential to consistently implement internal process controls within analysis.

3.5.5 Conclusions

Definition and recommendation of the appropriate target tissue is crucial to enable accurate pathogen detection in both diagnostic and surveillance applications to shrimp aquaculture using real-time PCR analysis. This study reports tissue-specific qPCR detection of genetic viral loads in pleopod, gill, hepatopancreas, lymphoid organ, abdominal muscle tissue, hindgut and ventral nerve cord from *P. monodon* naturally infected with IHHNV, YHV-2/GAV or YHV-7. The results presented confirm detection of high load IHHNV from hepatopancreas tissue and highlight pleopod tissue as a comparably sensitive alternative tissue to gills for the detection of YHV. These findings indicate that current recommendations for target tissues of IHHNV and YHV provided in the WOAHA Aquatic Manual may require revision; however, extension of similar research to support broader review and modification of the Aquatic Manual is required. The results of this study will be valuable for understanding infection patterns of IHHNV and YHV, not only to improve their detection for surveillance and disease diagnosis, but also for consideration when devising risk management protocols to prevent the global transfer of shrimp pathogens within uncooked crustacean product.



Chapter 4.

Evaluating the consistency of pathogen detection from shrimp pleopods using real-time qPCR

PREPARED FOR PUBLICATION

Foreword

Chapter 3 developed critical evidence to support appropriate tissue sampling for the optimised detection of IHHNV, GAV and YHV-7, from naturally infected *P. monodon*. These results highlighted pleopod as a suitable and practical target tissue. Pleopod tissue is currently utilised broadly across shrimp aquaculture as a non-destructive tissue sample for pathogen screening. However, the consistency of viral detection among pleopods of the same shrimp is not well understood. Chapter 4 aimed to thoroughly investigate the detection of IHHNV and GAV from pleopod tissue and generate evidence to guide appropriate pleopod sampling strategies and associated detection result interpretation.

4.1 Abstract

Accurate and reliable pathogen detection is critical for effective shrimp disease management. Pathogen screening using qPCR is routinely conducted on pleopods given they are easily sampled, non-destructive and individually traceable. However, despite their routine use, few studies provide evidence to directly elucidate their suitability for this purpose, especially regarding the representativeness of detection from pleopod to other tissues, and the consistency of detection between multiple pleopods of the same animal. This study investigated the detection of gill-associated virus (GAV) and infectious hypodermal haematopoietic necrosis virus (IHHNV) among pleopods of naturally infected Australian giant black tiger shrimp, *Penaeus monodon* (19.2 ± 4.9 g). TaqMan qPCR detection of IHHNV and GAV was compared between hepatopancreas, gill and five pleopods sampled from individual shrimp. Additionally, various pleopod sampling and replicate testing scenarios were simulated to assess the relationship between sampling effort and the relative gain in detection reliability. Under the conditions examined here, our analysis revealed that quantitative IHHNV detection between pleopods of the same shrimp were consistent ($CV = 0.54 \pm 0.41$) and had strong linear correlation to detection from gill ($R^2 = 0.87$) and hepatopancreas ($R^2 = 0.77$). Comparatively, detection of GAV was significantly more variable between pleopods of the same shrimp ($CV = 0.88 \pm 0.60$), and detection correlation between pleopods and gill ($R^2 = 0.39$), or hepatopancreas ($R^2 = 0.14$) were low. Unlike GAV, IHHNV detection was increasingly consistent between pleopods of shrimp with higher overall loading. However, since infection progression was not controlled for, appropriate caveats should be considered when interpreting this analysis. Based on statistical pleopod sampling simulations, analysis of IHHNV and GAV from a single pleopod may yield false negative results from animals with naturally acquired low viral titre. Therefore, sampling and analysis of multiple pleopods are recommended to improve the reliability of such detections. These findings are important for developing sensitive sampling strategies from non-destructive samples, as is necessitated by SPF-type programs or for screening of valuable stock for rare infections of high consequence. Similarly, ensuring highly representative viral quantification is critical where viral titre is used to inform management decisions or stock exclusion thresholds. The trade-off between improved data quality and the commensurate increases in sampling effort and costs required for its acquisition must be critically evaluated. Further research to define the relationships between detected viral loading for GAV and IHHNV and their relative impacts on productivity and shrimp health are required to facilitate this evaluation.

4.2 Introduction

Global shrimp aquaculture is impacted by both large-scale episodic losses due to highly infectious disease outbreaks (Asche et al., 2021; Shinn et al., 2018) and yield limiting chronic conditions which reduce productivity (Valappil et al., 2021). Mitigating or managing disease impacts is therefore requisite to the sustainable growth and development of global shrimp aquaculture (Asche et al., 2021; Flegel, 2019; Kumar et al., 2021b; Seethalakshmi et al., 2021). Current shrimp disease management heavily emphasises prevention of pathogen entry into production systems, including strict sanitation and biosecurity practices, active pathogen surveillance throughout all phases of hatchery and growout, and the use of specific pathogen free (SPF) or resistant/tolerant (SPR/SPT) selected stock (Alday-Sanz et al., 2018; Flegel et al., 2008; Lightner, 2005; Moss et al., 2012; Poolkhet et al., 2022). Implementation of these strategies, launching heavily in the early 2000's, transformed global production and resulted in rapid expansion of the industry (Flegel, 2019). The development and success of these strategies has been facilitated by improved understanding of host-pathogen interactions in tandem with the development of accurate and reliable diagnostic and surveillance tools (Flegel et al., 2008; Lightner, 2005). Molecular detection tools, such as conventional polymerase chain reaction (PCR) and real-time quantitative PCR (qPCR) provide rapid, sensitive, specific and cost-effective pathogen surveillance and diagnostic capability (WOAH, 2023b). Accordingly, the World Organisation for Animal Health (WOAH, formally OIE) recommends qPCR for the detection and diagnosis of a range of aquatic pathogens, including those impacting shrimp aquaculture (WOAH, 2023b).

Pathogen screening using qPCR is routinely conducted for shrimp based on pleopod samples (Arbon et al., 2022; Chayaburakul et al., 2004; East et al., 2004; Noble et al., 2018; Remany et al., 2012). Excised pleopods provide a non-destructive and individually traceable sample type, that is relatively easy to collect. Therefore, pleopod samples are favourable for large-scale sampling, or sampling of valuable animals such as broodstock. However, despite their routine use, few studies provide evidence to directly elucidate their suitability for this purpose, especially regarding the representativeness of detection from pleopod to other tissues, and the consistency of pathogen detection between pleopods of the same animal (Arbon et al., 2024a; Hou et al., 2023; Noble et al., 2018). The importance of assessing within-host detection variability has been demonstrated for a diversity of host-pathogen models, such as barley and cereal yellow dwarf virus (*Luteoviridae*) detection between leaf or tiller samples of individual

plant specimens (Lacroix et al., 2016; Pereira, 1989) and influenza A detection between left and right nostril swabs of humans (Van Wesenbeeck et al., 2014). For viral detection in shrimp, initial efforts have demonstrated inconsistent pathogen loading among pleopods of the same shrimp can yield misleading detection results or misdiagnosis when a single pleopod is analysed (Noble et al., 2018). Considering the significant consequences of misdetection or misdiagnosis, further research extending these findings is required to improve sampling strategy and appropriate interpretation of pathogen detection results from pleopod samples.

This study investigated the detection consistency among pleopods of the two most commonly detected pathogens in Australian giant black tiger shrimp (*Penaeus monodon*) aquaculture (Arbon et al., 2022; Condon et al., 2020), including gill-associated virus (GAV, *syn.* yellow head virus genotype 2; YHV-2) and infectious hypodermal haematopoietic necrosis virus (IHHNV, *syn.* *Penaeus stylirostris penstyldensovirus* 1; PstDV1). Detection of IHHNV and GAV from naturally infected *P. monodon* was compared between hepatopancreas, gill and five pleopods sampled from individual shrimp, and various pleopod sampling scenarios were simulated to evaluate the relationship between sampling effort and the relative gain in qPCR detection reliability.

4.3 Methods

4.3.1 Sample collection

The shrimp used for this study were overtly healthy *P. monodon* originating from a commercial shrimp farm in Queensland, Australia. Shrimp had been reared in earthen ponds for 100 to 150 days of culture (DOC) and were collected during routine stock assessment throughout the grow-out period within the 2021-22 production season. Shrimp were frozen immediately after collection and stored at -20 °C until required. Shrimp tissue sampling is demonstrated graphically in Figure 4.1. For pleopod sampling, whole shrimp were thawed at 4 °C and all 10 pleopods were excised from each shrimp at the first joint. Excised pleopods were placed directly into 95% ethanol. The sampled shrimp were stored again at -20 °C.

Preliminary qPCR pathogen screening of a single pleopod from each shrimp (N = 219) was used to guide selection of shrimp for further analysis. The guided selection aimed to capture shrimp within a range of detection values for IHHNV and GAV, specifically qPCR cycle threshold (Ct) values between 25 and 40. This Ct range was selected to include individuals with viral loadings typical of those observed in naturally infected Australian *P. monodon* in the absence of disease (Arbon et al., 2022; Condon et al., 2020), and critically, including those approaching and surpassing each assay's theoretical limit of detection (see section 3.3.5, Table 3.2; Cowley et al., 2018; De La Vega et al., 2004). Briefly, a single pleopod was randomly subsampled from each pool of 10 pleopods and dissected to a standardised length of 5 mm from the distal end of the pleopod. The subsampled pleopod clips were immediately transferred into 2 mL lysing matrix D tubes™ (MP Biomedicals) with 350 µL of MagMAX™ CORE Lysis Solution (Applied Biosystems). Nucleic acid extraction and qPCR analysis of the pleopods used for preliminary screening was conducted as described in sections 3.3.3 and 3.3.4 (data not shown).

From the shrimp selected for further analysis (N = 44, 19.22 ± 4.93 g), five pleopods were randomly selected from the remaining nine pleopods stored in 95% ethanol and sub-sampled to a standardised length of 5 mm. Each 5 mm pleopod clip was placed individually into 2 mL lysing matrix D tubes™ (MP Biomedicals) with 350 µL of MagMAX™ CORE Lysis Solution (Applied Biosystems). The respective frozen shrimp were thawed at 4 °C. The right lobe of the hepatopancreas was dissected from each thawed shrimp after dorsoventral dissection. Gill

tissue was also sampled from a subset of 35 shrimp, out of the 44 selected for further analysis. The hepatopancreas and gill tissues were mechanically homogenised, and 100 mg of tissue homogenate was immediately transferred to 2 mL lysing matrix D tubes™ (MP Biomedicals) with 350 µL of MagMAX™ CORE Lysis Solution (Applied Biosystems).

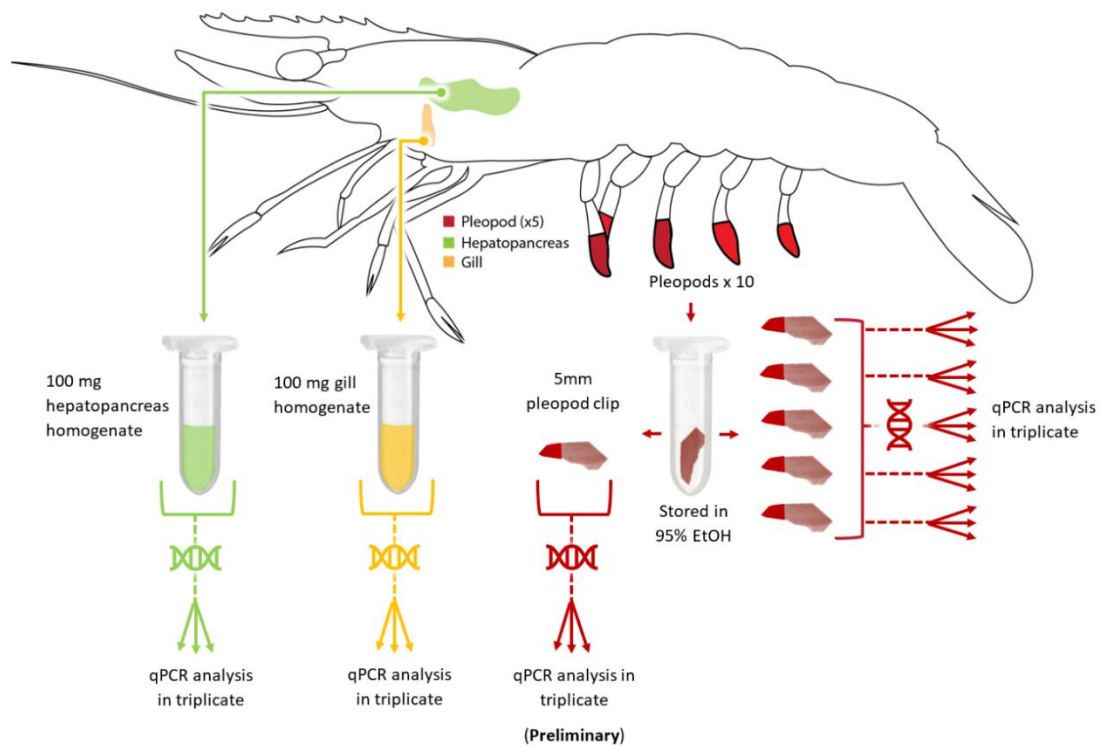


Figure 4.1. Penaeus monodon tissue sampling, demonstrating sampling of all 10 pleopods into 95% ethanol, followed by length standardised (5 mm) subsampling of a single pleopod for preliminary pathogen screening (Preliminary). Subsequent samples collected from selected shrimp, including 5 mm pleopod tips (n = 5 per shrimp, 44 shrimp), 100 mg of the left hepatopancreas lobe (n = 44 shrimp) and 100 mg of gill tissue (n = 35 shrimp), were processed for nucleic acid extraction (represented by a nucleic acid double helix symbol) and triplicate analysis using (RT-)qPCR.

4.3.2 Nucleic acid extraction

Nucleic acid extraction of the five subsampled pleopod, hepatopancreas and gill homogenates from each selected shrimp was conducted as described in section 3.3.3.

4.3.3 Quantitative real-time PCR (qPCR) target detection

IHHNV DNA and GAV and *P. monodon* Dicer-1 gene (Dicer-1) RNA amounts in each TNA extract were quantified using real-time qPCR analysis, as per section 3.3.4 using the relevant primer and probe sequences listed in Table 2.1. Three 1.25 µL aliquots of each TNA extract were tested as technical replicates for each target. Within each analysis run, a non-template

control and positive nucleic acid control for each target were included. Samples which did not amplify beyond the relative threshold within 45 PCR cycles were categorised as undetermined. The generation of a standard curve for conversion of cycle threshold value (Ct) to target copy number μL^{-1} TNA extract was conducted per section 3.3.5.

4.3.4 Statistical analysis

Test results categorised as undetermined were assigned a viral copy number μL^{-1} of zero. Zero-value copy numbers were retained throughout subsequent analysis as their occurrence was exclusively associated with technical replicate detections of <9 copies μL^{-1} for IHHNV, <18 copies μL^{-1} for GAV, or repeated zero-value results, providing confidence that their occurrence was not due to a failure to amplify at an earlier true cycle threshold (McCall et al., 2014).

All statistical analysis was conducted using R (R Core Team, 2022). Coefficient of variation (CV) in qPCR detection was calculated as the standard deviation divided by the mean of untransformed calculated viral copy number μL^{-1} for each group. Untransformed calculated copy numbers were used to compute CV, as per Canchola et al., (2017) and Forootan et al., (2017). CV of qPCR detection from triplicate aliquots of each TNA extract was used to assess the technical reproducibility and precision of qPCR analysis. Technical triplicate detection results from aliquots of the same tissue TNA extract (mean viral copy number μL^{-1} TNA extract) were averaged to produce indicative detection results for each tissue sample prior to comparative analysis between tissue samples. Descriptive statistical parameters including the mean and standard deviation of detection were calculated for target copy number μL^{-1} TNA extract for each tissue type for each viral target. CV of qPCR detection (as previously described) between pleopods of each individual shrimp were calculated for each viral target. Significant difference between GAV and IHHNV CV values among pleopods of individual shrimp was determined using a Student's t-test. All subsequent analysis was conducted on $\log_{10}$ transformed calculated target copy number μL^{-1} TNA, after zero values were adjusted to one.

4.3.4.1 Pleopod detection representativeness

For comparison of target detection between tissue types, one out of five pleopods sampled and tested from each shrimp was randomly selected ($n = 44$ out of 220). This selection yielded a single tissue sample for pleopod, gill and hepatopancreas from each shrimp which were statistically compared. Random pleopod selection and statistical comparison between tissues

was conducted in triplicate to ensure the random selection of pleopods did not affect the outcome of analysis. All analyses were also repeated with ($n = 44$) and without ($n = 35$) the additional nine shrimp with only pleopod and hepatopancreas samples to ensure the inclusion of these shrimp did not affect the outcome of analysis. In either case, statistical outcomes remained consistent. Therefore, analysis for comparison of detection between tissue types included data for all available shrimp. Significant differences in $\log_{10}$ target copies μL^{-1} TNA between the various tissue types were determined using an ANOVA with Tukey HSD post-hoc test. The linear statistical relationship of target detections between the various tissue types for individual shrimp was determined using Pearson's correlation analysis (Harrell, 2023).

4.3.4.2 Pleopod sample simulations

Various pleopod sampling scenarios were simulated to evaluate the relationship between sampling effort and the relative gain in qPCR detection reliability. For the sampling simulations, triplicate qPCR test results from the 5 pleopod samples from each experimental shrimp were used, where all shrimp were known to be positive for either IHHNV or GAV and had at least one test replicate positive from at least one pleopod. Three sampling scenarios were simulated to investigate the trade-off between sampling and testing effort, and the reliability of target detection using (RT-)qPCR with respect to quantitation of indicative target load and 'true' positive detection. The three scenarios were based on the number of pleopods sampled per individual shrimp (1, 2 or 3) and further differentiated by the number technical test replicates (qPCR detection results from aliquots of the same tissue TNA extract) that were considered per pleopod, ranging from a single test to triplicate tests of the same TNA extract (e.g. ScenX_Y = sampling scenario X, where X is the number of pleopods 'sampled' for individual TNA extraction from each individual shrimp and, Y is the number of qPCR replicates run for each TNA extract). Examples of sampling scenarios are graphically illustrated in Figure 4.2.

For analysis of 'true' positive detection, if any of the test replicates selected for any pleopod from the given shrimp returned a positive detection (copy number μL^{-1} TNA extract > 0), the individual shrimp was considered positive. Given all shrimp included in the sampling simulation were known to be positive, the number of positive shrimp detected in the simulated scenario was compared to the total number of shrimp in the dataset to generate a rate of 'true' positive detection (%). Random sampling for each scenario was iterated 10 times. The rate of

‘true’ positive detections from the iterated sampling scenarios were compared by ANOVA with TukeyHSD post-hoc analysis. For assessment of quantitative detection, an ‘indicative’ ($\hat{y}$) quantitative detection was generated using the average of all test replicate detection results across all pleopods sampled from an individual shrimp, within a specific scenario. The ‘indicative’ detection results were then compared to the ‘ground truth’ (y) detection result for each shrimp, which comprised the average of all detections from all test triplicates of all five pleopods sampled from the individual shrimp ($n = 15$ qPCR test results). Comparison of the ‘indicative’ simulated detection result and the ‘ground truth’ shrimp detection result yielded a ‘residual’ value ($\text{residual} = y - \hat{y}$; Figure 4.2) which was used to assess the accuracy of the simulated scenario result. Random sampling for each scenario was iterated 10 times. The mean detection residuals from the iterated sampling scenarios were compared using an ANOVA with TukeyHSD post-hoc analysis.

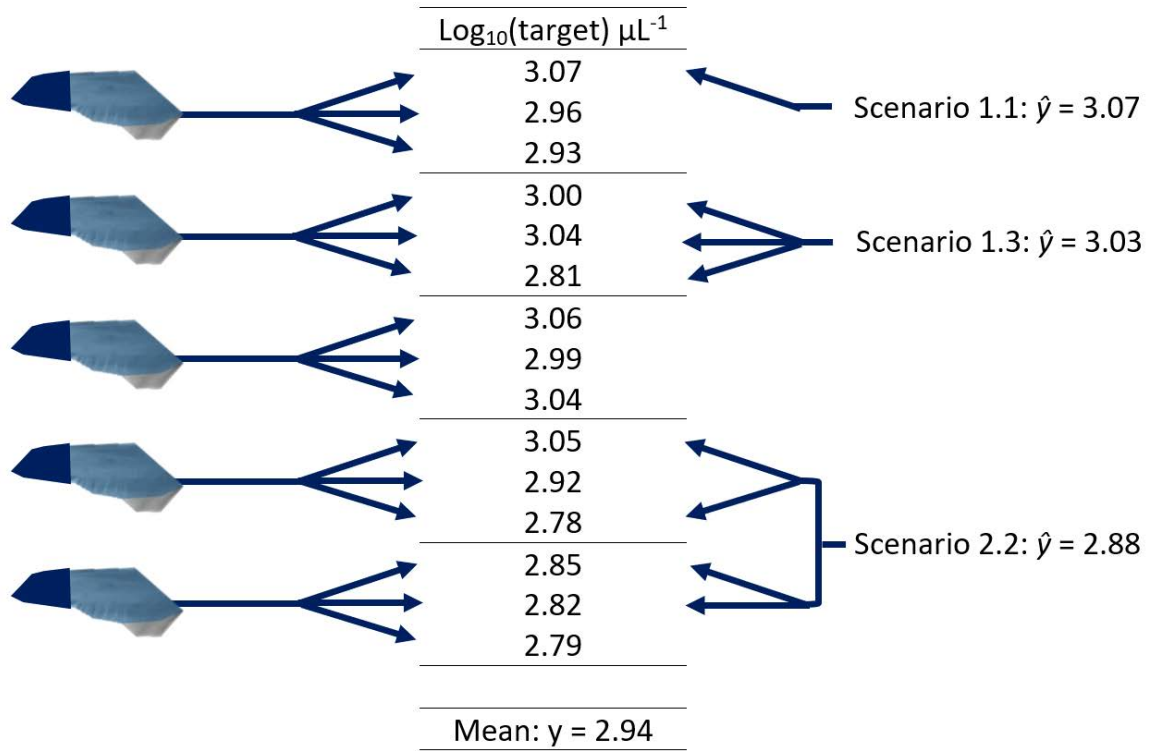


Figure 4.2. Examples of simulated sampling designs used to determine the effect of increasing sampling and qPCR testing effort on the quantitative accuracy of detection from pleopods. Scenario X_Y = sampling scenario X , where X is the number of pleopods ‘sampled’ for individual TNA extraction from each individual shrimp and, Y is the number of qPCR replicates sampled for each TNA extract within the simulation. The ‘indicative’ ($\hat{y}$) scenario detection is the average of all detection results captured within the scenario selection. The ‘ground truth’ (y) detection comprises the average of all triplicate pleopod detection results from the individual shrimp ($n = 15$ qPCR test results). The absolute difference between ‘indicative’ simulated detection result and the ‘ground truth’ shrimp detection result yields the ‘residual’ value ($\text{residual} = y - \hat{y}$), e.g. residual of Scenario 1_3 = $|2.94 - 3.03| = 0.09 \log_{10} \text{copies } \mu\text{L}^{-1}$.

4.4 Results

4.4.1 Overall tissue detection

GAV RNA and IHHNV DNA was quantified in tissues sampled from naturally infected *P. monodon* shrimp using TaqMan (RT-)qPCR. Viral target detection was compared between gill, hepatopancreas, and five individual pleopods collected from the same shrimp. IHHNV was detected most consistently from gill (100%, 35/35), followed by hepatopancreas (95.5%, 42/44) and pleopod (95.0%, 209/220) (Table 4.1). Average detected IHHNV was highest in gill ($3.47 \times 10^5 \pm 9.87 \times 10^5$ copies μL^{-1}), followed by hepatopancreas ($2.77 \times 10^4 \pm 9.14 \times 10^4$ copies μL^{-1}), and pleopod ($5.04 \times 10^3 \pm 1.39 \times 10^4$ copies μL^{-1}). Overall, $\log_{10}$ IHHNV copy number μL^{-1} detected from gill tissue was significantly higher than detections from hepatopancreas ($p = 0.003$) and pleopod tissue ($p < 0.001$) (Figure 4.3). GAV was detected most consistently from hepatopancreas (100%, 44/44), followed by pleopod (99.1%, 218/220) and gill (97.1%, 34/35). Average detected GAV was comparable across hepatopancreas ($4 \times 10^3 \pm 1.2 \times 10^4$ copies μL^{-1}) and gill ($3.05 \times 10^3 \pm 1.23 \times 10^4$ copies μL^{-1}) samples and was highest in pleopod ($6.36 \times 10^3 \pm 7 \times 10^4$ copies μL^{-1}) (Table 4.1); however, this average was highly influenced by detection from a single pleopod (Appendix 15). Overall, $\log_{10}$ GAV copy number μL^{-1} was significantly higher in hepatopancreas ($p < 0.001$) than detections from pleopod tissue (Figure 4.3).

Table 4.1. Summary of (RT)qPCR IHHNV and GAV target detection from tissues samples of naturally infected *Penaeus monodon* shrimp (Proportion positive = proportion of tissue samples across all sampled shrimp (N) that returned a positive detection result).

Tissue	N	IHHNV		GAV	
		Proportion positive	Mean $\pm$ SD copies μL^{-1} TNA	Proportion positive	Mean $\pm$ SD copies μL^{-1} TNA
100 mg Gill	35	100%	$3.47 \times 10^5 \pm 9.87 \times 10^5$	97.1%	$3.05 \times 10^3 \pm 1.23 \times 10^4$
100 mg HP	44	95.5%	$2.77 \times 10^4 \pm 9.14 \times 10^4$	100%	$4 \times 10^3 \pm 1.2 \times 10^4$
5 mm Pleopod (n = 5 per shrimp)	44	95.0%	$5.04 \times 10^3 \pm 1.39 \times 10^4$	99.1%	$6.36 \times 10^3 \pm 7 \times 10^4$

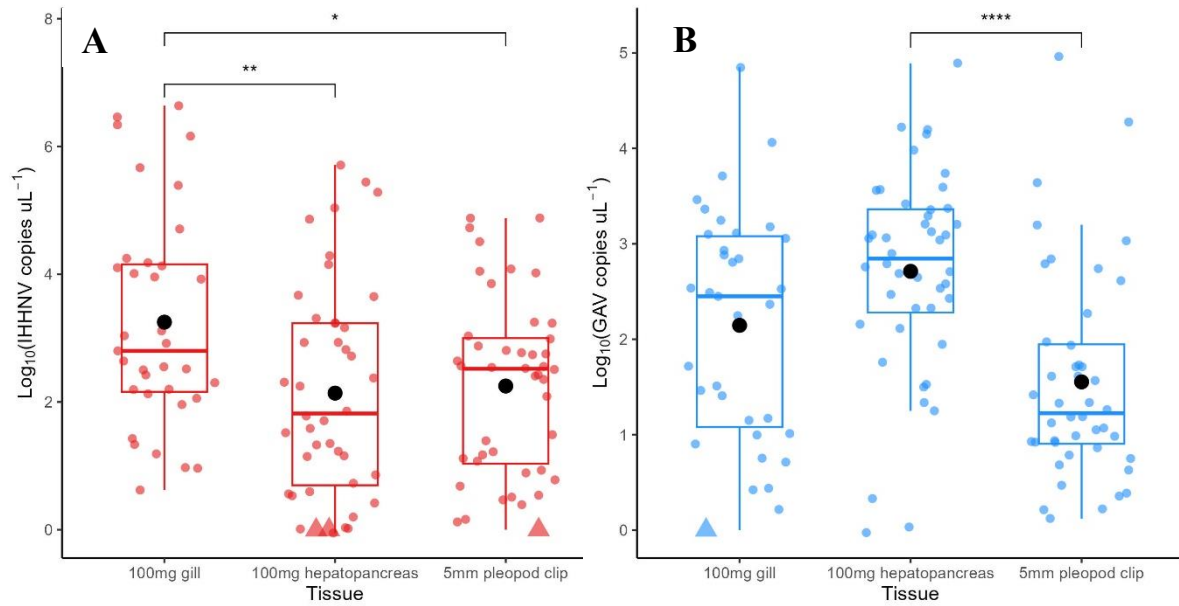


Figure 4.3. Tissue specific detection of (A) IHHNV and (B) GAV. Each point represents the average of $\log_{10}$ transformed target copy number uL^{-1} TNA extract detected from a single shrimp. Tissue samples with undetermined target detection (zero copies uL^{-1}) are displayed as triangular points. Detection results from a single pleopod (out of five) was randomly selected for each shrimp ($n = 44$ out of 220) for comparable representation to hepatopancreas ($n = 44$) and gill ($n = 35$) tissue samples. Significant difference in detection between the different tissues was determined using ANOVA with Tukey HSD post-hoc analysis ($\alpha = 0.05$). Random selection of pleopods and statistical analysis was repeated in triplicate. All analyses yielded the same statistical outcome.

4.4.2 Pleopod detection representativeness

IHHNV detection from single pleopods of individual shrimp were highly correlated with detection from hepatopancreas ($R^2 = 0.77$, $p < 0.01$) (Figure 4.4A and Appendix 8) and gill tissue ($R^2 = 0.87$, $p < 0.01$) (Figure 4.4B and Appendix 8). IHHNV detections between gill and hepatopancreas were similarly highly correlated (Appendix 10; $R^2 = 0.87$, $p < 0.01$). These results demonstrated that quantitative IHHNV detections from pleopod tissue were representative of detected loading from gill and hepatopancreas of the same shrimp.

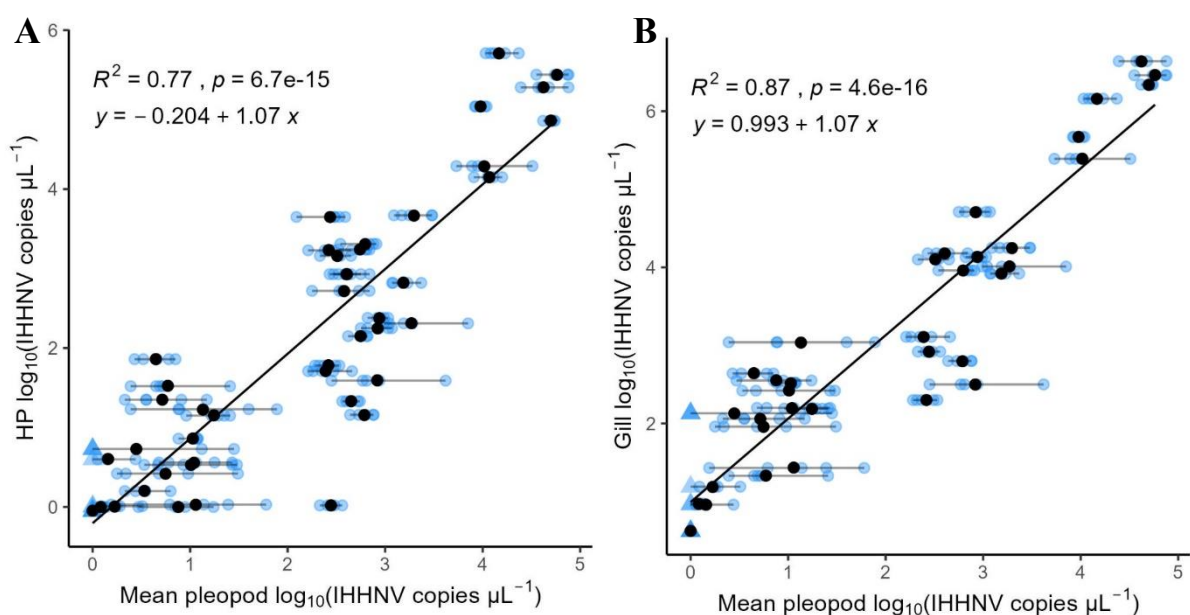


Figure 4.4. Correlation of the mean $\log_{10}$ IHHNV copy number μL^{-1} detected from pleopods, and (A) hepatopancreas (HP, $n = 44$) or (B) gill tissue ($n = 35$) sampled from individual shrimp. For each shrimp, five pleopods were sampled and analysed, individual pleopod detection results are displayed as blue points, connected by a horizontal line. Pleopod samples with undetermined target detection (zero copies μL^{-1}) are displayed as triangular points. The mean detection from pleopods of an individual shrimp are displayed as a black point. The linear coefficient of determination (R^2) describes the relationship between mean IHHNV detection across all five pleopods and IHHNV detection from (a) HP or (B) gill of an individual shrimp. R^2 values were determined using Pearson's correlation analysis.

GAV detections from single pleopods were poorly correlated with detection from hepatopancreas ($R^2 = 0.14$), however, the correlation was statistically significant ($p = 0.013$) (Figure 4.5A and Appendix 9). GAV detected from single pleopods were moderately correlated with detection from gill tissue ($R^2 = 0.39$, $p < 0.01$) (Figure 4.5B and Appendix 9). GAV detections between gill and hepatopancreas were also moderately correlated ($R^2 = 0.26$, $p < 0.01$) (Appendix 10). These results implied that quantitative GAV detections from pleopod

tissue were not strongly indicative of detected loading from hepatopancreas of the same shrimp and were only marginally more indicative of detected loading from gill tissue.

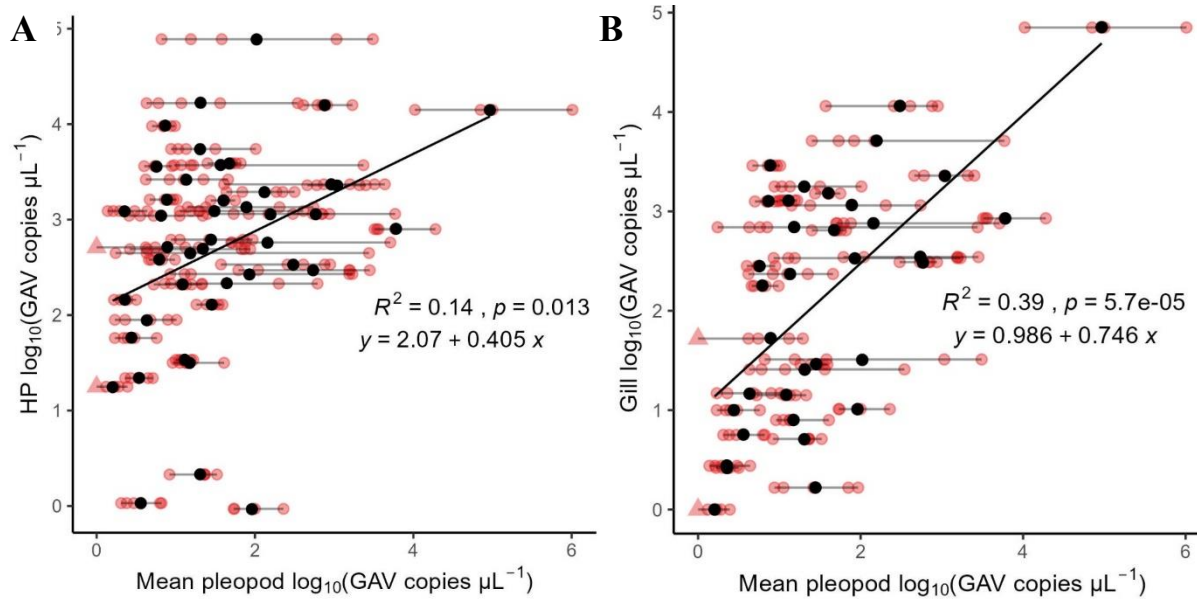


Figure 4.5. Correlation of the mean $\log_{10}$ GAV copy number μL^{-1} detected from pleopods, and (A) hepatopancreas (HP, $n = 44$) or (B) gill tissue ($n = 35$) sampled from individual shrimp. For each shrimp, five pleopods were sampled and analysed, individual pleopod detection results are displayed as blue points, connected by a horizontal line. Pleopod samples with undetermined target detection (zero copies μL^{-1}) are displayed as triangular points. The mean detection from pleopods of an individual shrimp are displayed as a black point. The linear coefficient of determination (R^2) describes the relationship between mean GAV detection across all five pleopods and GAV detection from (a) HP or (B) gill of an individual shrimp. R^2 values were determined using Pearson's correlation analysis.

4.4.3 Pleopod detection consistency

There was no correlation observed between whole shrimp weight (g) and Dicer-1 detection (cycle threshold; Ct) from pleopods ($R^2 = 0.027$, $p = 0.29$), indicating that extracted TNA from length standardised pleopod samples was not influenced by shrimp size (Appendix 5). The observed CV in Dicer-1 detection was generally very low between pleopods ($n = 5$) of individual shrimp ($\text{CV} = 1.4\% \pm 0.5\%$; mean $\pm$ SD), and comparable to the CV of mean Dicer-1 detection for individual shrimp in the experimental cohort ($\text{CV} = 1.65\%$), indicating that the extraction of TNA from length standardised pleopod samples was performed consistently between pleopods and between individual shrimp.

Overall, the observed CV of untransformed copy number μL^{-1} TNA extract across the 5 pleopods sampled from each shrimp was significantly higher for GAV detections (mean $\pm$ SD; 0.88 ± 0.60) than the CV observed for IHHNV detections (mean $\pm$ SD; 0.54 ± 0.41) (Figure 4.6A; $t_{75} = -3.07$, $p = 0.003$). The CV of IHHNV detection between pleopods decreased with increasing mean shrimp loading, with a moderate negative linear correlation ($R^2 = 0.28$, $p < 0.01$) (Figure 4.6B and Appendix 6). In contrast, GAV detection between pleopods was more variable, yielding a negligible positive correlation between CV and mean shrimp loading ($R^2 = 0.1$, $p = 0.033$) (Figure 4.6B and Appendix 6). For comparison to previous studies (Noble et al., 2018), Figure 4.6 was replicated using the CV of detection between pleopods on $\log_{10}$ transformed copy number (Appendix 7). These results cumulatively indicated that, under the conditions analysed here, IHHNV detection between pleopods of individual shrimp was more consistent than GAV detection. Moreover, unlike GAV, IHHNV detection was increasingly consistent between pleopods of shrimp with higher overall IHHNV loading.

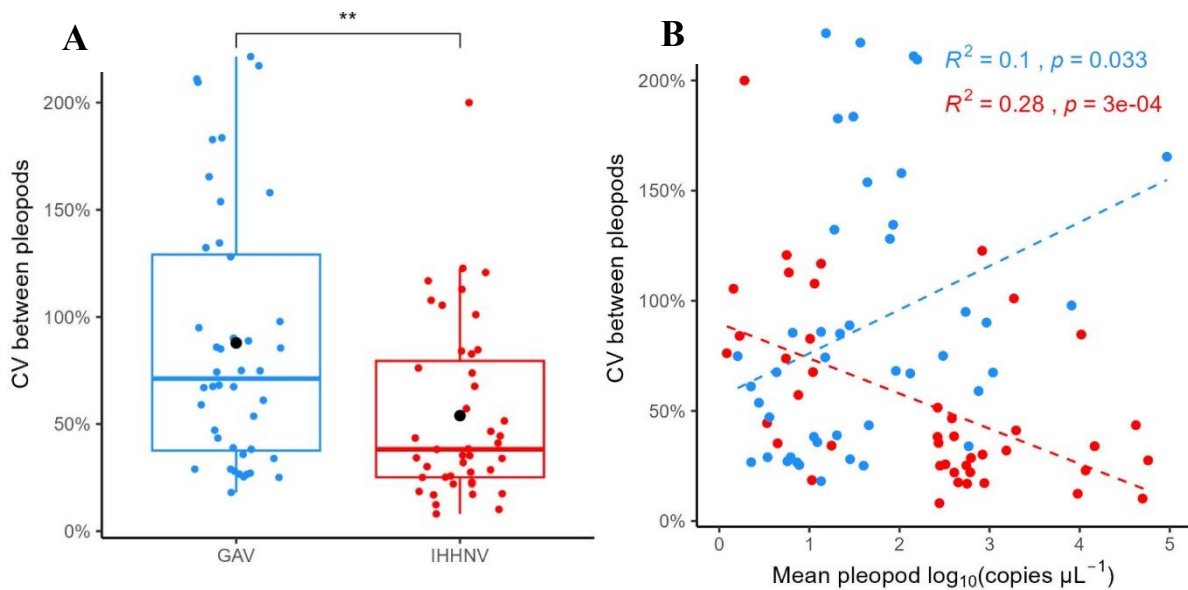


Figure 4.6. Coefficient of variation of untransformed copy number μL^{-1} TNA extract between pleopods ($n=5$) of individual shrimp. (A) Overall differences in CV for GAV (blue) and IHHNV (red) detection and their significance as determined by a student's t -test ($\alpha = 0.05$). (B) CV of GAV (blue) and IHHNV (red) detection as they relate to the $\log_{10}$ transformed mean detection from all 5 pleopods of an individual shrimp.

4.4.4 Pleopod sample simulations

The pleopod sampling simulations compared the 'indicative' average detection results from specified scenarios of pleopod sampling and qPCR test replication to the 'ground truth' averaged detection results across all triplicate qPCR results from all five pleopods sampled

from each individual shrimp. For each virus, all shrimp used within the sampling scenario were known to be positive for the specific viral target by qPCR detection from pleopod tissue.

For IHHNV, detection results from 40 shrimp were included in the sampling scenario dataset, comprising 600 qPCR detection results (40 shrimp x 5 pleopods x 3 qPCR replicate tests) with 94.33% of the results representing positive IHHNV detections (copy number $\mu\text{L}^{-1} > 0$). Across the range of IHHNV qPCR detections, the estimated limit of detection with the sample matrix, specified as the lowest copy number detected from pleopod samples at a positive call rate of 100% from triplicate tests (i.e. $> 95\%$ limit of detection; $\text{LOD}_{95\%}$), was approximately $10^{0.52}$ (~ 4) copies μL^{-1} (Appendix 3). The estimated limit of quantification, specified as the lowest copy number detected from pleopod samples where triplicate qPCR tests consistently yielded a $\text{CV} \leq 35\%$ (Forootan et al., 2017) was estimated at $10^{1.41}$ (~ 26) copies μL^{-1} (Appendix 3). For GAV, detection results from 41 shrimp were included in the sampling scenario dataset, comprising 615 qPCR detection results (41 shrimp x 5 pleopods x 3 qPCR tests) with 93.66% of the results representing positive GAV detections. Across the range of GAV qPCR detections, the estimated limit of detection (as previously defined; $\text{LOD}_{95\%}$) was approximately $10^{0.75}$ (~ 6) copies μL^{-1} (Appendix 4). The estimated limit of quantification (as previously defined; triplicate qPCR $\text{CV}_{\leq 35\%}$) was estimated at $10^{1.38}$ (~ 24) copies μL^{-1} (Appendix 4). Considered together, these results demonstrated that the applied assays shared a comparable capacity to determine the presence and quantify the loading of each pathogen within the sample matrices. This consistency suggests that the observed differences in detection patterns in subsequent analysis are more likely attributable to biological variation of each virus, rather than differences in detection performance.

4.4.4.1 IHHNV

Within each scenario absolute residuals between the average ‘indicative’ IHHNV detection and the ‘ground truth’ detection from individual shrimp were generally inversely related to mean shrimp virus loading (Appendix 11). This finding reflects the general reduction in CV between pleopods at higher shrimp viral loading (Figure 4.6) and further supports that the detection of IHHNV becomes more consistent and reliable when there is a higher overall shrimp loading. Residuals were flattened as the ‘true’ IHHNV mean pleopod detection approached zero copies μL^{-1} TNA extract. This observation is likely due to the assignment and retention of undetermined samples with zero-value copy numbers, and the increased proportion of

undetermined samples upon approach of the limit of detection within the sample matrix (Appendix 3).

‘Indicative’ IHHNV detections from pleopods converged towards the ‘ground truth’ IHHNV detection values for individual shrimp with increasing biological replication across scenarios (multiple pleopod tissue samples) (Figure 4.7). Absolute IHHNV detection residuals also reduced with increasing technical replication within scenario groups (multiple qPCR test replicates of the same pleopod tissue extract), although non-significantly. These results are as expected, based on the statistical law of large numbers, such that the observed estimate of the average improves with increasing sample size. Regardless of the number of qPCR replicates considered, sampling and analysis of two pleopods yielded significant reduction in IHHNV detection residuals compared to sampling and analysis of only one pleopod. However, even under the lowest input scenario (Scen1_1) the largest scenario residual was less than 15 copies μL^{-1} . Analysing three pleopods from the same shrimp did not significantly improve quantitative accuracy compared to scenarios Scen2_2 or Scen2_3 when using only a single qPCR test for each pleopod (Scen3_1). However, conducting qPCR analysis of three pleopods in duplicate (Scen3_2) or triplicate (Scen3_3) yielded significant reduction in IHHNV quantitation residuals compared to all testing scenarios when only two pleopods were sampled. Collectively, these results demonstrated that sampling of two or three pleopods improved the accuracy of IHHNV quantitation compared to sampling a single pleopod. However, the variation in IHHNV levels detected between replicate tests or multiple pleopods of the same shrimp was minimal, and single pleopod analysis yielded IHHNV detection results that closely reflected overall shrimp pleopod loading.

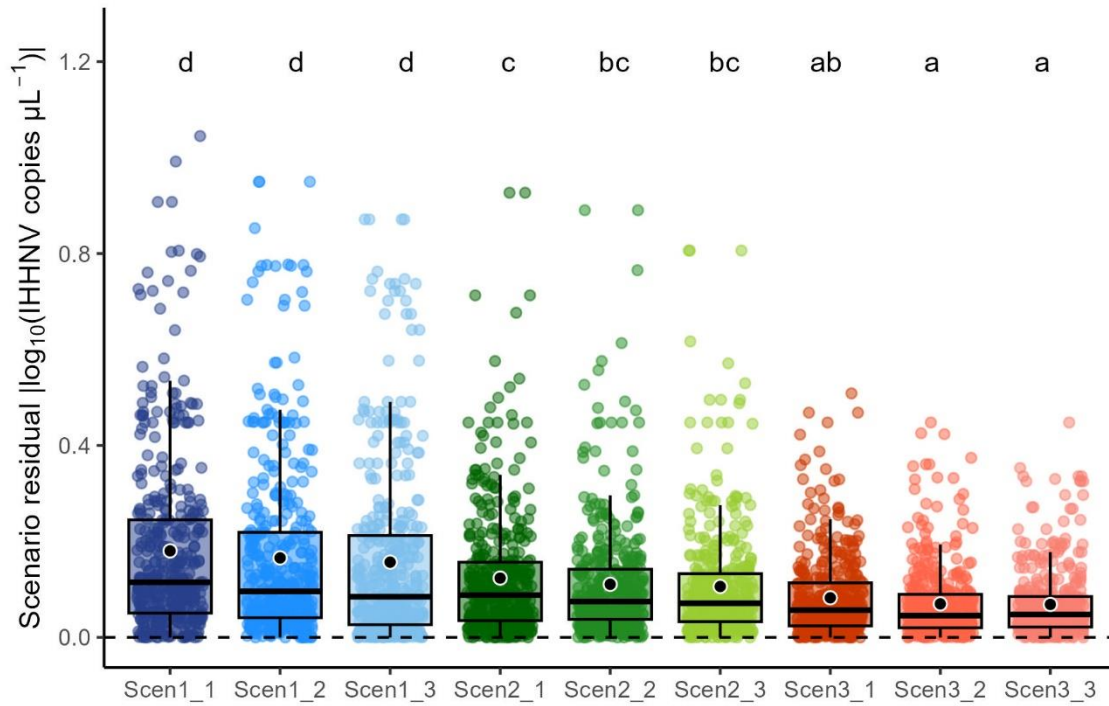


Figure 4.7. Absolute residual (difference) between the ‘indicative’ averaged IHHNV detection [$\log_{10}(\text{IHHNV copies } \mu\text{L}^{-1} \text{ TNA extract})$] from pleopods randomly selected within simulation sampling scenarios and the ‘ground truth’ average IHHNV detection across all sampled pleopods ($n = 5$) for each individual shrimp. (ScenX_Y = sampling scenario X, where X is the number of pleopods ‘sampled’ for individual TNA extraction from each individual shrimp and, Y is the number of qPCR replicates run for each TNA extract, i.e. sampled pleopods). Significant differences in mean residuals between scenarios, as determined by ANOVA with Tukey HSD post-hoc test ($\alpha = 0.05$), are indicated by letters.

The proportion of ‘true’ IHHNV positive shrimp detected did not significantly increase with increased sampling or qPCR test replication beyond sampling of a single pleopod with duplicate qPCR testing (Scen1_2) (Figure.4.8). However, consistent detection of 100% of the ‘true’ positive shrimp only occurred when three pleopods were sampled and tested in duplicate (Scen3_2) or triplicate (Scen3_3).

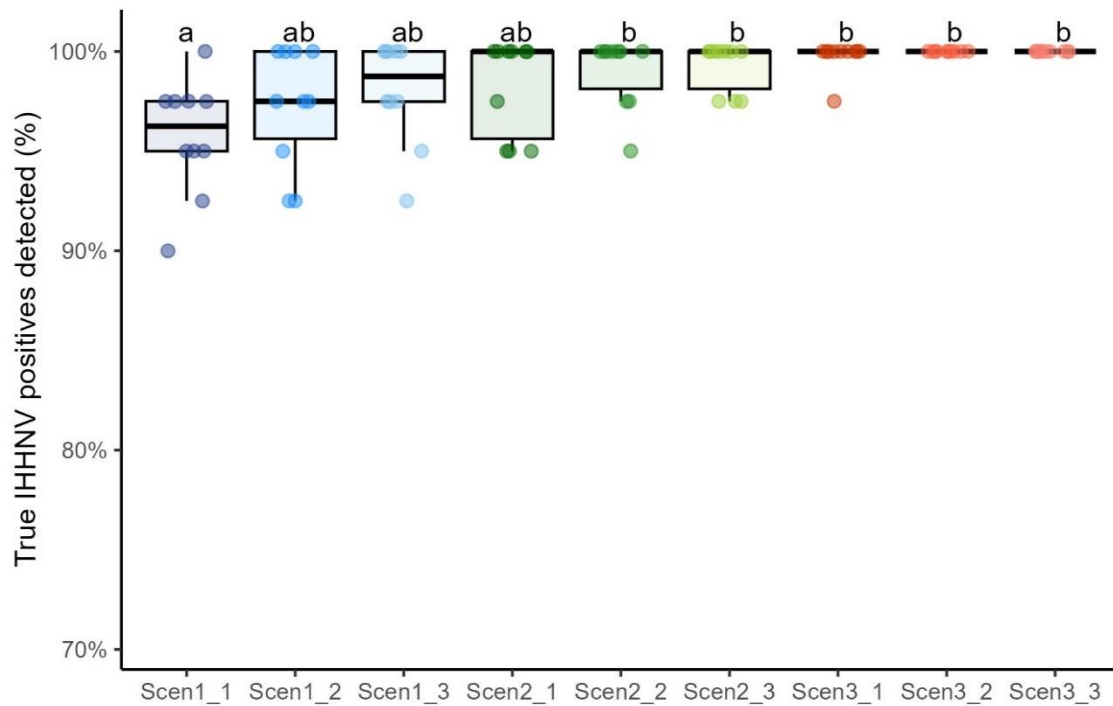


Figure.4.8. Rate of 'true' IHHNV positive shrimp detected from sampling scenario simulations. Where all shrimp ($n = 40$) within the experimental cohort were known to be positive for the detection of IHHNV. Individual shrimp were considered positive if any of the test replicates from any of the sampled pleopods within a sampling scenario had a positive IHHNV qPCR result. (ScenX_Y = sampling scenario X, where X is the number of pleopods 'sampled' for individual TNA extraction from each individual shrimp and, Y is the number of qPCR replicates run for each TNA extract, i.e. sampled pleopods). Significant differences between scenarios, as determined by ANOVA with Tukey HSD post-hoc test ($\alpha = 0.05$), are indicated by letters.

When the rate of detection of 'true' IHHNV positive shrimp was considered against the mean IHHNV loading of individual shrimp, the observed loss of positive detection reliability was attributed to shrimp with low viral load within the experimental dataset (Appendix 3, Appendix 13). Therefore, the scenario sampling analysis was repeated using shrimp with average pleopod detection ≤ 100 (10^2) copies μL^{-1} TNA extract, to investigate the effect of more rigorous sampling and testing scenarios under such conditions. From the subset of shrimp selected ($n = 16$), 240 qPCR detection results were included in the sampling scenario dataset (16 shrimp x 5 pleopods x 3 qPCR tests), with only 85.83% of the results representing positive IHHNV detections, compared to 94.33% from the original dataset.

Unlike the prior simulation, when only the low load shrimp were sampled under the various simulation scenarios, replicate qPCR testing yielded significantly increased rates of 'true' IHHNV positive detection within pleopod scenarios (Figure 4.9). For example, testing a single

pleopod in triplicate (Scen1_3) achieved significantly improved rates of ‘true’ positive detection compared to analysis of only one qPCR test (Scen1_1). Sampling of only a single pleopod and testing in triplicate yielded statistically similar rates of detection as sampling of two pleopods, each with singular, duplicate, or triplicate qPCR tests. No significant improvement in the rates of ‘true’ IHHNV positive detection were observed with additional sampling effort beyond Scen2_1. However, 100% of the ‘true’ positive low load shrimp were detected consistently only when three pleopods were sampled and tested individually in either duplicate (Scen3_2) or triplicate (Scen3_3). When considered together, these results clarify that increased qPCR test replication improved the reliability of IHHNV detection from pleopods of shrimp with low overall loading. Nevertheless, sampling of multiple pleopods was required to consistently detect all known positives.

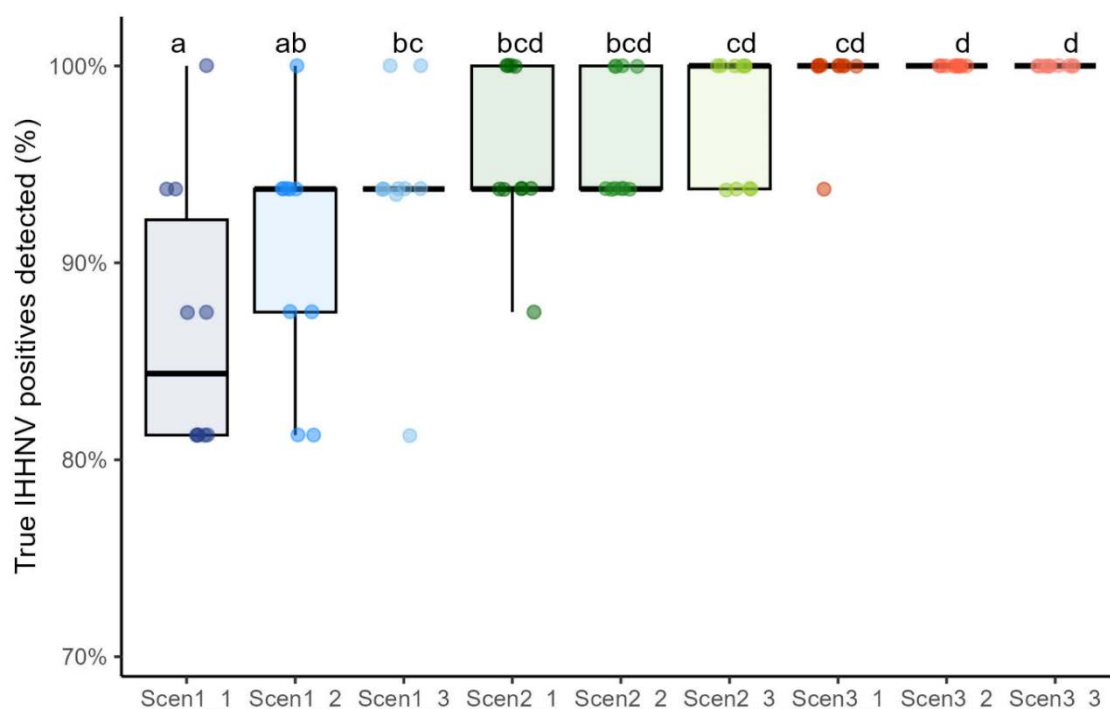


Figure 4.9. Rate of ‘true’ IHNV positive shrimp detected from sampling scenario simulations where random sampling was conducted from IHNV positive shrimp with an average pleopod IHNV $\log_{10}$ copy number μL^{-1} TNA extract < 2 ($n = 16$). Individual shrimp were considered positive if any of the test replicates from any of the sampled pleopods within a sampling scenario had a positive IHNV qPCR result. (ScenX_Y = sampling scenario X, where X is the number of pleopods ‘sampled’ for individual TNA extraction from each individual shrimp and, Y is the number of qPCR replicates run for each TNA extract, i.e. sampled pleopods). Significant differences between scenarios, as determined by ANOVA with Tukey HSD post-hoc test ($\alpha = 0.05$), are indicated by letters.

4.4.4.2 GAV

Convergence of quantitative GAV detection to the ‘indicative’ pleopod loading was not observed with increasing replication of RT-qPCR test replicates of the same pleopod tissue extract within scenario groups (Figure 4.10). However, simulated GAV detection results were significantly more indicative with increasing biological replication of pleopods sampled per shrimp, across scenarios. Specifically, regardless of the number of RT-qPCR test replicates, detection was significantly more indicative of the shrimp ‘ground truth’ GAV load when three pleopods were sampled, compared to two, and two, compared to one (i.e. Scen3 > Scen_2 > Scen1). Within each scenario, absolute residuals between the average ‘indicative’ GAV detection and the ‘ground truth’ detection from individual shrimp were generally independent of mean shrimp loading (Appendix 12). However, residuals were flattened as the ‘true’ mean GAV pleopod detection approached zero copies μL^{-1} TNA extract, given the assignment and

retention of undetermined samples with zero-value copy numbers, and the increased proportion of undetermined test results at low overall shrimp viral load (Appendix 4). Cumulatively, these results demonstrated that replicate tests of pleopod extracts yielded consistent GAV quantification, however, GAV loading between pleopods of individual shrimp was variable. Moreover, unlike IHNV, GAV detection between pleopods was not necessarily more consistent or reliable from shrimp with higher overall GAV loading. Given the high biological variability in pleopod GAV loading across the range of shrimp loadings analysed here, aligned with the statistical law of large numbers, the results showed that multiple pleopod samples were required to obtain GAV quantification representative of the average pleopod loading.

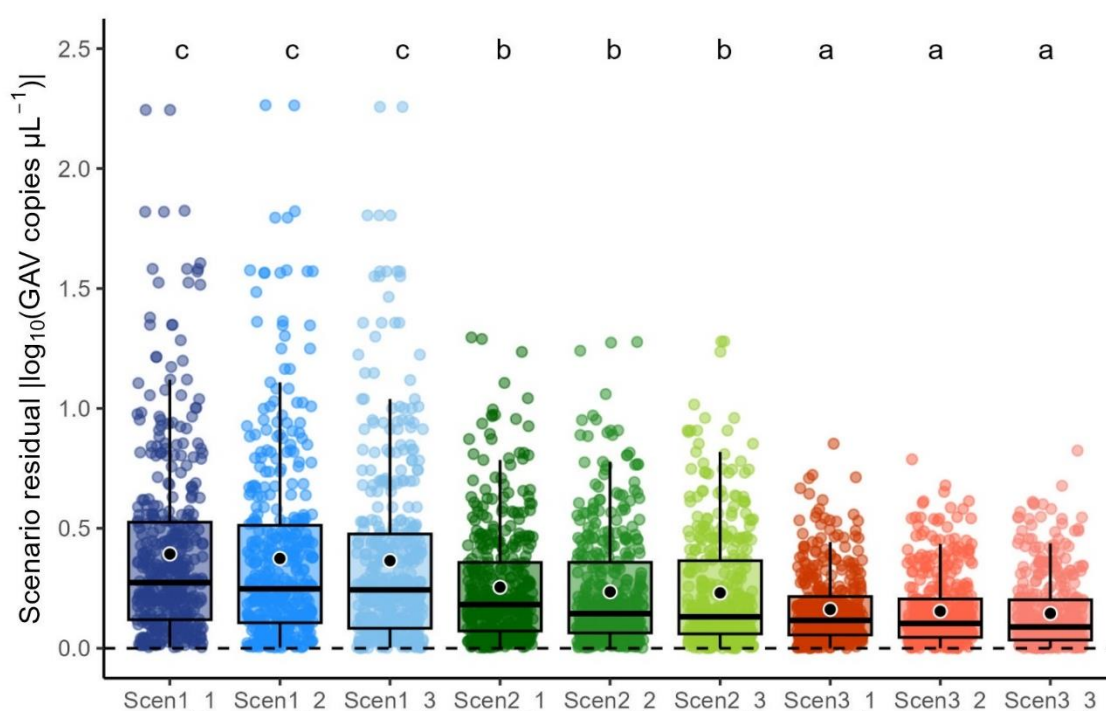


Figure 4.10. Absolute residual (difference) between the ‘indicative’ averaged GAV detection [$\log_{10}(\text{GAV copies } \mu\text{L}^{-1} \text{ TNA extract})$] from pleopods randomly selected within simulation sampling scenarios and the ‘ground truth’ average GAV detection across all sampled pleopods ($n = 5$) for each individual shrimp. (ScenX_Y = sampling scenario X, where X is the number of pleopods ‘sampled’ for individual TNA extraction from each individual shrimp and, Y is the number of qPCR replicates run for each TNA extract, i.e. sampled pleopods). Significant differences between scenarios, as determined by ANOVA with Tukey HSD post-hoc test ($\alpha = 0.05$), are indicated by letters.

For GAV, the proportion of ‘true’ positive shrimp detected was generally high, but significantly increased with increased pleopod sampling and qPCR test replication within scenarios (Figure 4.11, Appendix 14). Testing a single pleopod in duplicate or triplicate significantly increased

the rate of ‘true’ GAV positive shrimp detected compared to testing singularly. Testing a single pleopod in triplicate yielded a statistically similar likelihood of ‘true’ positive detection as the highest input scenario (Scen3_3). However, consistent detection of 100% of the ‘true’ GAV positive shrimp only occurred when either two or three pleopods were sampled and each tested in either duplicate (Scen2_2 and Scen3_2) or triplicate (Scen2_3 and Scen3_3). Together, these results demonstrated that increased qPCR test replication improved the reliability of GAV detection from pleopods of shrimp, however, sampling of multiple pleopods was required to consistently detect all known positives.

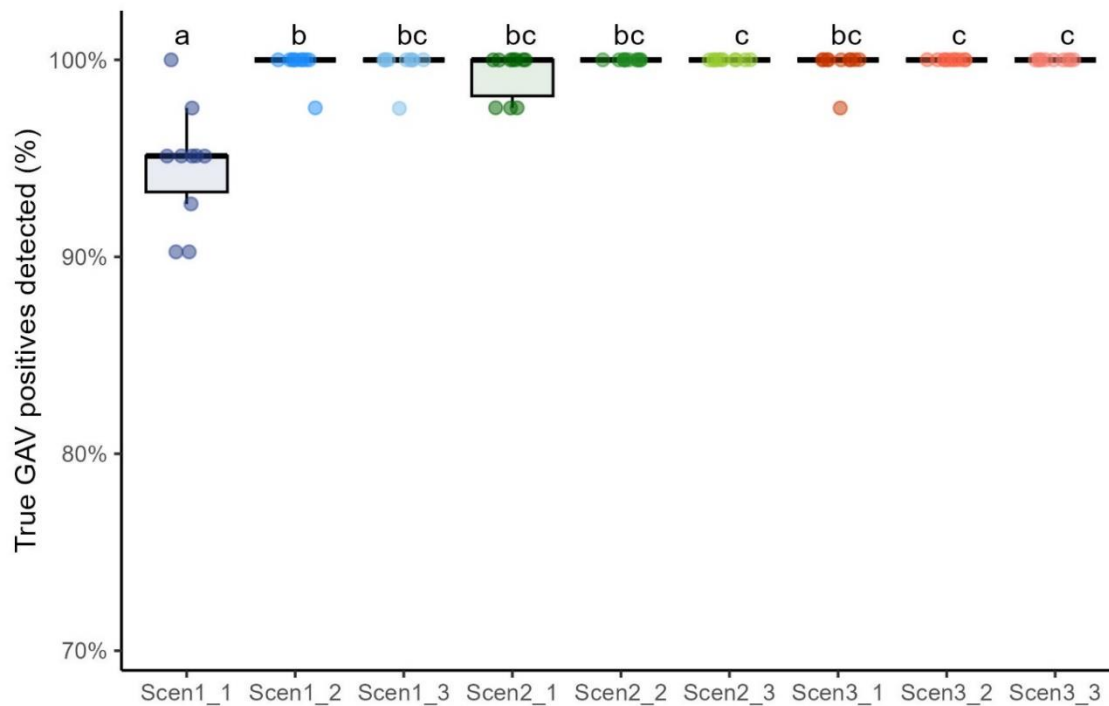


Figure 4.11. Rate of ‘true’ GAV positive shrimp detected from sampling scenario simulations. Where all shrimp ($n = 40$) within the experimental cohort were known to be positive for the detection of GAV. Individual shrimp were considered positive if any of the test replicates from any of the sampled pleopods within a sampling scenario had a positive GAV qPCR result. (ScenX_Y = sampling scenario X, where X is the number of pleopods ‘sampled’ for individual TNA extraction from each individual shrimp and, Y is the number of qPCR replicates run for each TNA extract, i.e. sampled pleopods). Significant differences between scenarios, as determined by ANOVA with Tukey HSD post-hoc test ($\alpha = 0.05$), are indicated by letters.

4.5 Discussion

Pleopod tissue is routinely used for pathogen screening of shrimp (Arbon et al., 2022; Chayaburakul et al., 2004; East et al., 2004; Remany et al., 2012). Despite their routine use, limited studies have applied modern detection technologies to assess the utility and suitability of pleopods for this application (Arbon et al., 2024a; Hou et al., 2023; Noble et al., 2018). This study aimed to address these gaps by investigating the reliability of qPCR detection from pleopod tissues for determining pathogen target presence and for attaining a representative quantitative shrimp viral loading. The reliability and consistency of detection from pleopods was investigated in adult *P. monodon* shrimp, naturally infected with a range of viral loadings of IHHNV and GAV, and is discussed herein.

4.5.1 Detection of IHHNV from pleopods

Excised pleopods are a recommended target tissue for non-lethal sampling of shrimp for the detection of IHHNV (WOAH, 2023b). In studies comparing detection of IHHNV across multiple tissue types, pleopods are demonstrated to reliably yield sensitive detection in experimentally challenged *Litopenaeus vannamei* shrimp (Hou et al., 2023) and naturally infected *P. monodon* (Arbon et al., 2024a). In naturally infected *P. monodon*, quantitative IHHNV detection from pleopod and various other tissues in individual shrimp including gill, hepatopancreas, lymphoid organ, hindgut, ventral nerve cord and tail muscle, were also observed to be highly linearly correlated ($R \geq 0.75$) (Arbon et al., 2024a). The present study provides further evidence to support the strong linear correlation of IHHNV detection between pleopod, gill and hepatopancreas tissue of individual shrimp ($R \geq 0.88$). Considering the corroborative evidence of Hou et al., (2023), Arbon et al., (2024a), and the present study, pleopod tissue is demonstrated as suitable for providing reliable IHHNV detection that is representative of loading in other tissues. This conclusion strongly supports the systemic nature of IHHNV infection, and current WOA recommendation of pleopod as a suitable, fit-for-purpose target tissue (WOAH, 2023b).

Sampling simulations conducted within this study implied that quantitative detection highly representative of the mean overall IHHNV detection in pleopods could be achieved with the lowest input sampling and testing conditions. For example, the maximum absolute difference between IHHNV detection from a single pleopod, and the mean IHHNV detection across five pleopods from the same individual shrimp, tested in triplicate, was less than 15 copies μL^{-1} .

Under the conditions analysed, our results suggest that when IHHNV is detected, quantitation from a single pleopod is likely to be reasonably indicative of overall pleopod loading, especially at higher shrimp loading. Moreover, loading of IHHNV in pleopod is highly correlated with loading in other tissues throughout the individual shrimp. However, when considering the rate of ‘true’ positive IHHNV detection, 100% detection was not consistently achieved until three pleopods were sampled and tested individually in duplicate or triplicate. Therefore, considered together, our findings support current industry sampling practices involving excision and qPCR testing of a single pleopod for the purpose of reliable IHHNV quantitation; however, determining the presence of IHHNV in pleopods of shrimp with low levels of infection may require further biological and technical replication beyond current industry standard practices.

4.5.2 Detection of GAV from pleopods

GAV, *syn.* yellowhead virus (YHV) genotype 2, is not listed within the WOAHA Aquatic Animal Health Code, therefore, no recommendations for its detection are specified by WOAHA (WOAHA, 2023b, 2023a). However, given the phylogenetic relationship of the YHV genotypes, and their current classification as a single species in the genus *Okavirus* (ICTV, 2023), the WOAHA recommendations for detection of YHV genotype 1 are considered in proxy here (WOAHA, 2023b). For the detection of YHV, gill is recommended as the most suitable non-lethal tissue sample (WOAHA, 2023b). The use of pleopod tissue as an alternative to gill for the detection of GAV is supported by the findings of Noble et al., (2018) and Arbon et al. (2024a), where similar levels and variability of GAV detection in gill and pleopod tissue were observed, suggesting the two tissue types are highly comparable in utility for determining GAV presence. The results of the present study further support the findings of Noble et al., (2018) and Arbon et al. (2024a).

Within the experimental cohort of the present study, pleopod sampling simulation demonstrated that increased technical replication of RT-qPCR analysis from the same pleopod TNA extract improved the rate of ‘true’ positive detection, regardless of the number of pleopods sampled. However, detection of 100% of the ‘true’ positive shrimp was only achieved consistently when two or three pleopods were sampled and each pleopod extract tested in duplicate or triplicate. These findings suggest that current industry sampling practice to collect and analyse a single pleopod for pathogen screening may fail to detect all GAV positive

animals. Therefore, sampling of two or more pleopods, with replicated RT-qPCR testing would significantly improve detection reliability, mirroring the conclusions of Noble et al., (2018) for both pleopod and gill tissue sampling.

However, despite the similar overall levels and rate of GAV detection between gill and pleopod, limited correlation of GAV loading among tissues including pleopods, gills and hepatopancreas detected within individual shrimp has been observed from naturally infected adult *P. monodon* (Arbon et al., 2024a). The results of the present study provide further evidence demonstrating low correlation of GAV loading between pleopod, gill and hepatopancreas tissue. The variability of GAV detection among pleopods in the present study was also high across the range of shrimp loadings captured within the experimental cohort. Noble et al. (2018) similarly described variable GAV detection among both pleopods and gill filaments of individual shrimp, across a range of GAV infection levels similar to those detected in the present study. Given the variability of GAV loadings detected among pleopods, simulated sampling scenarios demonstrated that sampling and analysis of multiple pleopods was required to improve the representativeness of detection to reflect the overall GAV loading in pleopods. However, considering the low correlation of GAV loading in pleopods to other tissue types within individual shrimp, even an accurate quantitation of overall GAV loading in pleopods is likely to be misrepresentative of GAV loading in other shrimp tissues.

4.5.3 Implications for pathogen screening using pleopods

The analysis and findings from this study are limited to the specific experimental conditions, including factors related to the natural acquisition of infection, infection progression, shrimp hosts and laboratory sample processing and analysis conditions. As such, the capacity to define a specific sampling strategy that will be optimal for all conditions from our analysis is limited. With that said, the overall results of this study suggest that detection of IHNV and GAV from a single pleopod is likely to yield false negative results from animals with naturally acquired low viral titre. Therefore, screening of shrimp using single pleopod samples may underestimate IHNV and GAV prevalence. Additionally, while detection of IHNV between pleopods was more consistent and quantitatively correlated with detection from other tissue types, detection of GAV across pleopods of individual shrimp was highly variable. These findings indicate that significant increases in replication of tissue sampling and qPCR testing is required to improve the reliability of IHNV and GAV detection, especially from low load animals. Achieving the

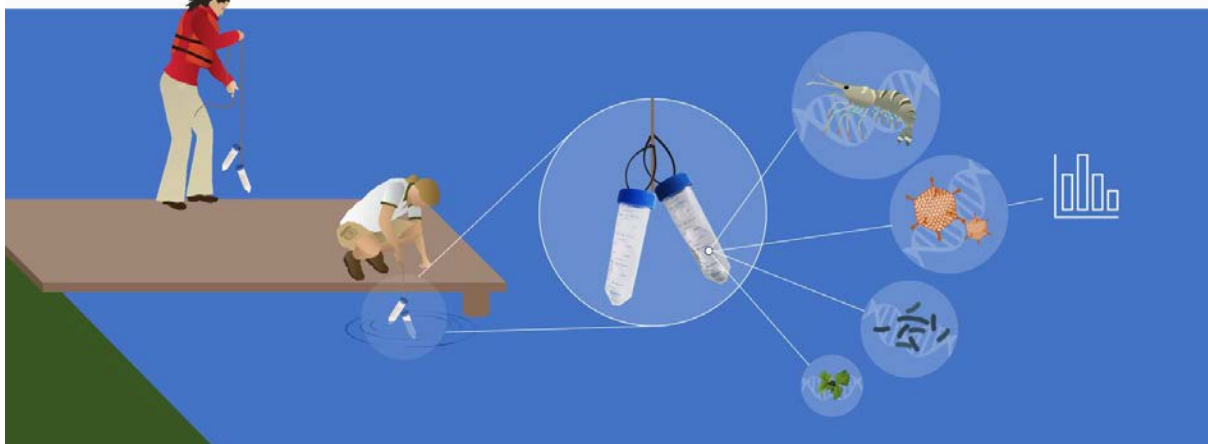
utmost reliability of ‘true’ positive detection from non-destructive samples is necessitated by SPF-type programs (Moss et al., 2012) and for surveillance of rare infections of high consequence (Kim et al., 2023). Similarly, ensuring highly representative viral quantitation is critical where viral titre is used to inform management decisions, for example, in shrimp selective breeding efforts where viral titre has been suggested as an effective selection criterion to confer pathogen resistance (Knibb et al., 2015; Phuthaworn et al., 2016; Trang et al., 2019b). However, based on our findings, achieving such reliability and accuracy, especially for GAV, requires significant biological and technical replication. Such requirements would inevitably involve commensurate increases in the financial and temporal cost of analysis, in addition to increased cuticle damage and stress of the sampled animals with related risk of secondary welfare implications (Corteel et al., 2009; Mitchell et al., 2023). To reduce costs, multiple pleopods may be pooled, however, the impact of pooling on the sensitivity of viral detection, and the subsequent interpretation of quantitative detection results from pooled samples, has yet to be clearly established (Laurin et al., 2019; Moody, 2024).

To enhance detection reliability at low copy number, increasing qPCR technical replication is more cost-effective and time-efficient than increasing pleopod sampling. This approach minimises stock damage while improving detection reliability by capitalising the increased probability of amplifying a few target copies within the sample, considering the stochasticity of the PCR process defined by Poisson uncertainty (Asogawa, 2022; Forootan et al., 2017; Tellinghuisen, 2020). However, to achieve more representative quantitative detections, increasing the number of tissue sample replicates is still required, especially for the detection of GAV which is demonstrated to yield highly variable detection between pleopods and gills of individual shrimp (Noble et al., 2018). Nonetheless, for endemic shrimp viruses within Australia there is currently no evidence to support differential management of individuals based on detected viral titre, especially at low copy number, for example, for selecting based on viral titre to confer resistance to GAV induced mortality (Noble et al., 2020a, 2020b). Thus, the trade-off between the value of improved representativeness of quantitative detection and the commensurate increases in sampling costs must be critically evaluated. Understanding the balancing point of this trade-off will require further research to define the relationships between detected viral loading for GAV and IHHNV and their relative impacts on productivity and shrimp health. Understanding the biological relevance of molecular detections is essential to ensure that impaired interpretation is not carried forward to misinform risk assessments, or

detrimentally affect overall surveillance outcomes and management decisions (Stärk et al., 2019).

4.5.4 Conclusion

This study provides evidence to support improved interpretation of IHHNV and GAV qPCR detection results from pleopods of naturally infected *P. monodon*. Overall, quantitative detection of IHHNV from pleopods was found to be highly representative of other tissues within individual shrimp, and relatively consistent among pleopods of the same animal, conferring the systemic nature of IHHNV infection. Conversely, quantification of GAV from pleopods was poorly representative of loading in other tissues and detection between pleopods was highly variable. To achieve more accurate quantification of IHHNV and GAV from pleopods, multiple pleopods need to be sampled, and to improve the reliability of ‘true’ positive detection from low load shrimp, pleopods should be tested with adequate technical replication to overcome the Poisson distribution limitation.



Chapter 5.

Passive eNA sample collection to monitor viral pathogens in farmed giant black tiger shrimp (*Penaeus monodon*) pond aquaculture systems

PREPARED FOR PUBLICATION

Foreword

Chapter 3 and Chapter 4 developed foundational knowledge to support improved pathogen detection and detection result interpretation from shrimp. However, to conduct monitoring and surveillance at large-scale, such as across a farm, the requisite shrimp sampling effort increases commensurately with the size of pond populations, the number of ponds per farm, the level of desired detection sensitivity, and the frequency of monitoring intervals. With this increasing effort, the cost-efficiency and ease of sampling decreases rapidly, becoming prohibitive to adoption. Therefore, a more efficient, alternative sampling approach is required to facilitate large-scale pathogen surveillance and monitoring programs. Chapter 5 aimed to assess the utility of a novel environmental passive sampling approach for this purpose, including validation across a commercial shrimp farm and optimisation for improved deployment strategy.

5.1 Abstract

Collection and analysis of environmental nucleic acid (eNA; including eDNA and eRNA) is proposed as an effective and efficient approach for monitoring and surveillance in aquatic biosecurity applications. Passive eNA collection involves the passive settlement or binding of nucleic acid to filter material during a period of environmental incubation. Passive eNA sampling achieves comparable performance to active eNA sampling but is considerably simpler and more cost efficient. Therefore, passive eNA collection has significant potential where large-scale sampling is required to be undertaken by nonexperts, for example to conduct routine surveillance of pathogens in commercial aquaculture systems. Despite the potential benefits, few studies have investigated the use of passive eNA sampling for pathogen monitoring in aquaculture systems and, as such, sampling approaches have yet to be validated and broadly applied in this field. This study aimed to evaluate the utility of passive eNA sampling to detect and quantify infectious hypodermal and haematopoietic necrosis virus (IHHNV, syn *Penaeus stylirostris* penstyldensovirus 1) and gill-associated virus (GAV, syn yellowhead virus 2) from giant black tiger shrimp (*Penaeus monodon*) production ponds. Briefly, passive eNA samplers were deployed across 31 production ponds (~1 ha) and incubated for 24 h. To evaluate the optimal submersion duration for passive eNA collection, an additional study was conducted with passive eNA samplers deployed across 5 production ponds, and submerged for 1, 2, 4, 6 or 24 h. The capacity for eNA samples to provide a proxy measurement of host infection was assessed using paired shrimp tissue samples collected from each pond, across both studies. Viral detection using TaqMan qPCR analysis from all eNA samples were compared to detection from shrimp tissues collected from the same ponds. Both IHHNV and GAV could be detected from passively collected eNA samples. Comparison of qPCR analysis demonstrated passively collected eNA yielded IHHNV quantifications that were highly indicative of those detected from shrimp collected from the same pond ($R^2 = 0.82$). Indicative IHHNV eNA results could be obtained with only 1 hour of filter submersion. Within the ponds analysed, quantitative eNA-based detection of GAV did not show concordance with host tissue detection. However, both IHHNV and GAV loading detected from eNA samples increased with increasing submersion time, indicating that longer submersion (24 h) of passive samplers would likely be more suitable for surveillance of rare or low-level infections. The findings of this study provide strong evidence to support further application of passive eNA sample collection for efficient and cost-effective pathogen monitoring in aquaculture production systems and provides insight for optimised sampling strategies.

5.2 Introduction

Infectious disease is a significant challenge to the ecological and economic sustainability of global shrimp aquaculture (FAO, 2022; Stentiford et al., 2012). Timely, accurate and cost-effective detection of pathogens is critical to effective shrimp disease mitigation and management (Major et al., 2023). Molecular technologies, primarily qPCR, form the basis of current pathogen detection and disease diagnosis in shrimp aquaculture (Kumar et al., 2021b; WOA, 2023b). Current practices for shrimp pathogen detection rely on the collection and analysis of host tissue samples. While host tissue sampling remains a requisite of disease diagnosis (i.e. for confirmation of a pathogenic agent in clinically affected or apparently healthy animals) (WOA, 2023b), this practice can present challenges for non-diagnostic purposes, such as for surveillance and monitoring. Specifically, pathogen surveillance programs can rapidly become expensive due to associated costs and requirements of extensive host tissue sampling and analysis (Huerlimann et al., 2020; Reich et al., 2018). For example, shrimp production ponds may be stocked at densities ranging between 20-60 shrimp m⁻² (Eid et al., 2020; Mena-Herrera et al., 2006), translating to a theoretical pond (~0.5 - 1 ha) population of 100 000 – 600 000 shrimp (e.g. Zeng et al., 2020), with farms often comprising tens, to hundreds of ponds. Consequently, to conduct a farm-scale pathogen monitoring program, which may aim to detect a target occurring at a prevalence of 10% within a large pond population, a total of 39 shrimp per pond must be sampled, assuming a reasonable test sensitivity (0.8 for pooled tissue samples of 3 individuals; epitools.ausvet.com.au). Multiple tissue types per animal may also be required for the optimised detection of multiple pathogen targets (WOA, 2023b). This sampling effort must be scaled for the number of ponds on the farm, and repeated at regular intervals, for the monitoring program to provide early detection capability and valuable spatiotemporal insights for improving pathogen management. Under such a scenario, even when pooling is used to reduce analysis costs, sample numbers and sampling effort increase commensurately to thousands of samples, rapidly becoming cost and time-prohibitive (Brunner, 2020). Therefore, a more efficient, alternative sampling approach is required to facilitate farm-scale pathogen analysis and monitoring programs.

5.2.1 Environmental sampling for pathogen surveillance

Collection and analysis of genetic material obtained from environmental samples, termed environmental DNA (eDNA) or RNA (eRNA), collectively hereafter eNA, is proposed as an alternative to tissue sampling for monitoring of pathogen presence in aquatic systems (Bass et

al., 2023; Bastos Gomes et al., 2017; Bowers et al., 2021; Brunner, 2020; Sieber et al., 2024). Existing in various physical states, eNA may be derived from entire organisms embedded within biofilms to extracellular nucleic acid, including all intermediate variations (Power et al., 2023). In the context of pathogen discovery, surveillance and monitoring, eNA is defined as nucleic acids of pathogenic agents extracted from ‘true’ environmental samples, such as water, soil, sediment or biofilm (WOAH, 2022). While positive molecular detection from an eNA sample cannot confirm infection, as positive detection from the environment does not demonstrate the infection of a susceptible host (WOAH, 2022), detections from eNA samples may serve as a proxy to indicate infection of host animals (Bass et al., 2023). As a proxy, eNA samples are a valuable tool for viral surveillance and monitoring, and provide considerable advantages over sampling of host tissue (WOAH, 2022). Converse to individual tissue sampling, eNA sample size decreases with an increasing population size given a fixed viral target prevalence, as more infected individuals shed eNA into the system (Brunner, 2020). This quality affords increased spatiotemporal scope, specificity, sensitivity, and efficiency of surveillance efforts, as a small number of eNA samples can represent multiple host animals or whole populations (Bass et al., 2023; Brunner, 2020; Childress et al., 2024; Hall et al., 2018). The capacity for a single eNA sample to simultaneously capture multiple pathogen targets further eliminates the requirement to sample different target tissues or host taxa, greatly reducing sampling requirements and enabling more comprehensive analyses (Sieber et al., 2024). Given this greatly improved efficiency, eNA sampling can be applied routinely throughout the production cycle to monitor pathogen presence in the absence of overt clinical signs, enabling improved scope for early detection and proactive, rather than reactive, disease management (Farrell et al., 2021). Additionally, non-invasive eNA sampling avoids the requisite of handling, stressing or sacrifice of host animals during monitoring (Bowers et al., 2021). This is particularly advantageous for monitoring of high-value populations such as broodstock, or in pathogen introduction pathways such as intake canals, where the host species (e.g. shrimp) are not cultured (WOAH, 2022). Furthermore, eNA collection is less likely to spread pathogens than traditional sampling approaches, as direct handling of infected host animals is not required (Strand et al., 2019).

Given the significant benefits associated, eNA monitoring is increasing in popularity, and has been applied to a diversity of environmental systems for targeted pathogen and parasite monitoring (e.g. those reviewed in Bass et al., 2023). Although eNA sampling offers significant

benefits for these applications, conventional methods can become challenging due to the need for active filtering, concentrating or storage of large volumes of environmental samples (e.g. water) (Tsuji et al., 2019; Verdier et al., 2022). Active collection methods require specialist equipment, including peristaltic pumps or in-line filters (Rusch, 2021). This equipment is often difficult to operate, time and cost inefficient, easily contaminated, and result in an inability or reluctance from non-experts to conduct field sampling (Bessey et al., 2022; Chen et al., 2022; Villacorta-Rath and Burrows, 2021; Zaiko et al., 2018). These processes may also represent a risk of cross-contamination across multiple sample sites if insufficient decontamination and sterilisation of equipment occurs. While this presents issues for data quality and the introduction of false positives, more critically, under a pathogen monitoring context in an aquaculture environment, such cross-contamination could result in the translocation of viable pathogens across a farm system. Active filtration methods also face challenges in standardisation of sampling. For example, collection and filtration of the same sample volume from clear or turbid water sites may require a disparate number of filter membranes to complete filtration, or complete filtration may not be possible, as filters can become blocked with high particulate loading (Bessey et al., 2022; Chen et al., 2022; Goldberg et al., 2016; Huerlimann et al., 2020; Ip et al., 2024). Such disparities can affect downstream processing and analysis, or limit the capacity for relative comparison between samples.

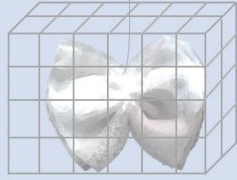
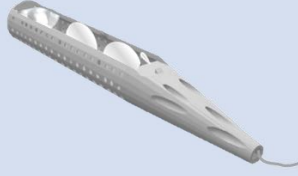
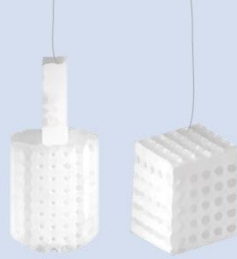
5.2.2 Passive environmental sampling

An emerging alternative approach, termed ‘passive eNA collection’, is increasing in popularity as a suitable eNA sample collection method with comparable performance to active eNA collection (Bessey et al., 2022, 2021; Kirtane et al., 2020; Rafiee et al., 2021; Verdier et al., 2022; Vincent-Hubert et al., 2017). Passive eNA collection involves incubation of a filter medium within the environmental matrix to enable passive settlement or binding of genetic material to the filter (Bass et al., 2023). The filter medium is then collected, placed directly into a preservative solution, and processed for nucleic acid extraction and further analysis. Passive eNA collection is considerably more time and cost efficient than active collection methods and requires minimal equipment, hence reducing the risks of cross-contamination and the level of expertise required for operation (Chen et al., 2022). These qualities render passive eNA collection highly amenable for application to large-scale monitoring efforts, and remote deployment by non-experts (Bessey et al., 2022).

Passive sampling for environmental pathogen surveillance has a well-established history, originating from Moore and colleagues in 1948 for the detection and tracing of *Salmonella* in sewage using gauze swabs submerged for 48 h (Moore, 1948). The application of ‘Moore’ swabs have seen recent renewal, for example for the detection of SARS-CoV-2 (Rafiee et al., 2021; Schang et al., 2021) and typhoidal *Salmonella* bacteria (Sikorski and Levine, 2020) in human wastewater. Recently, a diversity of passive collection materials have been applied to aquatic systems (see Table 5.1), including demonstration of utility for pathogen monitoring (e.g. Vincent-Hubert et al., 2017). These recent studies highlight the promise of passive eNA collection for application to pathogen monitoring in aquaculture.

Although eNA approaches provide substantial advantages for sampling scope and efficiency, the disconnect between the infected host and the environmental sample presents challenges for translating eNA detection results to evaluate potential host disease risk and to inform biosecurity management decisions (Bass et al., 2023; Bessey et al., 2022). Beyond accurate presence/absence determination, for target detection from eNA samples to be valuable for monitoring host infection in any quantitative capacity, calibration studies are required to correlate eNA detection with detection from target host samples (Bass et al., 2015). Given the array of biotic and abiotic factors influencing eNA generation and persistence in the environment (Wang et al., 2021), such calibration studies are likely required on a site-, species- and pathogen target-specific basis. To date, few studies that have applied active or passive eNA collection approaches in aquatic contexts have generated statistically supported associations between eNA detections and confirmed infection in the host organism (e.g. Rusch, 2021; Strand et al., 2014; Wittwer et al., 2019). As such, despite their increasing popularity and application, the integration of eNA approaches for pathogen detection into key reference documents, such as the World Organisation for Animal Health Manual of Diagnostic Tests for Aquatic Animals (herein WOAHA Aquatic Manual), are limited. Currently, only one study, involving eNA detection of a WOAHA listed aquatic disease agent (*Gyrodactylus salaris* hosted by Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*)), has demonstrated sufficient validation for inclusion in the Aquatic Manual (Rusch et al., 2018; WOAHA, 2022, 2021b).

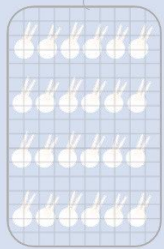
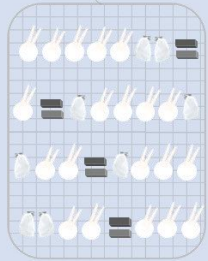
Table 5.1. Modern examples of aquatic field-deployed passive eNA samplers

Filter material	Submersion time	Target	Field environment	Sampling unit design adapted from cited publication (right)	Reference
Montmorillonite clay ⁴ Granular activated carbon ⁵	7 and 21 d	<i>Lampsilis siliquoidea</i> (mussel) eDNA and universal mitochondrial 12S rRNA fish eDNA	Freshwater creek		(Kirtane et al., 2020)
Gauze (Moore swab)	16 h	SARS-CoV-2 eRNA	Human wastewater		(Rafiee et al., 2021)
Gauze (Moore swab) Cellulose nitrate filter membranes Cotton buds	3-7 and 24 h	SARS-CoV-2 eRNA	Human wastewater		(Schang et al., 2021)
3D-printed hydroxyapatite	24h	<i>Asellus aquaticus</i> (isopod) eDNA	Freshwater pond		(Verdier et al., 2022)

⁴ Material contained between two 40 µm cell strainers

⁵ Material contained within a polyester mesh bag

Table 5.1 Continued.

Filter material	Submersion time	Target	Field environment	Sampling unit design <i>adapted from cited publication</i>	Reference
Cellulose ester filter membrane (CEFM) Electropositive nylon filter membrane	4, 8, 12, 24h	Universal mitochondrial 16S rDNA fish eDNA	Open ocean (tropical and temperate)		(Bessey et al., 2021)
CEFM CEFM with 1% chitosan or 3% chitosan CEFM overlayed with electrospun nanofibers and 1% chitosan Cotton fibres ⁶ Hemp fibres ⁶ Sponge with embedded zeolite or activated carbon	5 min, 10 min, 30 min, 1 h and 18 h	Universal mitochondrial 16S rDNA fish eDNA	3 million L marine mesocosm (Main tank; The Aquarium of Western Australia [AQWA])		(Bessey et al., 2022)
Glass fibre filter membrane ⁷	0.5, 8, 24, and 72 h	Universal mitochondrial 12S rRNA fish eDNA	Freshwater lake (~2 ha)		(Chen et al., 2022)

⁶ Material contained within a nylon bag

⁷ Material contained within polyethylene terephthalate bag

Accordingly, this study aimed to investigate the capacity of passive eNA sampling for determining viral pathogen presence in giant black tiger shrimp, *Penaeus monodon*, aquaculture ponds, and to evaluate its utility for proxy quantification of pathogen loading in shrimp hosts. We aimed to determine the association between eNA target detection and detection from shrimp tissue, initiating the validation of our passive eNA sampling approach for the detection of a WOAHP listed shrimp pathogen, namely infectious hypodermal and hematopoietic necrosis virus (IHHNV, *syn Penaeus stylirostris* penstyldensovirus 1; PstDV1), and an important viral pathogen in Australian farmed shrimp culture, that of gill-associated virus (GAV, *syn* yellowhead virus 2). Finally, we investigated the effect of submersion time on the detection patterns from passive eNA samples and the correlation to detection from shrimp tissue samples.

5.3 Materials and methods

Methodological details of this study are reported, where applicable, to align with reporting guidelines detailed by Goldberg et al. (2016) and by the WOAHA Aquatic Animal Health Standards Commission for validation of the use of environmental DNA methods for detection of WOAHA-listed aquatic animal diseases (WOAHA, 2022).

5.3.1 Experimental design

This study was conducted at a commercial *Penaeus monodon* grow-out facility in North Queensland, Australia. The earthen production ponds (~1 ha) had been stocked with shrimp for between 125 and 169 days of culture (DOC) at the time of sampling. Water quality parameters of temperature (°C), pH and dissolved oxygen (DO; ppm) were collected at ~05:00 and ~13:00 from each pond on the day of sampling to capture the approximate minimum and maximum range of parameters. Salinity data was also collected from each pond within a week of sampling.

5.3.1.1 Farm-scale monitoring

Initially, to investigate the capacity of eNA passive sampling for the detection of viral shrimp pathogens from aquaculture ponds, passive eNA sampling was conducted across a commercial farm. The sampling approach was conducted with the aim of replicating the approach of a farm-scale pathogen monitoring program. Farm-scale sampling comprised paired sampling of shrimp tissues and passively collected eNA from a total of 31 (1-31) earthen shrimp production ponds (~1 ha). Sampling from each pond included duplicate passive eNA samples immersed for 24 h approximately 30 cm below the water surface and 2 m from the pond edge. At the same period of the passive eNA sampling, 13 pools of three shrimp pleopod tissues were sampled from each pond, comprising samples from a total of 39 individual shrimp. The sample size was selected to provide 95% confidence of detecting a target prevalence of 10%, assuming test sensitivity of 0.8 from the pooled tissue samples (epitools.ausvet.com.au).

5.3.1.2 Submersion duration experiment

Following the farm-scale sampling, an additional experiment was conducted to investigate the optimal submersion duration of the passive eNA samplers. For the submersion duration experiment, paired shrimp tissues and passive eNA samples from five (labeled herein A - E) earthen shrimp production ponds (~1 ha) were sampled. A total of 10 passive eNA samples

were placed in each of the five ponds at the same location, approximately 30 cm below the water surface and 2 m from the pond edge. Duplicate eNA samples were collected from each pond after 1, 2, 4, 6 or 24 h of submersion. A total of 10 shrimp were sampled from each of the five ponds during the same period of eNA sample submersion. Fewer shrimp samples were collected within this experiment compared to the farm-scale monitoring study, as the 5 sampled ponds were known to be highly positive for both IHHNV and GAV based on previous qPCR analysis (data not shown). The number of shrimp sampled from each pond enabled theoretical detection of ~30% target prevalence from each pond population, assuming a test sensitivity of 0.9 from individual tissue samples (epitools.ausvet.com.au). From each individual shrimp, pleopod, gill and hepatopancreas tissue was sampled. Details of the sampling are described in Table 5.2.

Table 5.2. Sampling design utilised throughout the farm-scale monitoring study and the eNA sampler submersion duration experiment.

Purpose	Ponds	Shrimp sampled/ pond	Tissues sampled/ shrimp	eNA samples/ pond	eNA submersion duration (h)
Farm-scale monitoring	1-31 (n = 31)	39 (13 pools of 3)	Pleopod	2	24 (duplicate)
Submersion duration	A-E (n = 5)	10	Pleopod, Hepatopancreas, Gill	10	1, 2, 4, 6 and 24 (duplicate)

5.3.2 Passive eNA sampling

Passive eNA samplers comprised a hydrophilic, biologically inert, mixed cellulose ester (MCE) microdisc membrane filter composed of cellulose acetate and cellulose nitrate (0.22µm, 47 mm dia) (Cat. [MFMCE047022GWSW](#), Membrane Solutions, WA, Australia) placed within a plastic housing of 50 mL capacity to provide protection from damage caused by potential debris and biota (Figure 5.1). Each housing included six ~1cm² holes to allow water flow across the filter and a ceramic weight to ensure the unit submerged. Duplicate passive samplers were removed from each pond after 24 h of submersion in the production pond (eNA₂₄; farm-scale monitoring) or 1, 2, 4, 6 or 24 h (eNA₁, eNA₂, eNA₄, eNA₆, eNA₂₄, respectively; submersion optimization experiment). Upon removal from the pond, membrane filters were collected from the plastic housings and transferred individually into DNase and RNase-free tubes containing 2 mL of Ambion™ nuclease-free water (not DEPC-treated) (Cat. AM9932, Invitrogen™, Texas, USA) and 2 mL of MagMAX™ CORE Lysis Solution (Cat. A32702 Ref.

100043807, Applied Biosystems™, VIC, Australia). Filters were incubated at room temperature in the lysis solution for 5 days before a 350 µL aliquot was taken in a 1.7 mL micro-centrifuge tube for nucleic acid extraction.

A sterile technique was used for handling of eNA samples throughout the experiments during both field collection and laboratory processing and analysis. Contamination control included wearing of gloves to handle all materials and using sterile, single-use equipment. Membrane filters were certified sterile, and filter housings were bleach sterilised prior to use.

5.3.3 Paired shrimp tissue sampling

A total of 39 (farm-scale monitoring experiment) and 10 (submersion duration experiment) shrimp were collected from each of the ponds during the same period the passive eNA sample collection. For the farm-scale monitoring experiment, a single 5 mm pleopod clip was sampled from each shrimp, pooled into three, and placed immediately into 2 mL Lysing Matrix D tubes (Cat. 6913050, MP Biomedicals™, OH, USA) containing 350 µL of MagMAX™ CORE Lysis Solution (Cat. A32702 Ref. 100043807, Applied Biosystems™, VIC, Australia). For the submersion duration experiment, shrimp were collected from each pond and stored at -20 °C until further sampling. After thawing at 4 °C, pleopod, gill and hepatopancreas samples were collected from each individual shrimp. Tissue samples were weighed and placed immediately into 2 mL Lysing Matrix D tubes containing 350 µL of MagMAX™ CORE Lysis Solution. All tissue samples were stored at room temperature in lysis buffer for between ~24 h and 5 d prior to nucleic acid extraction.

5.3.4 Nucleic acid extraction

Nucleic acid extraction was conducted as per section 2.3.2 with minor adaptation. Briefly, from tissue samples, 5 µL of 20 mg mL⁻¹ MagMAX™ CORE Proteinase K (Cat. A32702 Ref. 100043773, Applied Biosystems™, VIC, Australia) was added to each 2 mL Lysing Matrix D tube (Cat. 6913050, MP Biomedicals™, OH, USA) containing the sample in 350 µL of

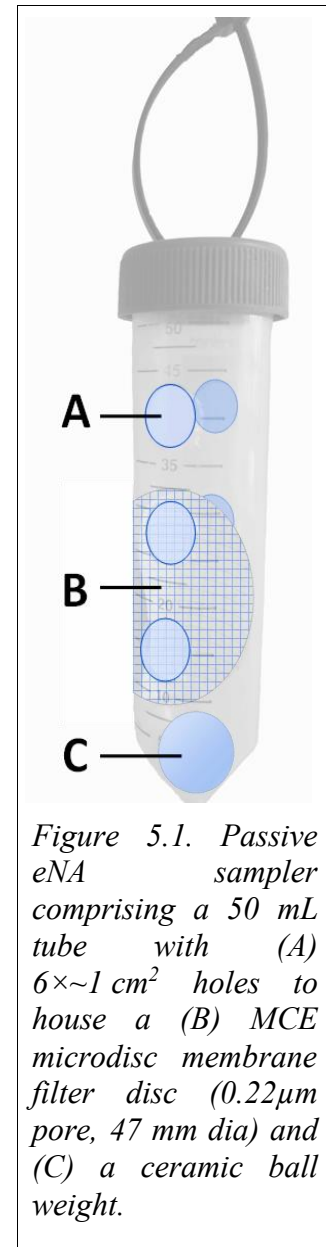


Figure 5.1. Passive eNA sampler comprising a 50 mL tube with (A) 6×~1 cm² holes to house a (B) MCE microdisc membrane filter disc (0.22µm pore, 47 mm dia) and (C) a ceramic ball weight.

MagMAX™ CORE Lysis Solution (Cat. A32702 Ref. 100043807, Applied Biosystems™, VIC, Australia). The sample was homogenized for 30 s at 5000 rpm using a Precellys® 24 tissue homogenizer (Cat. P000669-PR240-A, Bertin Technologies, France). From eNA samples, 5 µL of 20 mg mL⁻¹ MagMAX™ CORE Proteinase K was added to each 1.7 mL microcentrifuge tube containing the 350 µL aliquot of lysis solution after incubation with the eNA filters. The homogenised tissue samples and eNA samples were incubated at ~72 °C for 60 min. Total nucleic acid (TNA) extraction from the homogenised and digested samples was conducted using the MagMAX™ CORE Nucleic Acid Purification Kit (Cat. A32702, Applied Biosystems™, VIC, Australia) with a KingFisher™ Flex 96 Deep-Well Magnetic Particle Processor (Cat. A31508, ThermoFisher Scientific™, VIC, Australia) as per the manufacturer's instructions. Total nucleic acid was eluted into 100 µL of MagMAX™ CORE Elution Buffer (Cat. A32702 Ref. 100043817, Applied Biosystems™, VIC, Australia) and stored at -20 °C until required.

5.3.5 Quantitative real-time PCR (qPCR)

Quantitative polymerase chain reaction (qPCR) analysis was used for the detection of the two endemic pathogen targets, IHHNV and GAV. Additionally, the *P. monodon* Dicer-1 gene (Dicer-1) was targeted for detection using RT-qPCR as a protocol integrity control (Su et al., 2008). qPCR analysis was conducted as per section 3.3.4 using the relevant primer and probe sequences listed in Table 2.1. For the submersion duration experiment, three 1.25 µL aliquots of each TNA extract were tested as technical replicates for each target. Briefly, analysis was conducted using a QuantStudio™ 5 Real-Time PCR System, 384-well (Cat. A28140, Applied Biosystems™, VIC, Australia). Cycle conditions were standardised for both DNA and RNA targeting assays, consisting of a reverse-transcription (RT) step of 45 °C for 10 min, initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 10 s and annealing and extension at 60 °C for 30 s. Raw data were processed using QuantStudio™ Design & Analysis Software v1.5.2 (ThermoFisher Scientific™, www.thermofisher.com), using the ΔR_n method with a relative threshold of 0.05, to produce cycle threshold (Ct) values of positive detections. Samples which did not amplify beyond the relative threshold within 45 cycles were categorized as undetermined. A positive target control and non-template control were included in each analysis.

As discussed by Rafiee et al. (2021), unlike grab sampling approaches for eNA collection where the filtered sample volume is defined, there is little ability to determine the volume of

water that has directly contacted a passive eNA sampler during incubation. Consequently, our data was standardised to target copy number μL^{-1} TNA extracted from each passive eNA sample filter given each sample was collected and processed consistently, allowing for a relative comparative analysis of samples. To do so, conversion of Ct value to target copy number μL^{-1} TNA extract was conducted as per section 3.3.5, using serial 10-fold dilutions of synthetic linear dsDNA gBlock™ Gene Fragments (Integrated DNA Technologies™, NSW, Australia). Samples categorised as undetected were assumed to have a viral copy number of zero. For the submersion duration experiment, target copy number μL^{-1} TNA extract was converted to target copy number mg^{-1} tissue for pleopod, gill and hepatopancreas samples to enable comparison between the tissue samples.

5.3.6 Statistical analysis

Statistical analysis was conducted using R (R Core Team, 2022). Descriptive statistical parameters, including total number of samples tested, total number of positive detections, rate of positive detection (%) and the average (mean) and standard deviation (SD) of positive detections in copy number μL^{-1} TNA extract, or copy number mg^{-1} were calculated for averaged technical replicates of each target, between sample types and between ponds. Further statistical analysis to evaluate the differences in copy number and correlation of detection between sample types were conducted using $\log_{10}$ transformed copy number μL^{-1} , or copy number mg^{-1} , after zero values were adjusted to one.

Significant differences in overall IHNV detection between the sample types were determined using a Kruskal-Wallis test (R package: *stats*; $\alpha = 0.05$; R Core Team, 2022), due to significant deviation from homogeneous variances, as determined via a Levene's test (R package: *car*; $\alpha = 0.05$; Fox and Weisberg, 2019). Post-hoc analysis was run using a Dunn test with Holm's correction (R package: *FSA*; $\alpha = 0.05$; Ogle et al., 2022). Overall differences in GAV detections between sample types were determined using Welch's two sample t-test with equal variance (R package: *stats*; $\alpha = 0.05$; R Core Team, 2022). Linear regression analysis was used to define the relationship of IHNV detection from pleopod tissue and passive eNA samples collected from each pond. A single outlying datapoint (Pond A, eNA₂₄) was excluded from linear regression analysis. The correlative significance of the linear relationship was determined using Pearson's correlation analysis (R package: *Hmisc*; $\alpha = 0.05$; Harrell, 2023). Multiple linear regression analysis (R package: *stats*; $\alpha = 0.05$; R Core Team, 2022) was used to determine if

pond water quality parameters including temperature, pH, DO and salinity had statistically significant influence on IHHNV detection from passive eNA samples.

5.4 Results

5.4.1 Farm-scale monitoring

Analysis of pooled shrimp tissue samples yielded positive GAV detection from all 31 ponds, with positive detection rates of pooled samples ranging from 15% (2/13) to 100% (13/13) for individual ponds (Appendix 17; Appendix 19). Indicatively, 100% of the ponds were also GAV positive by duplicate eNA₂₄ samples, where a pond was considered eNA positive if one or both duplicate eNA samples yielded positive detection. There was no association observed between the prevalence or copy number of GAV detected from shrimp tissue and the consistency of positive detections from duplicate eNA samples for GAV. Specifically, three ponds yielded only one positive detection from the duplicate eNA samples. From these three ponds, 100% of the pooled pleopod samples were positive for GAV, and represented a relatively low, moderate and high average copy number μL^{-1} from shrimp tissue samples for the 31 ponds analysed.

Analysis of pooled shrimp tissue samples yielded IHHNV detection from all 31 ponds, with positive detection rates of pooled samples ranging between 31% (4/13) and 100% (13/13) for individual ponds (Appendix 17; Appendix 19). While 30 of the analysed ponds were IHHNV positive by one or both duplicate eNA samples, a single pond (pond 3) was negative for IHHNV detection across both eNA samples. This pond had low prevalence of positive tissue detections (5/13) and low IHHNV copy number (5.9 ± 9.8 copies μL^{-1}). Pond water quality was relatively consistent across the analysed ponds during the surveillance experiment, including pond 3 (Appendix 16; Appendix 21). Multiple linear regression analysis determined that IHHNV detection from eNA₂₄ samples was significantly associated with IHHNV detection from shrimp tissue ($t = 9.25$, $p < 0.001$), however, the measured water quality parameters did not significantly influence IHHNV detection from eNA₂₄ samples ($t < 1.2$, $p > 0.05$).

Overall, detected GAV copies μL^{-1} from each pond was low, averaging $2.25 \times 10^2 \pm 8.99 \times 10^2$ from pooled pleopod tissue, and $2.20 \times 10^2 \pm 2.84 \times 10^2$ from eNA₂₄ samples (Figure 5.2; Appendix 17; Appendix 19). GAV copy number was significantly higher in eNA₂₄ samples than pleopod tissue samples ($t_{df=60} = -3.81$, $p = 0.0003$). In contrast, detected IHHNV copies μL^{-1} from each pond averaged $4.62 \times 10^6 \pm 1.09 \times 10^7$ from pooled pleopod tissue, and $1.65 \times 10^4 \pm 4.17 \times 10^4$ from eNA₂₄ samples. IHHNV detections were significantly higher in pooled pleopod tissue than from eNA₂₄ samples ($H_{df=1} = 6.17$, $p = 0.013$).

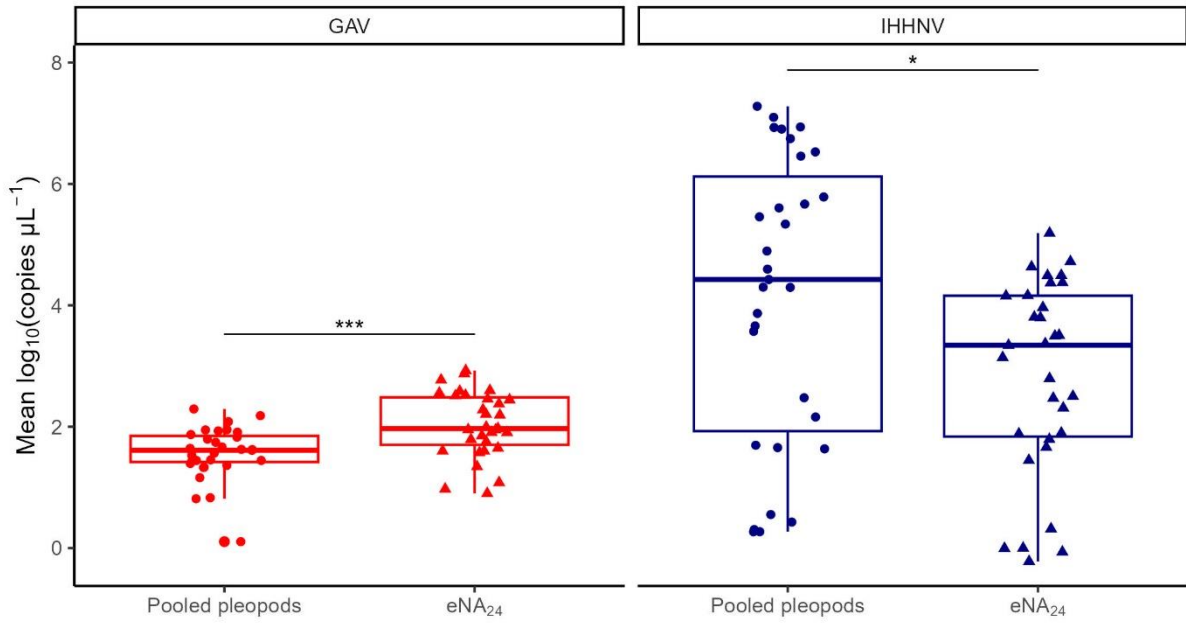


Figure 5.2. Comparative detection from pooled *Penaeus monodon* pleopod tissue samples and passive eNA₂₄ samples collected from the same ponds ($n = 31$). Each point represents the mean $\log_{10}$ transformed copy number μL^{-1} detected from each sample type.

Comparative pond-wise analysis between pooled pleopod tissue samples and passive eNA₂₄ samples demonstrated no significant correlation for GAV detection ($R^2_{adj} < 0.01$, $F_{1,29} = 0.339$, $p = 0.565$). However, a highly significant linear correlation was identified between mean IHNV detection from duplicate eNA₂₄ samples, and mean IHNV detection from the pooled pleopod tissue samples for each pond ($R^2_{adj} = 0.82$, $F_{1,29} = 136$, $p < 0.001$) (Figure 5.3).

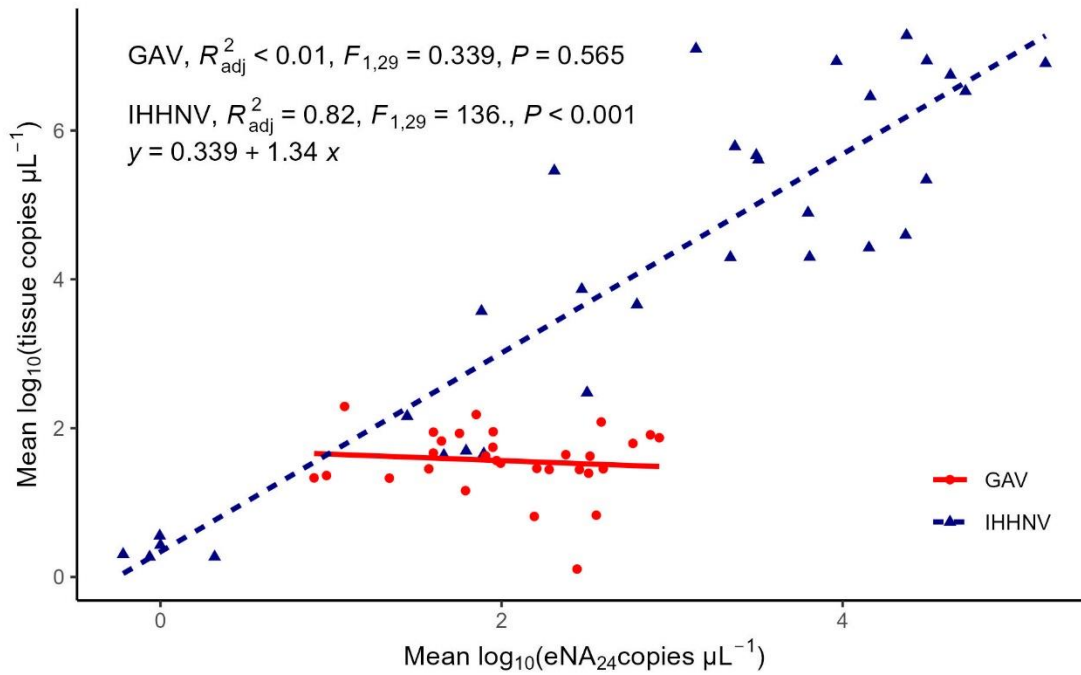


Figure 5.3. Comparative detection of IHHNV from passive $e\text{NA}_{24}$ samples and pooled *Penaeus monodon* pleopod tissue samples collected from the same ponds. Each point represents the mean $\log_{10}$ transformed copy number μL^{-1} detected from duplicate $e\text{NA}_{24}$ samples compared to the mean $\log_{10}$ transformed copy number μL^{-1} detected from 13 pools of three individual shrimp pleopods from the same pond.

5.4.2 Submersion duration experiment

All five ponds analysed for the submersion duration experiment were positive for the detection of both GAV and IHHNV (Appendix 18; Appendix 20). Detection of GAV across all samples was significantly lower than detection of IHHNV ($H_{df=1} = 134.92$, $p < 2.2\text{e-}16$). IHHNV was detected in all shrimp tissue samples and all $e\text{NA}_{1-24}$ samples. GAV was detected in all $e\text{NA}_{1-24}$ samples, however, was not detected in 2/50 (4%) of the pleopod and hepatopancreas samples, and 1/50 (2%) of the gill samples (Appendix 18; Appendix 20). Overall IHHNV detection between tissues were not significantly different, however, detection from each tissue type was significantly higher than detection from the $e\text{NA}_{1-24}$ samples ($H_{df=3} = 52.46$, $p = 2.4\text{e-}11$) (Figure 5.4). Overall GAV detection was significantly higher in pleopod tissue compared to hepatopancreas ($p = 0.049$) and $e\text{NA}_{1-24}$ samples ($p = 0.005$). Detection of GAV was consistent between the five ponds. Comparatively, detection of IHHNV between the five ponds was significantly different, and pond differences were consistently reflected in detection patterns from each sample type.

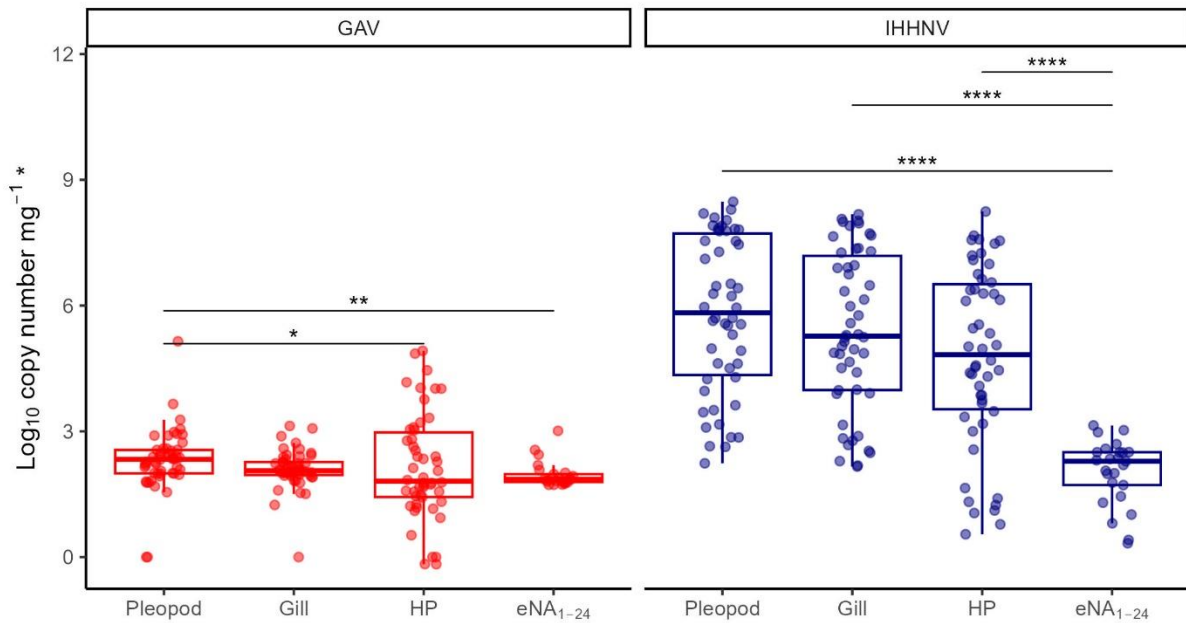


Figure 5.4. Overall detection of GAV and IHNV in *Penaeus monodon* pleopod, gill and hepatopancreas (HP) tissue and eNA passive samples collected at 1, 2, 4, 6 and 24 h of submersion (eNA₁₋₂₄). Each point represents data from a single shrimp or eNA sample collected from one of the 5 ponds (A-E) sampled. * eNA target quantification is representative of log₁₀ copy number μL^{-1} .

Quantitative detection of GAV and IHNV from the passive eNA samples increased with increasing submersion duration time, with submersion for 24 h yielding the highest overall detected copy number μL^{-1} for both targets (Figure 5.5). GAV detection from eNA₁₋₆ samples remained relatively constant, with a significant increase in detection from eNA₂₄ samples, compared to eNA₁ ($p = 0.04$) and eNA₂ ($p = 0.02$) samples. Comparatively, IHNV detection increased more linearly relative to eNA sampler submersion duration, with IHNV detection from eNA₂₄ samples significantly higher than eNA₁ samples ($p = 0.046$).

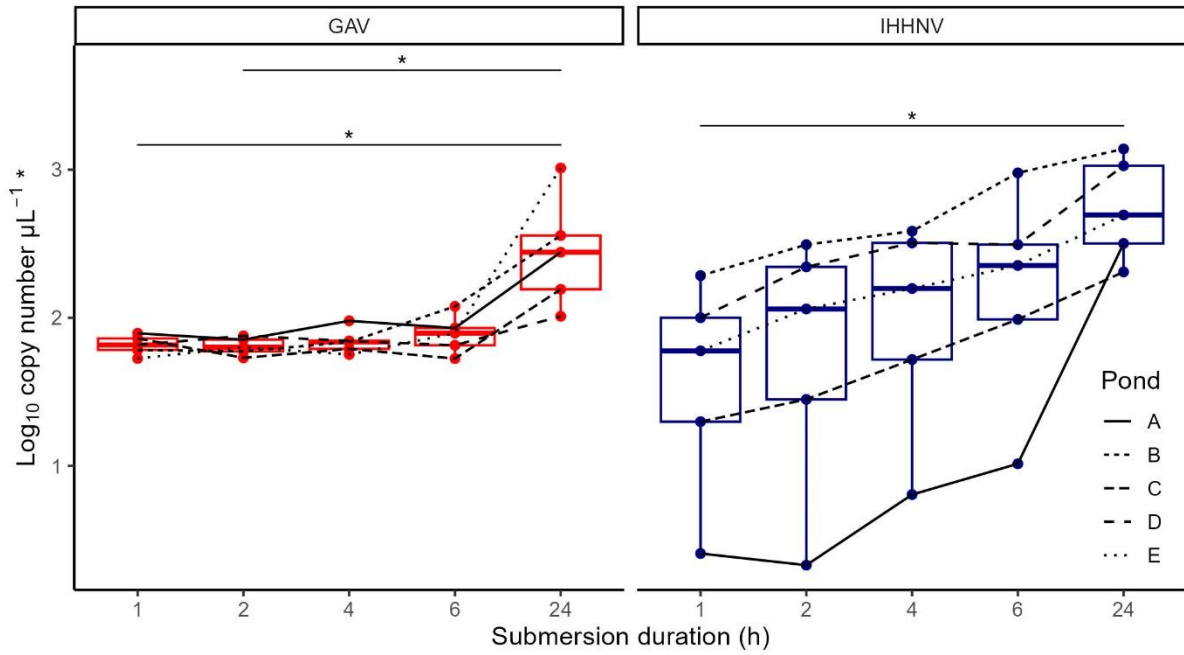


Figure 5.5. Detection of GAV and IHHNV from passive eNA samples after submersion for 1, 2, 4, 6 or 24 h in *Penaeus monodon* production ponds (A-E). Each point represents the mean detection from duplicate eNA samples from each pond after each submersion duration.

Detection of GAV between eNA₁₋₂₄ and shrimp tissue samples were not correlated (Appendix 20). However, detection of IHHNV from eNA₁₋₂₄ was highly significantly correlated with detection in shrimp tissues sampled from the same ponds (Figure 5.6; Appendix 20). IHHNV detection in pleopod and gill tissue were most highly correlated with the eNA samples collected after submersion for 1 h, while detection from hepatopancreas was most correlated with eNA samples collected after submersion for 4 h.

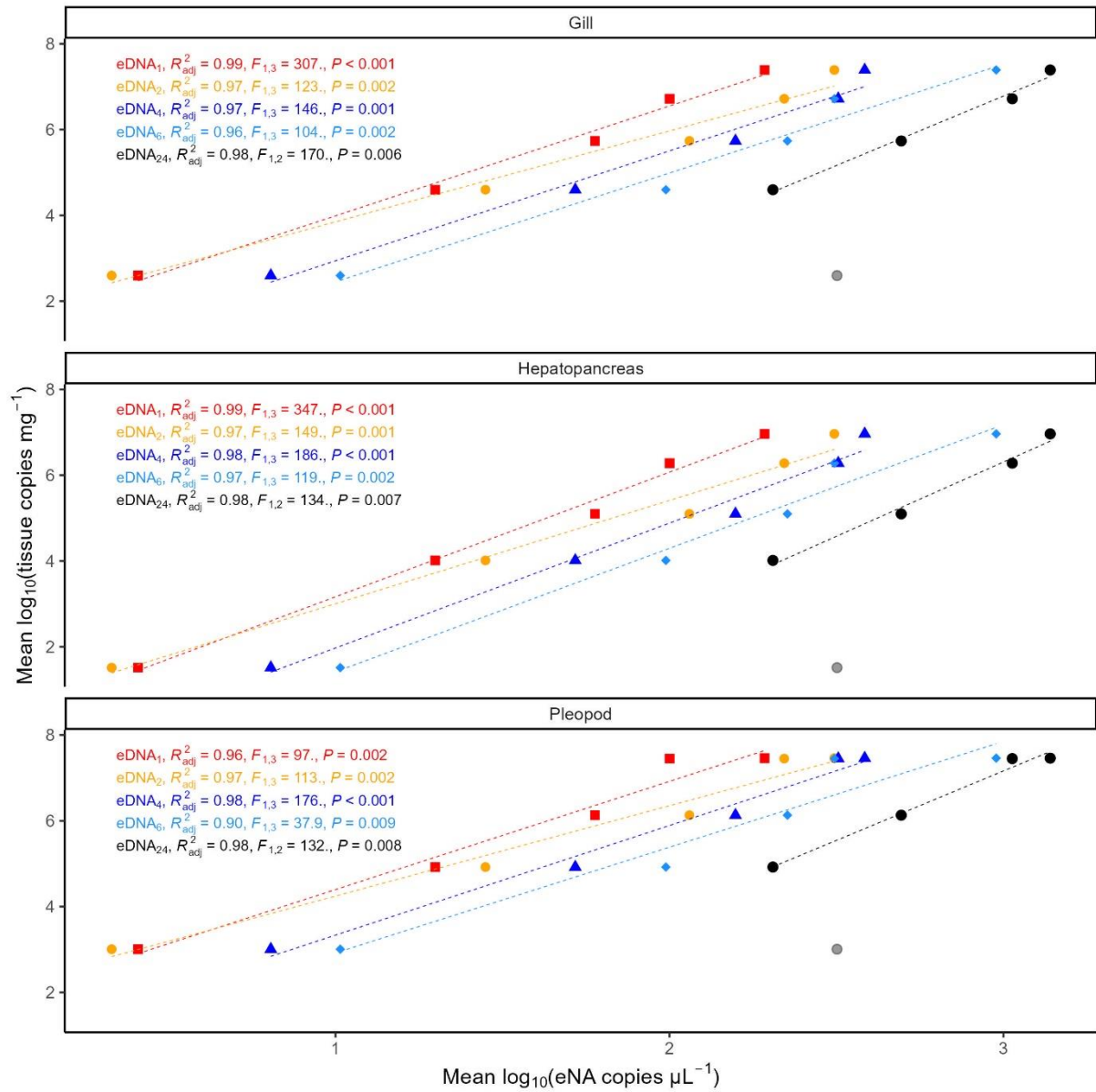


Figure 5.6. IHNV detection between passive eNA samples after submersion for 1, 2, 4, 6 or 24 h (eNA₁₋₂₄) and *Penaeus monodon* tissue samples (gill, hepatopancreas and pleopod) collected from the same production ponds. Adjusted Pearson's correlation coefficient (R^2_{adj} ; Holm corrected) are derived via linear correlation between the mean IHNV detection from duplicate eNA samples from each pond after each submersion duration, and mean detection from 13 pools of three samples for each tissue type. A single outlier pond (A) was excluded from regression analysis for eNA₂₄ but is shown in each plot (grey circle) for reference.

5.5 Discussion

The present study aimed to investigate the utility of passive eNA sampling for detection of viral shrimp pathogens from commercial *P. monodon* aquaculture production ponds. Our findings demonstrate that both IHHNV and GAV can be detected from passive eNA samples. The detection results of IHHNV indicate that adequate calibration studies may enable proxy quantification of shrimp host infection using eNA. This study also demonstrated that both targets could be detected from eNA samples immersed for only 1 h, and that detected copy number of each target increased with increasing submersion duration. These results highlight two potential application strategies for eNA sampling from shrimp ponds which are discussed herein.

5.5.1 Farm-scale eNA monitoring

5.5.1.1 IHHNV

The farm-scale eNA monitoring approach demonstrated that passive eNA collection for large surveillance-type sampling efforts was both practical and had comparable detection performance to current industry standard tissue sampling. Passively collected eNA samples incubated for 24h yielded representative positive detection of IHHNV, with the exception of a single pond with low tissue based IHHNV prevalence and copy number. Generally, ponds with average IHHNV shrimp tissue detection greater than 10 copies μL^{-1} consistently produced positive IHHNV detection from both passive samplers deployed. Below this threshold, environmental IHHNV eNA levels may be approaching the limit of quantification (LOQ) (Schang et al., 2021). While the lower limits of detection from passive eNA samples in this context are yet to be elucidated, the deployment of replicate samplers in shrimp production ponds is recommended to improve the likelihood of detection at low target presence. Although these findings provide compelling evidence that passively collected eNA samples are highly reliable for detection IHHNV, further research is required to assess the effectiveness of passive eNA sampling from ponds with lower environmental target pathogen levels. For example, research should be extended to analysis from ponds with low host prevalence, low level host infections, and from ponds early in the production cycle, where total infected biomass is low.

Calibration studies establish statistical associations between positive eNA detection and detection from host derived samples (Bass et al., 2015; WOA, 2022). Such studies are

required to determine the utility of eNA-based target detection in proxy-quantitative host infection monitoring. Few studies have included field-calibration analysis to validate the usage eNA for monitoring host infection in aquatic systems (e.g. Rusch et al., 2018). Applying active eNA collection, and building upon the findings of Strand et al. (2014), Wittwer et al., (2019) demonstrated a significant non-linear correlation between estimated spore concentrations detected via eNA of the crayfish plague agent *Aphanomyces astaci* and *A. astaci* detection from infected crayfish tissue samples collected from freshwater streams via traditional trapping methods. Similarly, Richey et al. (2020) demonstrated significant association between the detection of myxozoan-associated enteronecrosis agent *Ceratomyxa shasta* from actively filtered eNA samples and the prevalence of *C. shasta* detection from fish hosts within freshwater rivers. Using passive eNA collection, Schang et al. (2021) demonstrated a statistical correlation between the daily detected concentration of SARS-CoV-2 in human wastewater and detection from passive samplers, providing valuable evidence to validate passively collected eNA as an alternative sample type for environmental viral monitoring, however no direct correlation to detections from infected hosts was reported. Replicating a farm-scale monitoring program, our study demonstrated that passive eNA samples incubated for 24h in marine shrimp ponds yielded IHHNV detection that was significantly statistically associated with IHHNV detection from pooled pleopod tissue samples (linear $r^2 = 0.82$). These results provide valuable evidence to support the utility of passive eNA sampling as an alternative to tissue sampling for proxy-quantification of host viral pathogen loading in shrimp aquaculture systems. Furthermore, these results provide significant contribution towards the validation of eNA samples for IHHNV surveillance purposes.

Establishing the capacity of passive eNA samples to provide accurate relative quantification of shrimp tissue loading, using IHHNV as a proof of concept in this study, provides a promising indication for significant enhancement to aquaculture biosecurity using this tool. Pending appropriate calibration to the range of shrimp hosts, production environments and pathogen targets, application of passive eNA sampling will afford a simpler, more cost effective, non-destructive alternative, or complement, to current tissue sampling approaches for monitoring and surveillance purposes.

5.5.1.2 GAV

All 31 ponds were positive for the detection of GAV by shrimp tissue and passive eNA samples. However, unlike IHHNV where eNA detection became more reliable with increasing shrimp infection prevalence and copy number, there was no apparent association between shrimp tissue detections and the reliability GAV detection from duplicate eNA samples. Furthermore, despite the low copy number of GAV detection from shrimp tissues compared to IHHNV detections, eNA samples yielded GAV detection at a comparable copy number to environmental detection of IHHNV. Additionally, GAV quantification from passive eNA samples was not significantly correlated with detection from shrimp tissues (pleopod, gill and hepatopancreas), collected from the same ponds. The cause of this detection discrepancy remains unknown. Nevertheless, we speculate on potential factors that could result in a disproportionately high level of GAV detection from eNA samples, unrepresentative of detection levels from shrimp tissue samples. Primarily, we speculate that there may be an additional, high-biomass or high load, environmental host taxa of GAV within the production ponds, inflating the environmental levels of GAV beyond that caused by *P. monodon*. For example, the yellow head viral host range is known to include *Palaemon* sp. and *Acetes* sp. shrimp found cohabitating in shrimp production ponds (Longyant et al., 2005). A preceding study detected comparable prevalence of GAV (24%) and IHHNV (20%) from *Acetes* sp. sampled from Australian *P. monodon* production ponds (Condon et al., 2020). However, from these positive samples, GAV was detected at significantly higher levels (3.3×10^4 copies μL^{-1} TNA extract), compared to IHHNV (1.9×10^2 copies μL^{-1} TNA extract). Given *Acetes* sp. are the most common cohabitating epibenthic fauna found in Australian shrimp ponds (Coman et al., 2003), considered together, this evidence indicates a plausible additional origin contributing to elevated detection of GAV from environmental samples that could confound correlation between eNA and shrimp tissue detection.

Molecular surveillance using environmental sampling can achieve higher levels of sensitivity due to the presence of ‘legacy’ genetic material (extracellular or non-living) (Zaiko et al., 2018). Greater sensitivity may be advantageous for surveillance of pathogens in potentially present but untargeted sources, such as cohabitating host taxa, which is valuable for assessing pathogen transmission pathways, dynamics, and associated risk. However, this characteristic raises a critical limitation to be considered in the interpretation of eNA detection data.

Specifically, the possibility for ‘off-target’ positive detections may limit the accuracy or utility of eNA samples for estimating the extent of infection in the aquaculture host species.

5.5.2 Submersion duration experiment

The optimal submersion duration of the passive eNA sampling units was evaluated across five ponds, at 1, 2, 4, 6 and 24 h of submersion. Pathogen target detection from eNA samples increased with increasing submersion duration, with significantly higher detection of IHNV after 24 h of submersion, compared to 1 h, and significantly higher detection of GAV after 24 h of submersion, compared to 1 and 2 h. The increased detections from samples submerged for 24 h suggests that binding of target eNA continues to occur over time with prolonged exposure to the environment, potentially facilitated by biofilm formation on the filters (Power et al., 2023). Previous studies deploying cellulose ester membrane filters for passive eNA collection have investigated the effect of different submersion durations. When submerged for 5 min to 18 h in a 3 million L marine aquarium (Bessey et al., 2022) and 30 min to 72 h in freshwater tanks (Chen et al., 2022), no significant increases in target eNA detection with increasing submersion time were observed. However, in an open marine environment, increased target detection was observed between 4 and 8h of submersion, reaching equilibrium after 8h, until 24 h (Bessey et al., 2021). Together, these previous studies indicate that environment-specific factors, including both abiotic and biotic factors, likely influence the dynamics of eNA capture and degradation on the filter material, and thus, the effectiveness of different submersion durations for different settings (Kirtane et al., 2020; Wang et al., 2021). This lends further evidence to suggest eNA calibration studies must be carried out in an application specific manner.

Under the conditions of the present study, increasing submersion duration (up to 24 h) yielded the highest eNA target copy numbers detected. As such, with extended spatiotemporal exposure of the sampler, this longer submersion duration may be more suitable for surveillance applications where target infections are rare and presence/absence sensitivity at low-level host infection is required. Although overall eNA copy number detections were lower, submersion for as little as 1 h also produced highly representative quantitative estimates of IHNV loading compared to tissue detection ($R^2 = 0.96$ to 0.99). These findings suggest that shorter submersion durations can yield accurate estimation of host infection when host infection prevalence is high and host tissue loading is moderate to high. Accordingly, a shorter submersion duration of 1 h may be suitable for rapid, large-scale, proxy-quantification of host

infection where host infection within the pond is suspected or known. Such application would facilitate management approaches like risk-ranking of production ponds based on endemic pathogen presence for priority harvest planning.

A single pond produced anomalous eNA detection of IHHNV (eNA₂₄ Pond A), such that higher environmental IHHNV was detected than expected. This expectation assumes the consistent linear relationship between submersion time and IHHNV eNA detection level, established by the other analysed ponds, and from the previous four submersion duration time-points for Pond A (1, 2, 4 and 8 h). The causation of this discrepant result is unknown. Pond A had lowest shrimp IHHNV copy numbers detected from the ponds assessed within the submersion duration experiment. Extension of this analysis to capture ponds with similar or lower IHHNV loading is required to understand if this detection anomaly is a biological phenomenon (e.g. related to lower host infection levels or biofilm facilitated binding) or a spurious outlier arising from sampling error.

5.5.3 Material choice & sampler design

Selection of eNA collection material must consider both the capacity for the material to bind the environmental target, and the practicality of use (Bessey et al., 2022). Numerous materials demonstrate effective passive eNA collection, making practical considerations a key determinant for material selection (Bessey et al., 2022; Chen et al., 2022). While granular materials like granular activated carbon (GAC) exhibit a high capacity for eNA absorption and entrapment, challenges arise in downstream processing and aquatic deployment (Chen et al., 2022; Kirtane et al., 2020). Encasing granular materials, (e.g. in nylon bags or ‘teabags’), is necessary to for deployment of passive eNA filters in an aquatic environment. This can pose issues as the casing may inadvertently absorb eNA or limit the environmental exposure of the granular absorbent (Bessey et al., 2022; Kirtane et al., 2020). Comparatively, membrane filters, such as cellulose ester membranes, perform comparably well, are simple for handling and downstream processing, commercially produced and readily accessible, cost-effective, and can be deployed in housings with large pores, allowing for maximum exposure to the environment (Bessey et al., 2022; Chen et al., 2022). A mixed cellulose ester (MCE) membrane filter (0.22 µm pore), comprising cellulose nitrate and cellulose acetate, was selected for this study. Mixed cellulose ester membranes are electronegatively charged over a wide range of pH values and exhibit enhanced virus adsorption in the presence of salts due to the antichaotropic effect yielding increased hydrophobic interactions between the filter and viral particles (Junter and

Lebrun, 2017). MCE membranes were selected for the present study on the basis of previously demonstrated eNA collection performance, particularly in marine environments, in addition to their ease of use and availability (Bessey et al., 2022, 2021; Chen et al., 2022; Schang et al., 2021; Tsuji et al., 2019). To prevent damage or loss of the membrane filters from debris, biota and water current during field deployment, each filter was individually housed. The housings were designed to provide adequate protection, and be simple to deploy and sterilise, while maximising environmental exposure across the membrane filter. Similar housings have been described for successful field deployment of passive eNA samplers into freshwater creek systems (Kirtane et al., 2020) and human wastewater systems (Schang et al., 2021). While the filter housings described in this study were easy to use, able to be sterilised for re-use and performed well for their intended application, a simpler filter housing, such as the oyster rack design described by Bessey et al. (2021, 2022), may function similarly well for large-scale deployment in aquaculture settings.

5.5.4 Limitations

The results of this study provide promising evidence for the utility of passive eNA pathogen detection and monitoring for aquaculture. However, the disconnect between the environmental samples and the host organisms remains a challenge for quantitatively estimating host infection levels (Bessey et al., 2021). There are multiple factors which may limit the quantitative accuracy of eNA samples for estimating host infection, including sources of target eNA not derived from the aquaculture host species, such as additional cohabitating host species within the system shedding target eNA (Coman et al., 2003; OIE, 2019b; Zaiko et al., 2018), contamination with non-viable pathogen in treated feed products (Tacon, 2017; WOA, 2022) or from pathogen life-stages that aren't producing active infection in the host species (relevant to parasitic pathogens; Trujillo-González et al., 2020). Quantitative estimates derived from eNA signal may be further complicated by factors including taxon-specific eNA shedding rates (Power et al., 2023; Rusch, 2021), and site-specific water chemistry, inhibitor presence and flow dynamics affecting eNA persistence, decay and detection (Goldberg et al., 2016; Huerlimann et al., 2020; Kirtane et al., 2020; Wang et al., 2021). Considering these potential factors, application-specific calibration of eNA signal to host infection is required, to enable assessment and validation of eNA utility under the specific conditions of each system. Proxy-quantitative eNA monitoring results may only be useful for relative quantitative comparison between samples collected at the same location and at the same timepoint or season (e.g. within

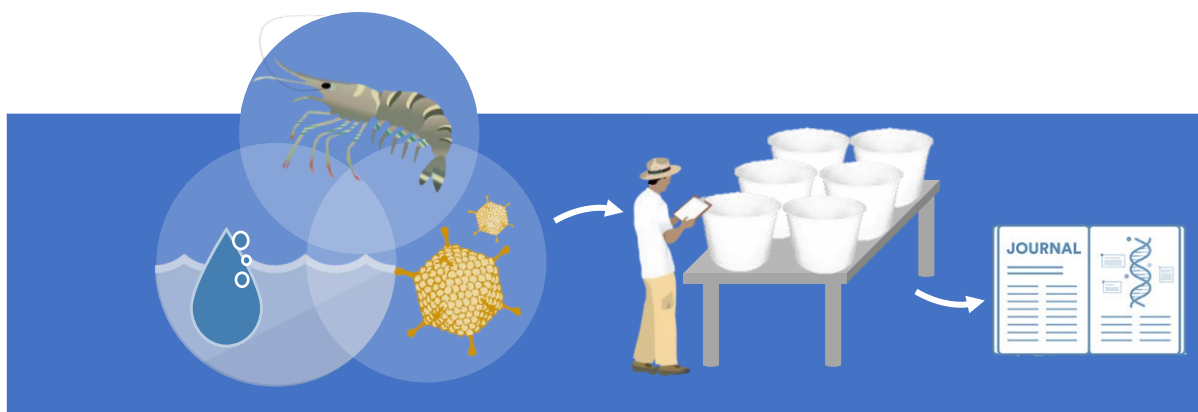
the same farm). Establishing a standardised approach for deployment and analysis, paired with accurate recording of metadata including water chemistry parameters, is required to facilitate both the enhanced integration of eNA detection data into farm biosecurity and more robust spatial and temporal analysis (Bessey et al., 2022; Bowers et al., 2021; Zaiko et al., 2018).

For quantitative analysis of endemic pathogens, such as those described in this study, the presence of low level or rare false positive detections are likely to have negligible impact on the results (Goldberg et al., 2016). Furthermore, confidence in the true positivity of the eNA samples was supported by paired tissue analysis, confirming the infected host species was present in the environment. While sterile equipment and techniques were utilised meticulously throughout this study, the absence of negative control field samples in our dataset and the potential for eNA field contamination with subsequent false positive detection are acknowledged (Forstchen, 2020). False positive detection of a rare, but significant, pathogen (e.g. WSSV) could arise through contamination of an eNA sample from non-viable sources (e.g. shrimp feeds; see Tacon, 2017). In commercial application, such a false positive could result in unnecessary additional sampling for diagnosis (WOAH, 2022), misled decision-making and substantial managerial and economic implications for aquaculture stakeholders (Bowers et al., 2021). Therefore, to avoid such issues, future field application of eNA sampling for pathogen surveillance and monitoring should include adequate field negative controls (Forstchen, 2020).

The strong linear association ($R^2 = 0.82$) between eNA and tissue sample detection of IHHNV demonstrated the eNA detections as strong proxy-quantifiers for shrimp IHHNV infection levels in ponds. However, within the portion of unexplained variability, the potential effect of inhibitory compounds present within the environmental matrix was not determined. The presence of inhibitors within environmental samples are considered a significant challenge for the application of eNA-based detection (Goldberg et al., 2016). Future studies applying eNA in this context would benefit from the addition of a quantitative exogenous internal positive control (IPC) within all samples. This would enable the assessment and potential calibration for inhibitor effects to improve the representativeness of eNA signal to host infection and determine appropriate data-associated caveats (Bowers et al., 2021).

5.5.5 Conclusions

The challenges posed by disease in global shrimp aquaculture necessitate rapid and accurate disease management tools. Large-scale or long-term pathogen detection programs across shrimp farms becomes impractical due to extensive sample collection requirements, prompting the exploration of passive eNA sampling as an alternative. The present study demonstrates the promise of passively collected eNA as a sensitive surveillance tool and proxy for indicating host infection. The application of this novel approach offers advantages such as greatly improved cost efficiency, sampling scope, sensitivity, and reduced handling stress on host animals. Extension of calibration studies generating statistically supported associations between eNA detections and confirmed host infections are required to understand the impacts of host and environmental conditions on detected signal, and the limits of detection from eNA samples; such calibration studies will likely be required on an application-specific basis.



Chapter 6.

Towards a ‘systems’ approach for viral challenge experiments in shrimp: reporting guidelines for publication

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Foreword

The prior chapters have developed a critical knowledge base to support improved understanding of the viral landscape of Australian shrimp aquaculture, and enhanced pathogen detection sensitivity, interpretation and scope. Looking forward, the management of these pathogens will require robust understanding of their impact on shrimp health and productivity, and the host, pathogen and environmental conditions which trigger disease outbreak. Pathogen challenge experiments (PCEs) will play a critical role in the development of this understanding. Chapter 6 aimed to review the existing published literature to highlight conditions relating to the host, pathogen and environment which influence shrimp disease outcomes. Building upon this, to understand the current state of research, a critical assessment of prior viral PCEs conducted with *P. monodon* was conducted. Synthesising this body of evidence, a set of guidelines were formulated to support the ‘best-practice’ development, reporting in publication, and translation of future shrimp viral PCE for improved shrimp disease management.

6.1 Abstract

The success of contemporary disease management strategies in shrimp aquaculture, such as the 'systems' approach, is predicated on robust knowledge of the conditions and interactions between the host, pathogen and environment that promote disease. Pathogen challenge experiments (PCEs) are a powerful tool for investigating these conditions and interactions. However, absence of accurately reported experimental detail in published PCEs limits scientific transparency, reproducibility, and the potential for the research to make progressive advancements contributing to contemporary shrimp disease management strategies. This review identifies and discusses key factors relating to the host (shrimp), pathogen (virus), and environment that should be carefully considered during the design and publication of PCEs. We offer substantial evidence of their impact on viral disease outcomes, drawn from the existing body of literature, to support their consideration. The prevalence of reported experimental details for these factors across 186 viral PCEs in shrimp was evaluated. The review highlights a concerning paucity of experimental detail reported in published shrimp PCEs. We propose a checklist for the minimum reportable information in the publication of shrimp viral PCEs, hereafter referred to as the Shrimp PCE Reporting Guidelines (SPERG). The guidelines aim to enhance the transparency and standardisation of reporting in published PCEs, ensuring that key factors pertaining to the shrimp, pathogen, and environment are adequately considered and documented. Adoption of SPERG is envisaged to empower researchers, reviewers, and readers to assess the internal and external validity of PCEs, facilitating critical evaluation and improved utility of PCE findings for contemporary disease management.

Keywords: best practice, bioassay, metadata, transmission trial, virus

6.2 Introduction

Since the initial discovery of viral a pathogen in shrimp aquaculture (Couch, 1974) periodic reduction in global production volume has historically aligned with the emergence of significant pathogens, including infectious hypodermal and haematopoietic necrosis virus (IHHNV, *syn* *Penaeus stylirostris* penstyldensovirus 1), Monodon baculovirus (MBV, *syn* *Penaeus monodon* nudivirus), Taura syndrome virus (TSV), yellow head virus (YHV), white spot syndrome virus (WSSV), acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* (VpAHPND), *Enterocytozoon hepatopenaei*, and Decapod iridescent virus 1 (DIV1) (Flegel, 2019; Kumar et al., 2021a; Shinn et al., 2018). Viral diseases continue to limit the growth and sustainability of global shrimp aquaculture, with the cost of annual losses estimated to be billions of dollars (Flegel et al., 2008; Shinn et al., 2018; Stentiford et al., 2012). The management of disease in shrimp production is challenged by the limited capacity for conventional disease prevention strategies such as vaccination (Johnson et al., 2008) which have proven pivotal to the success of other major production species including salmonids (Mondal and Thomas, 2022). Shrimp aquaculture is further challenged by a limited ability to effectively implement biosecure controls to prevent pathogen entry and disease outbreaks (Asche et al., 2021). Primarily contributing to this limited control is the concentration of shrimp production in warm coastal regions in pond-based production systems (Boyd et al., 2022), which are subject to climatic and environmental fluctuation and high levels of exchange with the external environment (Millard et al., 2021). In some production regions, farms are also in close proximity to each other or are connected via water source, increasing the risk of pathogen transmission among farms (Patanasatienkul et al., 2023). As a consequence, disease management in shrimp aquaculture must consider the collective influence of factors within the production system on disease outcomes. Recently, advanced molecular technologies have begun to illustrate how the various factors within aquatic production systems interact to manifest in disease (Fajardo et al., 2022; Palaikostas, 2021; Stentiford et al., 2017; Zenger et al., 2019). Contemporary strategies to advance disease management in shrimp aquaculture advocate a holistic ‘systems’ approach, which considers disease as the outcome of host, pathogen, and environmental interactions (Flegel, 2019; Stentiford et al., 2017).

Investigation to understand the relationships between the shrimp host, pathogen, and environment and how they interact to yield disease is required for the successful administration of a ‘systems’ approach to disease management (Flegel, 2019). Generally, disease

investigations and pathogen challenge experiments (PCEs) are the primary means for developing knowledge regarding disease and its associated or causal drivers. In some cases, such as infection with WSSV, a correlation-causal conclusion generated via *in situ* disease investigations or field studies is robust because the presence of WSSV in high copy number is consistently associated with rapid onset of mass mortality (Domínguez-Borbor et al., 2019). Studying the outcomes of viral disease *in situ* is relevant to understanding the impact of disease under the complex influence and interactions of factors within the whole system (Noble et al., 2020b). However, for many other viral pathogens, high copy number detection is not always associated with poor productivity or mortality (Condon et al., 2020). In complex cases where the disease outcome does not involve a rapid onset of disease, such as sub-optimal or retarded growth, the ability to define the impact of a specific pathogen within the system is further reduced. Under such conditions, poor control over the conditions in the ‘system’ limits the confidence and robustness of *in situ* studies, permitting only associative conclusions that should be cautiously considered (Munro et al., 2011; Valappil et al., 2021). For example, reduced or retarded growth in *P. monodon* (so-called *P. monodon* slow growth syndrome) has been associated with a range of viral pathogens including, but not limited to, hepatopancreatic parvovirus (Chayaburakul et al., 2004; Flegel et al., 2004, 1999; Lightner and Redman, 1985), MBV (Chayaburakul et al., 2004; Flegel et al., 2004, 1999), Laem Singh virus (Panphut et al., 2011; Taengchaiyaphum et al., 2021b), and IHHNV (Sellars et al., 2019). In this case, no conclusive causal relationships were able to be demonstrated by these investigative studies due to insufficient control over the conditions of the *in situ* study system. As a result, the cause of *P. monodon* slow growth syndrome remains elusive, limiting the capacity for the syndrome to be effectively managed (Valappil et al., 2021).

Considering the difficulty of evaluating the conditions driving disease *in situ*, due to the complexity of production systems, a complementary or alternative strategy is required for more robust investigations. Pathogen challenge experiments (PCEs) (*syn* disease transmission trials or bioassays) involve deliberate, standardised exposure (‘challenge’) of a study population with a disease agent. PCEs enable the study of host health and disease progression under controlled conditions, allowing for causal relationships to be defined and quantified (Lambkin-Williams et al., 2018). PCEs are an important and powerful tool for resolving the impact of pathogens on shrimp health and in the development and elucidation of novel disease prevention technologies, such as immunostimulants (Babu et al., 2013; Kumar et al., 2022), and genetic

selection (Noble et al., 2020a). Knowledge gained through PCEs may be used to supplement and support disease investigations, or aid in disease management applying a ‘systems’ approach. However, for the findings of PCEs to be effectively translated and applied to benefit commercial production, PCEs must be conducted and reported upon accurately and robustly. In the absence of accurate reporting of experimental detail from PCEs, the internal validity, relating to the experimental rigour of the study, and external validity, relating to the capacity for the research findings to be contextualised and integrated into a ‘systems’ framework, are severely minimised.

Guidelines for standardised reporting of challenge trials are well established within the fields of human medicine (Consolidated Standards of Reporting Trials) (Moher et al., 2001) and livestock veterinary studies (Reporting Guidelines for Randomised Controlled Trials for Livestock and Food Safety) (O’Connor et al., 2010; Sargeant et al., 2014, 2010). Such standards have been developed to encourage ‘best practice’ for the design and reporting of experiments, facilitating transparency and reproducibility, and improving the capacity for consumers of the research to evaluate the internal and external validity of the studies. Few guidelines of a similar nature with relevance to research on aquatic species are available (Bustin et al., 2009; Holt et al., 2021; Taylor et al., 2019), for example the MISA guidelines (Minimum Information required to support a Stimulant Assessment experiment) for studies investigating immunostimulants in crustaceans (Hauton et al., 2015). However, more widespread availability and adoption of such reporting standards is still required. For example, to maximise the value of PCE studies for disease management in shrimp production, guidelines of a similar principle with specificity to shrimp PCEs are needed. With this aim, the current review identifies characteristics pertaining to the shrimp, pathogen (virus), and environment, and provides evidence to support their influence on disease outcomes. Guidelines for reporting in publication of viral PCEs are proposed to incorporate these characteristics and reflect the current evidence to support integration of PCE findings into the multifactorial ‘systems’ approach for shrimp viral disease management. To underscore the need and significance of these guidelines, the prevalence of relevant reported experimental details from 186 PCE studies of viral diseases in the giant black tiger shrimp, *P. monodon*, published between 1997 and 2023 were evaluated.

6.3 Infectious disease triad

Disease outbreaks are the consequence of exposure of a susceptible host to a virulent agent, under permitting environmental conditions, as described by the infectious disease triad (*syn* epidemiological triad) (Frost, 1976; Macmahon and Pugh, 1970). The disease triad concept was translated to the domain of aquatic animal health by Snieszko in the 1970s, in the context of environmental stress influencing the incidence of infectious disease outbreaks in fish (Snieszko, 1974). The principles defined by the infectious disease triad underpin the contemporary adoption of a ‘systems’ approach to disease management (Flegel, 2019; Stentiford et al., 2020, 2017). The ‘systems’ approach acknowledges the complex interactions of host, pathogen and environment influencing disease outcomes, and predicates that disease management strategies must therefore be multifactorial. The development of current and effective multifactorial disease management strategies is dependent on the integration of new research findings within the context of the broader system, relating to the host, pathogen and environment of production (Flegel, 2019).

For research findings derived from PCEs, aspects pertaining to the experimental shrimp, pathogenic agent and study environment must be considered and defined to provide sufficient external validity and background for integration of the findings within the context of the broader system. Characteristics of the experimental shrimp, including genetic and geographical source (Escobedo-Bonilla and Rangel, 2014), life stage (Angthong et al., 2021; Yoganandhan et al., 2003), and pre-existing pathogen infections (Molthathong et al., 2013), may influence the dynamics of disease expression within PCEs and should be considered in the translation of findings to commercial production settings. Addressing the environmental components of the disease triad, factors including experimental replication and system design (Noble et al., 2020a), environmental conditions (Millard et al., 2021), acclimation (Joseph and Philip, 2020; Vidal et al., 2001), sampling techniques and scheduling (De La Vega et al., 2004; Noble et al., 2018), and feed inputs (Tacon, 2017) should similarly be considered in the translation of PCE findings to production systems. Pathogen attributes including genetic origin (Andrade et al., 2022; Aranguren et al., 2013), production and processing of the inoculum (Munro and Owens, 2007), method of inoculation (including delivery and dose) (Noble et al., 2017) and chosen control treatments are important factors for interpretation and integration of study findings within the context of the broader system.

These essential components within the ‘system’ of shrimp disease (Figure 6.1), and their influence on disease outcomes in shrimp viral PCEs are discussed herein.

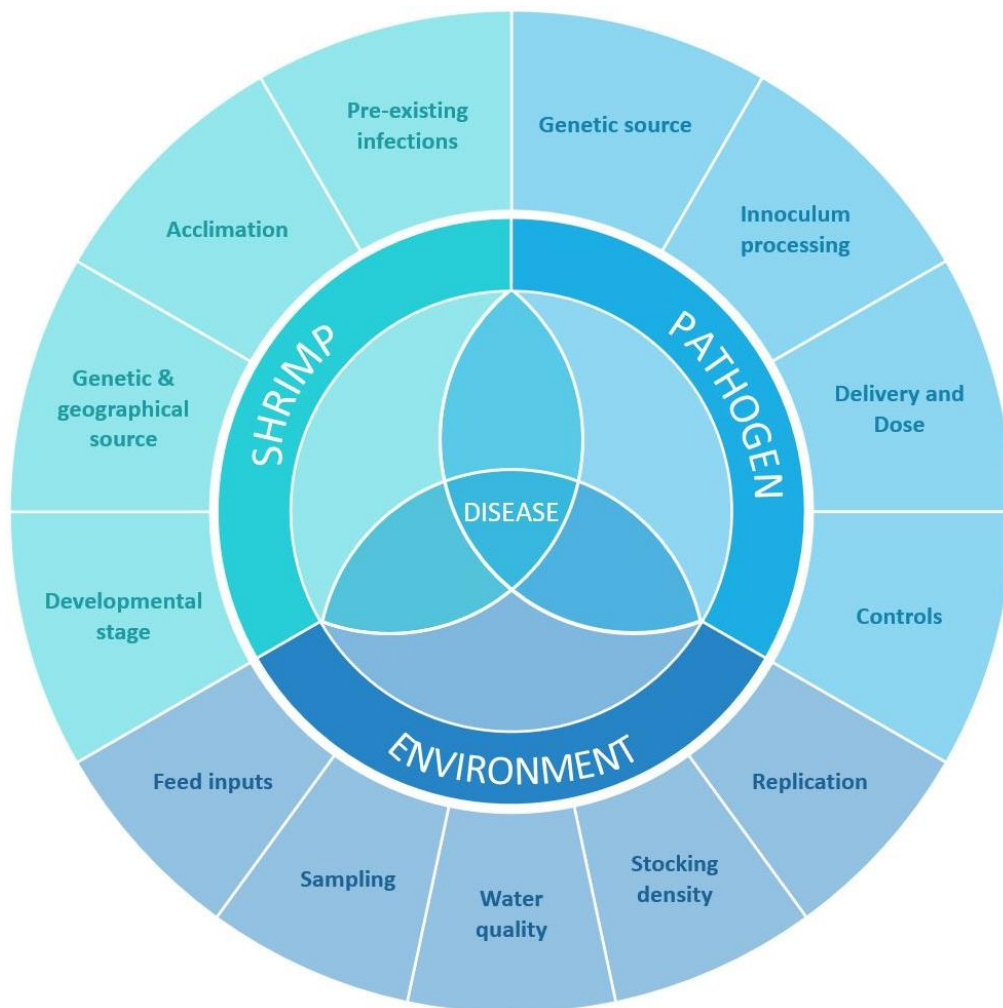


Figure 6.1. Disease outbreaks are the consequence of interactions between the susceptible host (shrimp), a virulent pathogen and conducive environmental conditions of the production system. Essential factors of each component within a viral pathogen challenge experiment on shrimp include those listed around the perimeter of the disease triad. Adapted from Snieszko (1974).

6.4 Essential components of viral pathogen challenge experiments (PCEs)

6.4.1 Host: The shrimp

Background information on the challenged shrimp is imperative for the interpretation of results of PCEs and to provide external validity to the findings, allowing for appropriate application to shrimp production systems. Characteristics of the experimental shrimp, including species, life stage, genetic and geographical source, stress and pre-existing pathogen infections can influence the dynamics of disease expression (Anghong et al., 2021; Dewangan et al., 2017; Escobedo-Bonilla and Rangel, 2014; Gardner et al., 2016). Detailed reporting of such metadata

in published PCEs will also enable progressive knowledge development for larger-scale comparative meta-analyses, such as for understanding species- and population-level responses to pathogen challenges.

6.4.2 Developmental stage – size

Differential susceptibility of shrimp to viral infection during ontogenesis has been firmly established (Pramod Kiran et al., 2002; Venegas et al., 1999; Yoganandhan et al., 2003). The operons of the reported differences include morphological and immunological development, immune gene expression, microbiome and moult-cycle dynamics (Angthong et al., 2021; Cornejo-Granados et al., 2018; Corteel et al., 2012; Yoganandhan et al., 2003). During the early stages of shrimp development, progression of nutrient and energy requirements are paralleled by changes in feeding behaviour, shifting from endogenous to various exogenous feed types (Kongkeo, 2005). Concurrently, morphological features including the branchial complex (gills) and digestive tract become developed (Motoh, 1984), facilitating nutrient and chemical exchange with the surroundings. Behavioural changes and growth of morphological features during the development of shrimp ensues progressive exposure and interaction with the external environment which may impact the capacity for viral entry and replication (Yoganandhan et al., 2003). For example, in *P. monodon* nauplii, protozoae, mysis, early post-larvae (PL1-10), late post-larvae (PL11-20) and juveniles challenged with WSSV via immersion under laboratory conditions, significant mortality only resulted for the later stages of post-larvae and juveniles (Yoganandhan et al., 2003). Increased susceptibility to WSSV at the later stages may have been related to the complete morphological and functional development of the branchial complex (gills). Specifically, because the gills represent a primary site for viral entry and replication, the infection and necrosis of this organ may be a key facilitator for disease and mortality during later development stages.

The immune capacity of shrimp also establishes throughout early development (Angthong et al., 2021; Jiravanichpaisal et al., 2007). Applying next-generation RNA sequencing, *P. monodon* immune transcripts including those related to pattern-recognition proteins (*c-type lectins*, *ficolins*), the prophenoloxidase system (*PPAF1*, *PPAF2* and *serpin3*), the immune deficiency pathway (*Relish*), anti-microbial peptides (*crustin Pm1*, *crustin Pm4*, *ALF* and *antiviral protein*), and heat shock proteins (*HSP70* and *HSP90*) were found to progressively increase during early development (Angthong et al., 2021). Expression of components from

these pathways has been associated with protection against viral infection (Janewanthanakul et al., 2020; Wang et al., 2020; Yuan et al., 2017), suggesting that earlier life stages lacking these components may be more susceptible to infection and disease. However, viral hijacking of shrimp immune pathways to facilitate viral replication may conversely be limited in early shrimp developmental stages, in the absence of the established immune pathways (Huang et al., 2012; Lin et al., 2012; Wang et al., 2014). Such an effect may have been involved within the aforementioned study (Yoganandhan et al., 2003), where WSSV disease progression in the later life stages may have been facilitated by both the morphological development of primary infection sites and immunological development enabling viral hijacking. Beyond immunological and morphological development, progressive exposure and interaction with the external environment associated with shrimp development also results in dynamic temporal succession of shrimp microbiota composition (Cornejo-Granados et al., 2018; Sha et al., 2022). The dynamics of the microbiome are associated with shrimp health and immune capacity (Butt et al., 2021; Infante-Villamil et al., 2021), thus, changes in its composition and diversity during ontogenesis may further influence disease susceptibility (Holt et al., 2021; Xiong, 2018; Xiong et al., 2018). Characterisation and analysis of the microbiome is yet to become a standard practice in PCE studies and currently remains cost-prohibitive in many instances. However, as research interest in the microbiome continues to expand, it is anticipated that the accessibility of this analysis will increase. In future, integrating microbiome analysis into PCE studies would enhance our understanding of disease outcomes and shrimp health from a ‘systems’ perspective.

The developmental progression of shrimp also drives changes in moult frequency and duration (Corteel et al., 2012; Dall et al., 1990). The dynamics of the moult cycle modulate large physiological changes which can result in increased susceptibility to environmental stressors and disease (Corteel et al., 2009; Lemos and Weissman, 2021). Throughout pre- and post-moulting the euryhaline osmoregulatory capacity of shrimp is largely reduced, and conformation to environmental osmolyte facilitates water uptake, increasing shrimp water content by up to 70%. Reduction in osmoregulatory capacity lessens shrimp resilience to fluctuating environmental stressors including low dissolved oxygen (DO), sudden salinity reduction, or increased ammonia, and may considerably alter the composition of shrimp haemolymph including the presence and concentration of immune factors (Lemos and Weissman, 2021). The large volumes of water incorporated into the body and changes to the

infrastructure of the exoskeleton during moulting increases the opportunity for viral acquirement, resulting in variable susceptibility to pathogen infection and disease as moult dynamics shift throughout shrimp development (Corteel et al., 2009; de Gryse et al., 2020).

Consistent reporting of shrimp age (days of culture [DOC] or days post hatch [dph]) as a standardised and quantitative measure for developmental stage should be practiced for publication of PCEs. Alternatively, depending on the purpose of the PCE, or if exact age is not known, it may also be appropriate to limit the description of size to a production group such as hatchery, grow-out, or broodstock. Where possible, when the cycle of synchronous moulting within the experimental population is known, or individual shrimp are assessed, reporting of moult stage should also be included. This information is essential for contextualising PCE results and enabling appropriate and specific application of PCE findings to commercial production systems.

6.4.3 Source

Variation between shrimp from different sources can strongly influence their susceptibility to pathogen challenge (Alcivar-Warren et al., 1997; Escobedo-Bonilla and Rangel, 2014). Genetic variation and pathogen pre-exposure are critical factors that may influence disease susceptibility and should be evaluated when considering the relevance of PCE findings to other shrimp stocks. Genetic variation between shrimp may arise naturally, for example, through random mutation or local adaption of geographically distanced stocks (Mondal and Mandal, 2020; Vu et al., 2020), or anthropogenically through the course of domestication and selective breeding (Prochaska et al., 2022; Trang et al., 2019a). Genetic variation associated with increased pathogen resistance and tolerance has been identified and targeted in studies for application in selective breeding programs to yield more robust production stocks (Chakrabarty et al., 2015; Dutta et al., 2014, 2013; Mohd Ghani and Bhassu, 2019; Noble et al., 2020a; Prochaska et al., 2022; Robinson et al., 2014). In the context of disease resistance, such genetic variation resulting in stock improvement can be slight. For example, a single nucleotide polymorphism (SNP) (g.1186A>G) in the anti-lipopolysaccharide factor 3 (ALFpm3) gene of *P. monodon* has been associated with higher rates of survival during WSSV challenge (Pooljun et al., 2020). This SNP was found to be variably present among three independent sources of farmed shrimp within the same province in Thailand, highlighting the importance of specific reporting of shrimp source for contextualisation of PCE findings.

Whilst rigorous genetic management and breeding strategy can effectively select for variants conferring reduced susceptibility to viral infection, poor genetic management of breeding populations can conversely result in genetic erosion (Moss et al., 2007). Considering over 50% of globally cultured shrimp originate from ‘copied breeding stocks’ (i.e., shrimp intended for grow-out production that are matured and used for breeding without appropriate consideration of genetic relatedness) (Doyle, 2016), considerable genetic erosion is expected in a large portion of farmed shrimp populations. Differences in the extent of genetic erosion between farmed shrimp sources can result in varying impacts on disease incidence, susceptibility, prevalence, and related mortality, further highlighting the importance of shrimp source for contextualisation of PCE findings.

Variable historic exposures to viral pathogens (Flegel, 2020, 2009) and origin-specific microbiome (Rajeev et al., 2021) may further diverge the viral susceptibility of shrimp between different sources. Viral pathogens in both wild and commercial systems are relatively ubiquitous, and pathobiome composition between geographical sources is diverse (Arbon et al., 2022; Chayaburakul et al., 2004; Condon et al., 2020). Excluding specific pathogen-free (SPF) lines, depending on the pathogen under investigation and the country or region the experimental shrimp are sourced from, the potential for historic exposure of shrimp stocks to the studied pathogen, or other endemic pathogens, is considerable. Such exposures are also likely unique to each stock. Historic and contemporary pathogen exposure of experimental animals, including their ancestral genetic lines, prior to PCEs may yield variable susceptibility or resilience to pathogen infection between stocks, giving rise to variation in clinical outcomes of PCEs. Accordingly, the source of experimental shrimp used in PCEs should be accurately reported in publication, including their geographical, temporal, and genetic origin where possible. Beyond exposure to known pathogens, the potential for experimental shrimp to be exposed to, or infected with unidentified but consequential pathogens should also be considered. In this case, while technologies such as next-generation sequencing remain cost-prohibitive, and targeted pathogen screening (see Section 6.4.4) is unable to detect unidentified pathogens, accurate reporting of shrimp source may allow for retrospective contextualisation of PCE findings if a pathogen of consequence is identified and confirmed to be present within the source region or population.

6.4.4 Screening experimental animals for additional pathogens

Pre-existing viral infections in overtly ‘healthy’ shrimp are commonly reported from both wild and cultured stocks (Arbon et al., 2022; Condon et al., 2020; Flegel et al., 2004; Yoganandhan et al., 2003). For example, in a large-scale investigation of pathogen presence in Australian *P. monodon* production systems, in the absence of overt disease events, 76% of shrimp analysed were found to be simultaneously positive for the detection of multiple (2 – 5) pathogen targets (‘co-infections’) by qPCR analysis (Condon et al., 2020). Such persistent, tolerated or chronic infections can be amplified by external stressors (De La Vega et al., 2004) and can influence host susceptibility to other co-infecting pathogens in a protective (Aranguren et al., 2012; Molthathong et al., 2013) or exacerbating (Yeh et al., 2009) manner. Co-infection of the host with multiple viral and bacterial pathogens, and microbiome dysbiosis (see definition in Bass et al., 2019) can further influence viral pathogenesis via immune overstimulation and annexing of the upregulated anti-bacterial shrimp immune pathways by viruses (de Souza Valente et al., 2020; Deepika et al., 2020; Qiu et al., 2021).

Commonly, the observed clinical signs during co-infection are attributed to the more virulent pathogen (Dewangan et al., 2017; Otta et al., 2014), despite limited understanding about the outcomes of the interactions between co-infecting pathogens and the host (Escobedo-Bonilla, 2021). In the absence of additional analysis to exclude potential co-infections from differential diagnoses, attribution of clinical signs to a pathogen on the sole basis of its detection provides limited grounds for drawing robust experimental conclusions (NACA, 2019). Deficient eliminatory analysis of possible co-infecting pathogens diminishes the rigour and validity of the research conclusions. In PCEs where the presence of co-infections is not considered, ambiguity arises in attributing the observed experimental outcomes to the specific study pathogen, particularly when the disease outcomes may be sub-clinical (Aranguren Caro et al., 2022).

Given the frequency and significance of multiple pathogen infections in production shrimp (Arbon et al., 2022; Condon et al., 2020), pre-screening experimental shrimp prior to introduction into the PCE system is critical to the validity of subsequent PCE results. Particularly, screening for a broad range of potential pathogens with focus on those that are endemic to the source of the shrimp, prior to and, at the conclusion of PCEs is necessitated. Pre-screening experimental shrimp would enable the preferential selection of shrimp free of

specific pathogens, or at a minimum, would allow for the consideration of viral burden in the interpretation of the experimental results (Hauton et al., 2015). Reporting of the screening conducted, and relevant pathogen detection in PCE publication would improve scientific transparency and thoroughness of PCEs.

6.5 Environment: The culture system

Aquaculture production systems are diverse in structure, composition and administration and represent complex, multi-trophic environments (Houston et al., 2020). With the majority of global shrimp production occurring in tropical coastal regions, volatile climatic conditions can result in large dynamic ranges of environmental culture conditions. The productivity of aquaculture is inherently dependent on abiotic and biotic factors within the aquatic environment, and the interactions of these factors with the cultured organism (Millard et al., 2021). Similarly, for pathogen challenge experiments, the design and maintenance of the experimental system can have considerable impacts on the outcome of the trials, and if conducted poorly or with limited foresight, can result in confounding effects and biases in PCE results. Factors such as experimental replication, system design, maintenance of environmental conditions throughout the PCE, sampling, and feed inputs are all significant for critical interpretation of results and contextualisation within the ‘systems’ framework.

6.5.1 Replication and sample size

To derive reliable and accurate results, PCE data must be generated in a manner that is robust, repeatable, and reproducible, applying assays and methods that are fit for purpose. Replication serves to measure and isolate sources of variation, limit the impact of spurious variation on effect estimates and hypothesis testing, and establish reproducibility (Blainey et al., 2014; Vaux et al., 2012). Clarity in reporting of sample or treatment replicates and independent repeats of PCEs are important for the transparency and interpretation of findings, as replication has an applied effect on inferencing errors and the power of subsequent analysis (Blainey et al., 2014). In PCEs, especially those investigating the genetic parameters influencing disease progression, families or groups are often reared or challenged in separate tanks, for example to physically facilitate maintenance of pedigrees (Moss et al., 2013). Under such conditions, non-genetic effects arising from separated rearing before or during experiments, including inconsistent water quality conditions, stocking density, or exposure to infected conspecifics, can significantly impact response to challenge (Noble, 2018). In the absence of replicated tanks for each family or group, the combined effects of environmental and methodological variation

become confounded with that of the treatment and/or genetic effect (Noble et al., 2020a). PCE details including the number of shrimp stocked per tank, the number of tank replicates per treatment group, the definition of the treatments groups and if the PCE was independently replicated should be clearly reported to enable critical evaluation of study robustness and scope.

6.5.2 Density

Stocking density can significantly influence disease susceptibility in shrimp (Lightner and Redman, 1998). Negative health effects associated with increased stocking density can be primarily attributed to elevated stress, reduced water quality, and greater opportunity for horizontal viral transmission through cannibalism and exposure to water-borne viral particles shed by infected conspecifics (Gao et al., 2017; Raja et al., 2015; Wu et al., 2001). For example, 14 days post-infection (dpi) with WSSV, increased cumulative mortality in *M. japonicus* (~1.1 g) was associated with increased stocking density, yielding 18% mortality at ~0.26 kg m⁻³, 46% mortality at ~0.48 kg m⁻³ and, ~72% mortality at ~0.92 kg m⁻³ (Wu et al., 2001). A similar trend was observed in WSSV infected *P. monodon*, *P. indicus* and *Litopenaeus vannamei* (~5 g), where mortality rates were highest for all three species when stocked at ~1.0 kg m⁻³, followed by ~0.50 kg m⁻³, and were lowest at the stocking density of 0.25 kg m⁻³, demonstrating a clear positive association with stocking density and mortality arising during PCEs (Raja et al., 2015). Reduced antioxidant capability and stress resistance associated with increased stocking density has also been reported in *L. vannamei* (~1.9 g), where shrimp stocked at higher density in cages within the same pond had reduced feed utilisation, growth and survival during the 60 day experiment (Gao et al., 2017). Direct reporting of stocking density, or parameters used to determine stocking density including the number of shrimp stocked per experimental unit, the weight of the shrimp stocked, and the volume of the experimental unit, should be included for context in publication of PCEs.

6.5.3 Environmental conditions

Abiotic environmental conditions including temperature, salinity, DO, and pH are operative upon both host and pathogen function, and their interactions. Dynamic fluctuation or sustained sub-optimal environmental conditions can lead to physiological stress and reduced ability for the host to resist disease (Millard et al., 2021). Reporting of abiotic environmental conditions in published PCEs is critical to the transparency and external validity of the associated findings.

6.5.3.1 Temperature

Temperature directly influences many processes, including shrimp metabolic and developmental processes, pathogen replication, and the solubility of other abiotic factors in the system (Chaitanawisuti et al., 2013; Le Moullac and Haffner, 2000; Rahi et al., 2021b). Penaeid shrimp can tolerate a wide range of temperatures, with optimal temperatures for growth and survival dependent on size, but generally ranging between 28 and 32 °C (Chaitanawisuti et al., 2013; Rahi et al., 2021b; Soundarapandian and Gunalan, 2010). Despite this wide tolerance range, numerous PCEs report an influence of variable or sub-optimal water temperature on infection dynamics in shrimp, via experimental manipulation of water temperature or as a coincidental finding. For example, comparing the replication rate of IHHNV in *L. vannamei* at different temperature treatments, Montgomery-Brock et al. (2007) demonstrated the replication of IHHNV in shrimp held at 32.8 ± 1.0 °C (1.20×10^5 virus copies/50 ng DNA at 17dpi) was reduced compared to shrimp held at 24.4 ± 0.5 °C (4.48×10^6 virus/50 ng DNA at 17 dpi). Similarly, in studies investigating the effect of water temperature on WSSV infection in *L. vannamei*, maintenance of shrimp at higher temperatures of 32 - 33 °C was demonstrated to significantly reduce mortality compared to maintenance of shrimp at temperatures of ~26-30 °C after WSSV challenge (Rahman et al., 2006; Vidal et al., 2001). Manipulation of water temperature was also determined to significantly influence mortality rates in *P. monodon* challenged with YHV-7 by various methods, including injection, cohabitation and feeding of infected tissue (Mohr, 2020). Experimental shrimp held at 30 °C experienced significantly reduced mortality due to YHV-7 infection compared to shrimp held at 25 °C (Mohr, 2020). Given the critical influence of temperature on PCE outcomes via interaction with host, pathogen and other environmental parameters, consistent reporting of temperature in publication of PCEs is required. Specifically, the experimental temperature maintained throughout the PCE, with inclusion of a measure of variation (e.g., a minimum to maximum range or mean with standard deviation of temperature) should be reported to enable contextualisation of experimental findings within a 'systems' framework.

6.5.3.2 Salinity

Penaeid shrimp are euryhaline, beginning as osmoconformers in larvae and transitioning to osmoregulators as adults (Chong-Robles et al., 2014; Karan et al., 2020). *P. monodon* are tolerant to salinities ranging from 1 to 57 ppt, but are optimally reared at 10 - 25 ppt (DPI&F, 2006; Wang and Chen, 2006; Ye et al., 2009). Despite the wide tolerance range, rapid

fluctuation in salinity is associated with increased susceptibility to pathogens and increased incidence of disease outbreaks (Mallik et al., 2016; Remany et al., 2012; Tendencia et al., 2010; Withyachumnarnkul et al., 2003). Beyond the physiological stress and energetic cost to maintain osmotic homeostasis resulting in reduced capacity for immune response (Chong-Robles et al., 2014; Yong, 2021), fluctuating salinity may also be a key mechanism of viral entry into shrimp (de Gryse et al., 2020). During conditions causing increased frequency of urination (i.e. osmotic stress following a sudden drop in environmental salinity), the recurrent opening of the nephropore (antennal gland) for urination to regulate haemocoel volume may increase the opportunity for pathogen exposure to the nephrocomplex (de Gryse et al., 2020). Following coordinated WSSV immersion challenge and hyposalinity exposure (35 g L⁻¹ to 5 g L⁻¹ over 5 h) in *P. vannamei*, cumulative mortality resulting from WSSV infection reached 100% within 48 h of exposure, while all WSSV-exposed shrimp not subjected to the hyposalinity conditions remained WSSV negative and survived (de Gryse et al., 2020). Higher salinity culture conditions can also result in the dominance of opportunistic pathogen communities in the microbiota of shrimp (Hou et al., 2020), potentially increasing disease susceptibility. In PCEs, fluctuation in salinity levels (i.e. during pre-experiment acclimation) may result in significant changes to the immune capacity of the shrimp or dynamics of pathogen transmission, confounding subsequent results of pathogen challenge. As such, consistent reporting of salinity levels and their maintenance throughout the experiment (i.e. range or mean with standard deviation) in publication of PCEs is critical.

6.5.3.3 Dissolved oxygen

Dissolved oxygen (DO) fluctuates in systems due to temperature, photosynthetic activity, decomposition of organic matter, and biotic respiration. Penaeids have osmoregulatory capacity, allowing for maintenance of internal O₂ levels, independent of environmental partial pressure (PO₂), up to a critical threshold. Below this threshold, O₂ consumption becomes limiting for metabolic activity (Herreid, 1980). For *P. monodon* the critical threshold to support normal function is approximately 3.7 ppm (Chen, 1985), with optimum conditions for growth at DO levels greater than 4-5 ppm (DPI&F, 2006). Shrimp exposed to conditions below the optimal range (< 4 ppm) become energetically stressed and thus have increased susceptibility to multiple other stressors, including pathogen infection (Allan et al., 1990; Le Moullac and Haffner, 2000; Lehmann et al., 2016). Given the fundamental impact of DO on shrimp health

and associated disease outcomes, inclusion of DO conditions in publication of PCEs is likely to improve the robustness and reliability of experimental findings.

Beyond the parameters of temperature, salinity, and DO, there are many other consequential environmental parameters including pH, nitrogenous compounds and contaminants. These factors all contribute to the stasis of the organism, the interactions between the host and the pathogen, and hence the dynamics and outcomes of infection (Chen et al., 2020; Fouzi et al., 2010; Hook et al., 2018; Li et al., 2023; Tapaamorndech et al., 2020). As such, their inclusion as reported parameters of PCEs are recommended, where possible.

6.5.3.4 Acclimation

Acclimation of experimental animals to the study system prior to the commencement of experimental activities (e.g. pathogen challenge) is important to enable return to homeostasis after stress induction from transport, handling, and fluctuations in environmental conditions (Wilson et al., 2021). The acclimation period may also serve to quarantine stock and enable pathogen screening prior to the experiment (Conour et al., 2006). Acclimation herein will be discussed with reference to variations in environmental conditions. Stress-related to handling is discussed below in Section 6.5.4 ‘Sampling and handling’.

Rapid changes in environmental conditions occurring during transfer of animals from source tanks or ponds into the artificial ‘captive’ conditions of an experimental system can reduce shrimp health and immunocompetence, leading to increased susceptibility to pathogens or acute disease states from chronic pre-infections (Joseph and Philip, 2020; Junprung et al., 2021; Millard et al., 2021). For example, the transfer of overtly healthy *M. japonicus* from ponds to controlled-environment tank-rearing systems induced elevated Mourilyan virus loads and increased associated mortality to 89% (Sellars et al., 2006). Additionally, when *L. vannamei* chronically infected with WSSV were cooled from 32.3 ± 0.8 °C to 25.8 ± 0.7 °C, acute disease was induced resulting in 100% mortality within 8 days of the temperature reduction (Vidal et al., 2001). Currently, there is limited evidence to support best practices for acclimation of shrimp when transferred into experimental systems. However, some studies have investigated return to homeostasis following salinity and thermal stressors (Rahi et al., 2021b, 2021a). For example, SPF juvenile (~ 0.2 g) *P. monodon* maintained at 20‰ were subjected to six different salinity levels (0, 2.5, 5, 10, 20, or 30‰) and monitored for 60 days (Rahi et al., 2021a).

Physiological, biochemical and gene expression parameters were measured at 0, 12 and 24 h, and 2, 3, 4, 5, 15, 30, and 60 days after reaching the target salinities by adjustment of 2.5‰ per day. By the 5th day, oxygen consumption, haemolymph serotonin and glucose levels, and expression of osmoregulatory genes had stabilised across all treatments. However, total haemocyte count and the expression of growth and immune related genes only stabilised by the 20th day (Rahi et al., 2021a). In a similar study, SPF *P. monodon* post-larvae (~25 dph) maintained at 28 °C were subjected to six different temperature levels (24, 26, 28, 30, 32 or 34 °C) and monitored for 60 days (Rahi et al., 2021b). Thermal stress (heat shock protein gene *HSP70*) was measured at 0, 12 and 24 h, and 2, 3, 4, 5, 15, 30, and 60 days after reaching the target temperatures by adjustment of 1 °C per 6 h. *HSP70* levels increased in all treatment groups up to 24 h, then declined until levels stabilised by day 3, although no treatments reached expression levels similar to the control group (28 °C) (Rahi et al., 2021b). While such findings provide an indication of physiological and biochemical responses of *P. monodon* to gradual change of a single environmental parameter, the effects of more sudden changes, or simultaneous change of multiple parameters are yet to be elucidated. In the absence of adequate acclimation, rapid changes in multiple environmental conditions could result in confounded disease outcomes within a PCE. Adequate duration and consistent conditions during acclimation of experimental shrimp prior to challenge are required to ensure that the observed outcomes of the PCE are not related to stress caused by prior exposure to environmental fluctuation, or differential acclimation conditions between treatment groups. Although there is limited evidence to currently support best practice for acclimation of shrimp to experimental systems, consistent reporting of the adopted acclimation practices and conditions for publication of PCEs is important to provide context of animal condition and may be of increased value and further relevance as more knowledge in this area is developed.

6.5.4 Sampling and handling

Beyond the critical importance of animal welfare maintenance when working with live animals, stress-induced from handling and sampling throughout an experiment can have significant impacts on shrimp physiology and the susceptibility of shrimp to disease (De La Vega et al., 2004; Mercier et al., 2009, 2006). These impacts may become additive with the experimental treatment or pathogen challenge, confounding experimental findings (McGaw and Nancollas, 2018). Stress due to handling or sampling of crustaceans can result in significant shifts in physiological, metabolic and immune responses (Mercier et al., 2009, 2006; Wilson et al.,

2021) and can yield significant differences in mortality during long-term experiments involving repeated handling (Wilson et al., 2022). Despite the lack of well-defined stress tolerance in shrimp related to handling and sampling, subtle changes in the captive environment or treatment of the shrimp, such as handling shrimp between tanks, may be sufficient to induce a stress response (Wilson et al., 2021) or elevate disease expression (De La Vega et al., 2004). In some studies, stress due to handling and sampling of experimental animals has been attributed to the triggering of acute disease states in *P. monodon* from chronic infection with gill-associated virus (De La Vega et al., 2004) and WSSV (Hsu et al., 1999). Post-stress settlement periods, based on return to normal physiological state after a stress event, would reduce the impact of handling/sampling stress on experimental outcomes, and have been advised for multiple crustacean species (McGaw and Nancollas, 2021, 2018; Wilson et al., 2021). However, in the case of PCEs where animals are pathogen challenged and immediately stocked at the commencement of the experiment, or when sampled animals are returned to the experimental system during repeat/time-series based sampling, such settlement periods are not possible. As such, it is important to include handling and sampling protocols in publication of PCEs, so that the conditions and potential impact can be considered when evaluating the reported disease outcomes and interpretations.

6.5.5 Feed

The influence of nutrition on shrimp immune capacity and disease susceptibility is well-established, for example, in studies highlighting the protective effects of feed additives on immune response and resistance to viral infections (Babu et al., 2013; Wilson et al., 2015; Yogeewaran et al., 2012). However, it is also essential to recognise that feeds can act as a potential pathway for pathogens to enter both experimental and commercial rearing systems (Tacon, 2017). Commonly used unprocessed feeds, including polychaete worms, shrimp, squid, contaminated *Artemia* and unpasteurised pelleted feeds, may introduce viable pathogens to the shrimp culture systems. Positive detection of pathogens including, but not limited to WSSV, IHHNV and other bacterial and parasitic pathogens, have been extensively reported from the aforementioned feed types (Tacon, 2017). The transmission of pathogens from the feed source to the shrimp has also been demonstrated in multiple studies (da Silva et al., 2015; Sivakumar et al., 2009; Vijayan et al., 2005), suggesting a plausible route for pathogen introduction into rearing and experimental systems. For pelleted feeds, extrusion at high heat or autoclaving of formulated pellets renders contaminant pathogens non-viable, removing the

risk of viable pathogen introduction into the system (Muñoz, 2011). However, in cases where studies or production processes rely on PCR-based analysis for pathogen detection and determination of infection status, remnant non-viable pathogen template introduced to the system and shrimp from feed may yield positive detection results, in the absence of infection (Elhafi et al., 2004).

While in many cases the content of the feed used in PCEs, such as commercial pelleted feed types, is not controlled directly within the study, the potential for pathogen introduction via this route should be considered. The use of gamma irradiated shrimp (Akuamoah et al., 2018) originating from the experimental source population, or a population of known health status, as feed within a PCE would eliminate the risk of introducing non-viable pathogen templates from commercial feed types, and additional, potentially viable pathogens via unprocessed feed-types, into the experimental system (Wesche et al., 2021). Nonetheless, where possible, pathogen screening analysis should be conducted for the experimental feed as with the experimental animals. Pathogen screening of feed would improve PCE robustness by allowing the risk of introduction of viable pathogens into the experimental system via unprocessed feeds to be considered and managed, or to enable the reductive comparison of non-viable viral template detected in the feed to be considered against the pathogens detected in the experimental animals prior to, and throughout the PCE.

6.6 Pathogen: The viral inoculum

The viral inoculum assumes fundamental importance in PCEs, with several key factors pertaining to the inoculum exerting significant influence on the outcomes and dynamics of the experimental disease. Understanding and accurately reporting on these factors, including the origin (genetic, geographical, temporal and host), processing, delivery, and dosage of the viral inoculum, is vital for proper interpretation and contextualization of the results in the broader context of shrimp disease research and management.

6.6.1 Genetic strain

The genetic composition of the virus used in a PCE is distinctively related to its genetic potential for pathogenicity and level of virulence (Gao et al., 2014; Li et al., 2017). Genetic variation yielding differences in pathogenicity has been widely reported for viruses of shrimp, including IHHNV (Rai et al., 2012), TSV (Aranguren et al., 2013; Robles-Sikisaka et al., 2002; Tang and Lightner, 2005), infectious myonecrosis virus (Andrade et al., 2022), and WSSV

(Gao et al., 2014; John et al., 2010; Marks et al., 2005a, 2005b). In these instances, subtle genetic variation can yield significant alteration of infection dynamics. For example, SNPs within wsv151 and wsv226 genes of the Chinese mainland WSSV strain (WSSV-CN, GenBank accession no. AF332093.1) were demonstrated to facilitate the avoidance of host siRNA-mediated RNAi immunity in *M. japonicus*, demonstrating that mutation of a single base can attenuate antiviral immune response in shrimp (Sun et al., 2019). Regular genetic monitoring is required to aid management of associated changes in virulence and transmissibility, given high rates of genetic diversity within viral genotypes, rapid evolutionary rate, and significant regional subdivision of viral strains arising from biogeographic drivers (Robles-Sikisaka et al., 2010). In the absence of indicative details for viral source, encompassing strain variation related to geographical, temporal and host species' origin, reproducibility of PCEs and contextualisation of PCE results within a relevant global perspective is limited. Hence, it is crucial to clearly document the origin of the viral inoculum, encompassing geographical, temporal, and host species details, and provide the genetic sequence whenever possible when publishing PCEs.

6.6.2 Inoculum processing

Given the prevalence of multiple viral infections in both farmed and wild shrimp populations (Arbon et al., 2022; Condon et al., 2020; Joseph et al., 2015), co-infections in tissue samples used to generate a viral inoculum for PCEs should be considered. Such co-infections may influence the clinical results of the PCE (also see Section 6.4.4 'Screening experimental animals for additional pathogens'). The possibility of multiple viral infections within the same shrimp or homogenate sample raises doubts about the reliability of using crude inoculums, such as unprocessed infected tissue or tissue homogenates, to assess the pathogenicity and virulence of a virus in a specific shrimp species (Munro and Owens, 2007). The presence of multiple viruses within the challenge inoculum can lead to issues in attributing clinical outcomes and interpreting experimental results accurately, affecting the assessment of the true pathogenicity and virulence of the studied pathogen. PCEs that neglect consideration of co-infections within the inoculum, along with any assumptions derived from the study, lack proper substantiation and should be interpreted with caution. Future PCEs should aim to analyse and disclose the presence of co-infecting pathogens within the study inoculum. In studies employing inoculation methods such as intramuscular injection, the generation and use of purified viral

inoculum should be targeted whenever possible. This approach would significantly minimise potential confounding factors associated with less-processed viral inoculums.

6.7 Inoculation method

Inoculation method can influence the quantity of viral particles delivered to the experimental animals resulting in varying infection consistency (Noble et al., 2017). Studies employing immersion-based inoculation may experience inconsistencies in viral delivery and infection efficacy due to fluctuations in salinity (de Gryse et al., 2020), differences in susceptibility to viral entry at different moult stages, or the presence of cuticular damage (e.g., excised pleopod) (Corteel et al., 2009). Similarly, inoculation via feeding of infected tissue (*per os*), may be influenced considerably by inconsistent viral distribution within the fed infected tissue (Arbon et al., 2024a; Noble et al., 2018), and variable feeding behaviour of the experimental shrimp (Maskrey et al., 2018). Intramuscular and intrabladder injection yield high consistency of viral delivery and infection, compared to oral inoculation and immersion (de Gryse et al., 2020; Noble et al., 2017; Raja et al., 2015). However, injection of virus directly into the target organ artificially avoids natural defence barriers to produce rapid infection of susceptible cells, and is misrepresentative of natural infection pathways probable to occur during production (Domínguez-Borbor et al., 2019; WOA, 2023d). Although less consistent and amenable to standardisation, oral inoculation, and immersion are more demonstrative of natural infection routes, including horizontal transmission of virus through cannibalism of infected conspecifics and viral shedding into the water (Wu et al., 2001). While the inoculation method used is dependent on each specific study aims and will not be standard across all PCE studies, reporting of inoculation method in publication should nonetheless be clear and detailed to enable critical evaluation. Secondary or ongoing exposure of experimental animals to infected material should also be considered in the design and implementation of PCEs. For example, in paired-contact cohabitation studies investigating the horizontal transmission dynamics of WSSV the prevalence of WSSV infection and resultant mortality was higher in non-inoculated ('healthy') shrimp when the cohabitating inoculated shrimp were not removed after they died, compared to those where dead inoculated shrimp were removed (Tuyen et al., 2014). The removal or retention of dead or moribund conspecifics from the challenge system should be detailed in the publication of PCEs to allow for consideration of secondary or ongoing re-infection via cannibalism.

6.7.1 Inoculum concentration/ dose

The quantity of viral particles delivered to an experimental shrimp, herein referred to as dose, is related to the speed and severity of disease onset within a PCE (Ngo et al., 2018). Critically, the quantified viral dose must be confirmed as viable. In the absence of crustacean cell lines to confirm inoculum viability and infectivity (WOAH, 2023b), pilot PCEs or LD50 trials (median lethal dose) may be alternatively conducted. Herein, discussion related to dose assumes viability of the viral particles of the inoculum has been confirmed.

Standardisation and reporting of dosage in PCEs are dependent on the method of inoculum delivery. For inoculation via intramuscular injection dose can be simply and clearly standardised to the total number of viral copies injected (e.g., $X \mu\text{L g}^{-1}$ shrimp weight of inoculum at X viral copies μL^{-1}) (Noble et al., 2020a; Spann et al., 2000). However, for inoculation via immersion, dose calculation must consider exposure as a product of viral particle concentration in the immersion bath and the length of time the shrimp is in contact with the particles (Noble et al., 2017). Precise dose calculation and normalisation for *per os* inoculation can be further challenged by inconsistent viral particle distribution within the tissue and variable feeding behaviour of the experimental shrimp (also see Section 6.7 ‘Inoculation method’). Maceration or homogenisation of tissue samples for *per os* challenge can minimise the influence of heterogeneous viral particle distribution on dosage (Abad-Rosales et al., 2019; Campos-Montes et al., 2023; Gudkovs et al., 2014). Additionally, implementing a starving period for experimental animals before *per os* challenge can increase shrimp receptivity to the inoculum feed, improving dose consistency (Joseph and Philip, 2023; Noble et al., 2017; Vijayan et al., 2005).

Regardless of inoculation method, and the approach used and reported for normalisation of dosage, standardisation and reporting of dosage necessitates accurate quantification of viral copy number within the inoculum. Quantification of viral copy number is commonly achieved using qPCR detection and standard curves generated using quantification standards. However, the use of diverse quantification standards, such as nucleic acid from specific biological samples, circular plasmid DNA constructs, or synthetic RNA or DNA oligonucleotides may yield variable quantification for the same sample (Bustin et al., 2009). For example, heterogeneity of plasmid stock, where plasmids may carry variable copies of the target sequence insert, or issues with target sequence amplification from circular plasmids due to the

secondary helical structure of the plasmid, compared to linearised forms (Beinhauerova et al., 2020), can yield highly variable quantification within the standard materials. Given these variabilities, clear and consistent reporting of inoculum dose and how the dose was quantified (see MIQE guidelines: Bustin et al., 2009) is essential to the internal and external validity of PCE results.

6.7.2 Control treatment

The control treatment of a PCE should constitute an absence of treatment, or a standard treatment of known effect, from which the challenge treatment can be compared against. In studies where a standardised solution can be used as a control treatment, there is certainty that the control group is unchallenged and the effects of inoculation and handling stress in the challenged group are accounted for in the control group conditions. For example, inoculation of the challenge group with a purified viral suspension in tris-buffered saline, and inoculation of the control group with tris-buffered saline only (Panphut et al., 2011). However, in studies where a non-standardised control treatment is used, such as PCEs with *per os* inoculation which uses tissue from overtly ‘healthy’ shrimp as the control treatment, there is less certainty in the standardisation of the treatment. As discussed in both Sections 6.4.4 and 6.6.2, unintentional introduction of viruses other than the principal virus being studied may influence the clinical results of the PCE. Absence of viral screening of the control tissue to rule out sub-clinical infections is an ongoing and critical issue that requires addressing before conclusive results from such challenge experiments can be justifiably drawn. Careful selection and standardisation of control treatments are essential to ensure the validity and reliability of PCE findings and should be detailed accordingly within publication of PCEs to enable critical evaluation of study findings.

6.8 Reported information in shrimp PCEs

Considering the substantial evidence supporting the multifactorial influence of host, pathogen, and environmental components on disease outcomes, it is important to examine the existing literature on shrimp PCEs within this context. We gathered data from 186 peer-reviewed publications of viral PCEs involving *P. monodon* related to the host, environment, and pathogen components of the disease triad, with reference to a ‘systems’ approach. This subset of studies was identified via a literature search of PubMed, conducted on 1 August, 2023, using the search terms ‘virus’ and ‘monodon’. The publications identified from this search were further refined to include only PCEs of *P. monodon*. Reported details related to the components

of the disease triad (Figure 6.1), were obtained from each study where available (Appendix 22). The prevalence of these reported details within the literature are displayed in Figure 6.2. The results of the literature survey highlighted a concerning paucity of reported experimental detail for viral PCEs in *P. monodon*.

Experimental details were most consistently reported for the host component, followed by pathogen and environment. Most studies reported the size of the experimental *P. monodon* (weight in grams; 91%, 169/186), gave reference to shrimp age or developmental stage (45%, 84/186), and provided shrimp source with respect to habitat (82%, 152/186) and origin country (85%, 159/186) or region (59%, 109/186). The use of shrimp declared as SPF was reported in a small portion of the studies assessed (8%, 14/186). Of the remaining studies that did not report the use of SPF shrimp (172 of 186), reports of pathogen screening (e.g., using PCR or qPCR) were alarmingly limited (43%, 75/172). Adding to this concern, very few of these studies (12%, 20/172) reported screening for possible co-infections.

Factors related to the challenge system (environment) were variably reported. The number of shrimp per replicate tank (83%, 155/186) was reported in most studies, however, tank volume (46%, 86/186) or stocking density (39%, 72/186; including studies that provided metadata from which density could be calculated) were reported in less than half of all PCEs evaluated. None of the studies assessed reported PCEs conditions for the core water quality parameters including salinity, temperature, pH, DO and nitrogenous wastes in combination. Salinity (82%, 152/186) and temperature (61%, 113/186) were reported most frequently, while pH (11%, 20/186), DO (8%, 14/186) and nitrogenous wastes (6%, 11/186) were rarely reported. Studies should aim to report a range or mean $\pm$ SD for water quality parameters, as it provides information as to the consistency of the conditions maintained throughout the experiment. For example, in PCEs where a temperature range is reported, the impacts of temperature fluctuation can be considered with respect to the outcomes of the study, especially of those that report temperature ranges across a large gradient ($\sim 4 - 6$ °C variation) (Catap et al., 2003; Oanh et al., 2011; Owens et al., 1998; Panphut et al., 2011; Patil et al., 2013, 2011; Soowannayan et al., 2013). Shrimp acclimation period was reported for more than half of the studies analysed (66%, 122/186). Feeding during the challenge experiments was uncommonly reported, with details of feed type, schedule, and ration reported in combination for only 12% (23/186) of PCEs.

Of concern was the apparent absence of reported viral (inoculum) data within the PCEs assessed. Most of the studies did not report the source of the study virus (71%, 132/186), with only 29% (54/186) directly reporting the geographic or genetic source, and 15% (28/186) reporting the temporal origin of the virus. The type of inoculum used (e.g., filtered homogenate or purified viral suspension) was more consistently reported (83%, 155/186) jointly with the inoculation method (100%, 186/186). The limited reporting of pre-screening experimental shrimp was mirrored in the screening of the viral inoculums. Screening for multiple viruses in non-purified viral inoculums or un-processed inoculum types was very rarely reported (4%, 8/186).

The authors of this review acknowledge that the absence of reported experimental detail within published PCEs does not necessarily mean that the factors were not managed or measured within the PCE. However, the utility of research and its value to directing successive studies is reliant on the information provided within the publication to assess the internal and external validity of the experiment and its findings (Sargeant et al., 2014). Consequently, the lack of reported detail in published PCEs severely limits the potential for the research to make progressive advancements and contribute to contemporary shrimp disease management strategies applying a ‘systems’ approach.

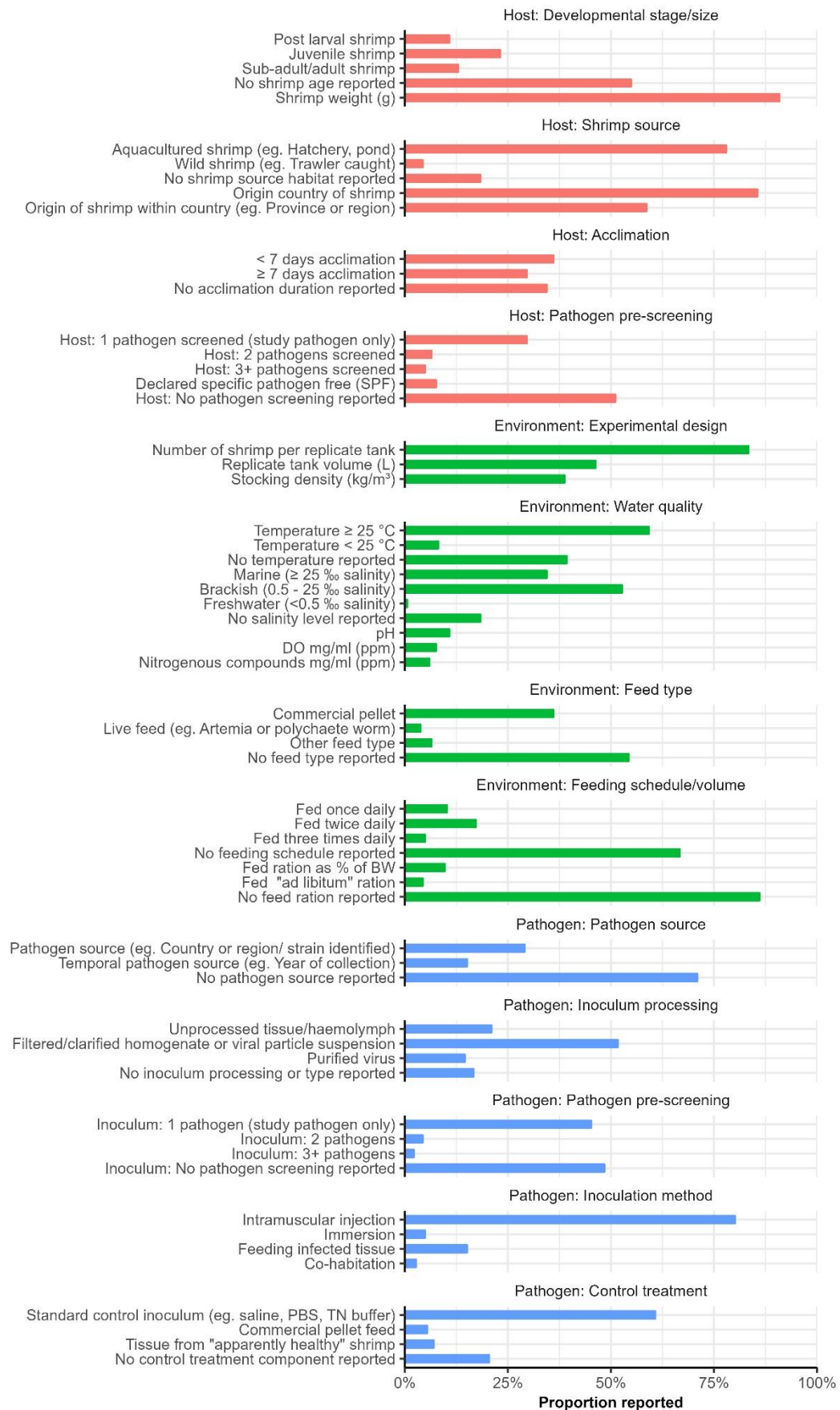


Figure 6.2. Prevalence of reported details from 186 peer-reviewed publications including viral pathogen challenge experiments on *Penaeus monodon*, published between 1997 and 2023. High resolution version available [online](#).

6.9 Minimum reportable information for publication

Considering the evidence to support a ‘systems’ approach to PCEs, and the scarcity of reported details from the existing body of literature (Figure 6.2), a more rigorous and repeatable strategy to improve the utility and translation of experimental findings from PCEs must be developed. We propose guidelines in the form of a checklist for the minimum reportable information in the publication of shrimp viral PCEs, hereafter referred to as the Shrimp PCE Reporting Guidelines (SPERG) (Table 6.1). The proposed guidelines include a core set of characteristics pertaining to the shrimp host, viral pathogen, and environment, and reflect current evidence supporting the multifactorial influence of components on disease outcomes. Given the complexity of disease as a manifestation of interactions within the ‘system’, the checklist does not account for all parameters which may impact the outcome of a PCE. As such, we encourage reporting of additional parameters beyond those listed in the checklist, where possible. However, we also acknowledge that in some instances, specific information included in the checklist may not easily be obtained by the researcher. Nonetheless, it is expected that reasonable adoption of the reporting checklist will improve the utility and transparency of future PCE-based studies, ultimately improving understanding of viral diseases of shrimp within a ‘systems’ framework.

Table 6.1: Shrimp pathogen challenge experiment reporting guideline (SPERG) checklist.

Factor	Experimental detail
Host	
Age	Stage, including days post hatch (dph) or days of culture (DOC)
Size	Weight (g) and/or length (mm)
Source	Habitat (farmed or wild, hatchery, pond, etc.)
	Genetic/geographic/temporal source (country, region, date collected)
Acclimation	Duration (days)
	Conditions (see System: environmental conditions)
Pathogen pre-screening	Sample size screened (proportion of experimental cohort)
	Pathogens screened
	Method of screening (PCR, qPCR, etc. See MIQE)
	Screening results (pathogen load & prevalence)
Environment	
Experimental design	Definition of experimental and control groups
	Duration of the experiment (days post infection - dpi)
Replication	Shrimp per tank
	Tank size (L)
	Stocking density
	Tanks per treatment
	Treatments within the experiment
	Repetition of the experiment
Sampling & analysis	Sample tissue type
	Sampling technique
	Sample storage conditions
	Sampling schedule (hours post infection - hpi or dpi)
	Shrimp replacement or sacrifice (during sampling and for mortalities/moribund shrimp)
	Details of laboratory and statistical analysis performed (see MIQE)
Environmental conditions/ water quality	Temperature
	Salinity
	pH
	DO
	Nitrogenous compounds (e.g., NH ₃ /NH ₄ , NO ₃ , NO ₂)
	Aeration provisions
Feeding	Filtration and water treatment provisions
	Feed source and pathogen screening results
	Feed treatment provisions
	Feeding schedule (BW% per feeding event)

Table 6.1 Continued

Factor	Experimental detail
Pathogen	
Source (strain)	Genetic/geographic/temporal source (country, region, date collected) Genetic sequence and strain identification (if available)
Processing method	Tissue sampling Homogenisation, filtration, clarification, purification etc.
Pathogen pre-screened	Pathogens screened Method of screening (PCR, qPCR, etc. See MIQE) Screening results (pathogen load)
Volume and Concentration	Volume of inoculum used Viral copies per unit volume
Inoculation method and conditions	Intramuscular injection: injection site Immersion: bath duration and concentration, washing post bath <i>Per os</i> : starvation period, feeding period, volume of tissue (%BW) Co-habitation: exposure duration, removal of mortalities, holding configuration
Control inoculum	Control treatment used If non-standard control is used; pathogens screened, method of screening, screening results (pathogen load)

6.10 Conclusion

Successful contemporary disease management strategies applying a ‘systems’ approach must be predicated on current and robust knowledge of the host, pathogen, and environment. PCEs are a powerful research tool for resolving the impact of pathogens on shrimp health and identifying solutions to the pervasive threat of viral disease in shrimp production. While defined experimental hypotheses can be robustly tested using well-designed PCEs, the high levels of control and ‘artificial’ nature of PCEs pose inherent limitations. As such, for these findings to provide realised value to global shrimp production, they must be contextualised to the broader ‘system’ framework. To achieve this effectively, the conditions of the experiment, including but not limited to the items described within the SPERG checklist, must be accurately and thoroughly detailed. In the absence of thorough reporting of experimental detail, the evaluation of both internal experimental validity and external validity to a ‘systems’ framework is limited, and the potential for the research to contribute to contemporary disease management is reduced. The SPERG checklist is proposed to improve the utility and transparency of future shrimp viral PCEs. We further envisage that adoption of SPERG will not only aid individual studies but also enable more robust and insightful meta-analyses from future shrimp PCEs.

While we targeted our review of reported experimental details in PCEs to viral challenge of *P. monodon* shrimp, the wider concepts posed are applicable to shrimp, and perhaps crustacean PCEs more generally, and may be adaptable to other pathogens beyond viruses. Ultimately, the SPERG checklist is aimed at improving the accessibility and application of PCE findings to viral disease management in commercial production for the benefit of animal health and the productivity of shrimp aquaculture.

Chapter 7.

General Discussion

Aquaculture is the fastest growing food production sector globally and is set to play a critical role in future food and nutrition security amidst global resource challenges (FAO, 2022). Farming of marine shrimp is an important aquaculture industry, representing production of one of the most extensively traded seafood commodities and an industry that has demonstrated continual multi-decadal growth (FAO, 2022). However, global shrimp aquaculture faces particularly severe economic and social challenges due to impacts of infectious diseases (Asche et al., 2021; Stentiford et al., 2017). Consequently, effective disease management is paramount to the maintenance and sustainable growth of this industry (Cain, 2022; Nguyen, 2024; Stentiford et al., 2012). Global and domestic trends in aquaculture indicate that effective disease management will increasingly demand robust, accurate, reliable and cost-effective diagnostic and surveillance systems (Kumar et al., 2021b; MacAulay et al., 2022; Stärk et al., 2019). In contemporary shrimp aquaculture, the detection of pathogens for disease diagnosis and surveillance purposes are primarily achieved through the use of qPCR (WOAH, 2023b). Timely detection of pathogens using qPCR enables the prompt implementation of control measures, thereby minimising transmission and mitigating the impact of infections on production (Kumar et al., 2021b; Lightner, 2006; MacAulay et al., 2022). Pathogen detection data also provides highly valuable information for the assessment of spatiotemporal epidemiological patterns, and teasing apart the complex ‘systems’ interactions between the host, pathogen and culture environment that manifest in disease (Bateman et al., 2021; Richard et al., 2021; Sanguanrut et al., 2018; Stärk et al., 2019). Despite the immense value of both effective pathogen detection systems, and understanding pathogen dynamics for advancing disease management, there are several significant barriers and knowledge gaps that are currently impeding the realisation of this value within the Australian shrimp (prawn) aquaculture industry. Fundamentally, a substantially improved understanding of pathogen dynamics throughout shrimp production systems is required to facilitate effective disease management that adopts a ‘systems-based’ approach, applying the principles of the infectious disease triad (Arbon et al., 2024b; Snieszko, 1974). To achieve this, improved application of existing and emerging biosecurity tools is required to support accurate, reliable and cost-effective pathogen detection (MacAulay et al., 2022). The research reported within this thesis takes significant steps towards overcoming these barriers by establishing robust evidence to inform industry pathogen management strategies and to improve the utilisation and cost efficiency of modern and emerging pathogen detection technologies in Australian shrimp aquaculture. In a broader context, the collective findings outlined in this thesis are a valuable

resource to guide the application of the methodologies and concepts developed within, enabling their translation to global shrimp production, and other existing and emerging aquaculture species. The significance and outcomes of the research comprising this thesis are discussed below, complementing the discussions presented within each individual chapter.

7.1 Significance and major outcomes

Given Australia's national biosecurity regulations that prevent import of live shrimp, internationally developed, commercial lines of specific pathogen free (SPF) *L. vannamei* or *P. monodon* are not available to the Australian industry. Thus, the only genetic stocks of *P. monodon* available to the industry are from wild populations, or Australian-based domestication programs developed from wild-sourced endemic animals (e.g. SFG, 2023) (Coman and Preston, 2008). Collection of broodstock with lower viral burden reduces the likelihood and extent of pathogen exposure to receiving farms and is a critical component of entry-level biosecurity. Until now, the availability of large-scale, contemporary understanding of pathogen presence within wild sources of *P. monodon* broodstock has been limited and has hindered the strategic targeting and collection of wild broodstock with lower viral burden. In Chapter 2, the most comprehensive assessment to date of endemic pathogens in wild-sourced Australian *P. monodon* broodstock was conducted. The results of this analysis revealed the high prevalence of commercially important endemic viral pathogens within wild-sourced broodstock. The findings exposed striking trends in pathogen detection related to temporal and geographic factors, including the significantly higher viral prevalence of east-coast Queensland sourced broodstock, compared to those from the Northern Territory. The high prevalence of pathogens detected highlights the significant likelihood of viral pathogens being introduced into hatchery production systems through wild-sourced broodstock, supporting the critical need for strong pathogen detection systems when introducing wild broodstock. Moreover, these findings further support the Australian industry's ongoing intent to establish domesticated endemic SPF *P. monodon* lines to avoid continued annual, or seasonal, exposure to pathogens by intake of wild-sourced broodstock. Importantly, the insights into temporal and geographical trends of pathogen presence have provided understanding of the broader pathogen landscape, enabling evaluation and comparison of wild broodstock sources, and has delivered immediate advantages to the Australian shrimp aquaculture industry by directly facilitating more risk-averse collection of wild broodstock for ongoing production. In doing so, pathogen exposure of farms has reduced. The findings of Chapter 2 can be further applied to inform strategic

collection of founder individuals from wild populations during development of Australian SPF domestication programs. Further research is required to determine the relative importance of each pathogen for inclusion within an SPF breeding objective, and for defining best-practice for exclusion of pathogen-positive broodstock from a hatchery. To gain this understanding, the impact of each pathogen on shrimp health and productivity needs to be fully elucidated and should be a considerable focus of future research.

Since the publication of Chapter 2 (Arbon et al., 2022), additional studies have applied a similar rationale for undertaking pathogen screening in wild shrimp populations. In doing so, Krol et al. (2024) recently documented the first detections of IHNV and WSSV in wild invasive populations of *P. monodon* within the Gulf of Mexico and the Northwestern Atlantic Ocean, highlighting a significant risk to both wild fisheries and aquaculture. The findings of Krol et al. (2024), and those described in Chapter 2, demonstrate the importance of documenting and monitoring the virome of wild shrimp populations with regard to direct assessment of their health and the risk they pose to farmed populations. Many aquaculture farms are located within, or are highly connected to, aquatic ecosystems (Boyd et al., 2022). Therefore, successful production and robust biosecurity in aquaculture greatly benefits from knowledge of the pathogens that infect surrounding wild populations and other relevant sympatric taxa (i.e. vector and reservoir species), for which the aquaculture species are competent hosts (Nielsen et al., 2023; Raj et al., 2023). This was critically illustrated within the Argentinian Lake Moreno system, where introduced salmonids are now a major host species of parasitic *Acanthocephalus tumescens* within the lake, following acquisition from wild fish populations (Rauque et al., 2003). Undertaking ongoing studies to document and monitor pathogen presence in natural populations (developing comprehensive ‘inventories’ (Bouwmeester et al., 2021)) should be considered a critical area of study for the rapidly diversifying range of aquaculture species (Lafferty et al., 2015). Recent examples include studies establishing the baseline occurrence of parasites in wild reservoir fish on Australian Murray cod (*Maccullochella peelii*) farms (Shamsi et al., 2021), or the those documenting presence of betanodaviruses (nervous necrosis viruses) in wild fish species along the Turkish coast of the Mediterranean Sea (Kaplan et al., 2023).

Monitoring pathogen presence and loading patterns in wild populations may also be valuable from ecological and fisheries management perspectives. Baseline data of wild population

pathogen presence and abundance would assist evaluation of whether disease impacts on wild stocks result from natural variability, or are exacerbated by spillover, or spillback, from aquaculture (Kelly et al., 2009; Leung and Bates, 2013). This is an especially important area for continued research as the effects of aquaculture on disease dynamics in wild populations within surrounding ecosystems are poorly characterised (Bouwmeester et al., 2021; Lafferty et al., 2015). Monitoring data can also be used to indicate broader ecological shifts and ecosystem health (Sieber et al., 2024), for example, for indicating increasing disease occurrence in wild populations associated with anthropogenic activity, pollutants or with climatic change (Bojko et al., 2020; Caldwell et al., 2020). In this context, the data described here serves as a key baseline and reference point for future research.

Beyond the direct application of the findings of Chapter 2 to the Australian farmed shrimp industry and broader inferences for marine pathogen monitoring, this research highlighted the endemic viral pathogens detected from farmed *P. monodon* at the highest prevalence (GAV and IHHNV), representing the greatest likelihood of farm exposure, as well as the presence of emerging threats of significant concern (YHV-7). These findings were used to strategically guide further research within this thesis toward investigation which had the highest relevance to the Australian farmed shrimp industry.

Robust pathogen detection is required throughout all phases of aquaculture production to accurately inform disease mitigation and control strategy (Kumar et al., 2021b; MacAulay et al., 2022). This is especially relevant for tropical aquaculture, where proportionally greater losses are incurred during disease outbreaks and farms have less time to mitigate losses (Leung and Bates, 2013). Clear evidence is required to support the optimised application of modern molecular detection tools and ensure sensitive, accurate and practical application (Stärk et al., 2019). In some instances, the rapid evolution and adoption of molecular detection technologies, including qPCR, has outpaced the development and dissemination of such supporting evidence (Stärk et al., 2019). Chapter 3 developed evidence to support the optimised detection of IHHNV, GAV and YHV-7 from naturally infected *P. monodon* by determining tissue-specific patterns of viral tropism using (RT-)qPCR. Additionally, Chapter 4 examined the current industry practice of sampling pleopods for pathogen screening and experimentally evaluated the limitations and value of pleopod tissue detections of IHHNV and GAV in more depth. The cumulative findings of Chapters 3 and 4 modernise our knowledge of tissue-specific

distribution of endemic viruses within *P. monodon*. The viral distribution patterns observed here extend upon those previously described and offer updated evidence by leveraging the greatly enhanced detection capacity of modern molecular methods. Notably, the findings indicated strong detection of IHHNV from shrimp hepatopancreas, which was previously not considered appropriate for the detection of IHHNV. Furthermore, several critical deficiencies in understanding of GAV infection dynamics and detection were identified, emphasising crucial focal points for further research. Importantly, these findings provide foundational evidence to guide improved shrimp tissue sampling protocols and enable more robust interpretation of detection results from various tissue types. Improved tissue sampling strategies and result interpretation enhances the utility of qPCR in achieving sensitive, reliable, and practical pathogen detection, directly benefitting industry pathogen screening and disease diagnosis outcomes (Lacroix et al., 2016; Noble et al., 2018). Similarly, the findings in Chapter 3 and 4 are invaluable for supporting the rigor of future research where measurement of viral loading or viral presence/absence determinations are key experimental factors, such as in studies investigating the genetic basis of pathogen resistance where viral loading is used as a phenotype for estimating disease resistance breeding values (Bishop and Woolliams, 2014; Noble et al., 2020a; Yáñez et al., 2014), or for the validation of novel disease treatments and technologies (Seethalakshmi et al., 2021).

The evidence generated within Chapter 3 and 4 also serves as a useful resource when revising recommended target tissues for the detection of IHHNV and the YHV complex within global diagnostic standards, including the WOAHP Manual of Diagnostic Tests for Aquatic Animals (WOAHP, 2023b). However, extending similar research to support this review and modification is emphasised, particularly to assess detection patterns throughout infection progression, and to employ a more diverse array of sample processing and molecular approaches for broader relevance. Beyond diagnostics, the findings reported within both chapters are significant for devising risk management protocols to prevent the global transfer of shrimp pathogens through uncooked crustacean product (Jones, 2012; Scott-Orr et al., 2017). This is particularly relevant for the Australian national import risk analysis and importation regulations aimed at protecting against incursion of exotic viral pathogens (DAF, 2023a), given our findings indicate high levels of viral presence in tissue types currently permitted for importation into Australia within uncooked crustacean product. Considered together, the knowledge developed in Chapters 3

and 4 of this thesis holds substantial significance, with potential application and implications spanning industry, research, and government sectors.

The cost-effectiveness of rapid sampling and analysis remains a significant limitation for the implementation and maintenance of large-scale pathogen surveillance programs (Stärk et al., 2019). Faced with this challenge, collection and analysis of environmental DNA (eDNA) or RNA (eRNA), collectively eNA, is proposed as an alternative to tissue sampling for detection and monitoring of pathogen presence in aquatic systems (Bass et al., 2023). Passive collection of eNA involves the settlement or binding of eNA to a filter medium during deployment and incubation within the environment (Bessey et al., 2021; Chen et al., 2022; Schang et al., 2021; Verdier et al., 2022; Vincent-Hubert et al., 2017). Due to its simplicity, cost efficiency, and comparable performance to traditional methods, there is a strong theoretical basis for applying passive eNA sampling for pathogen surveillance at large-scale in commercial shrimp aquaculture. However, until now, the application of such technologies in this context had not been demonstrated. Chapter 5 established the utility of passively collected eNA samples for pathogen detection from commercial shrimp ponds and subsequently validated such detections with paired analysis of shrimp tissue samples. The findings of this study revealed passively collected eNA is a suitable alternative to tissue sampling and is capable of providing proxy quantification of pathogen loading of farmed shrimp populations within ponds. These findings offer compelling initial evidence of the potential inherent in this novel approach for quantitative detection of viral shrimp pathogens.

While eNA is not currently considered suitable for diagnostic purposes (WOAH, 2022), the findings in Chapter 5 provide strong evidence and a theoretical basis to support the broader application of passive eNA sample collection for efficient and cost-effective pathogen monitoring and surveillance in farmed shrimp production systems. The greatly improved surveillance scope of the methods outlined, and enhanced understanding developed within this study, can be leveraged to provide numerous benefits to future shrimp production and research. For example, passively sampled, eNA-based detection can be used to develop extensive long-term pathogen presence datasets (Bowers et al., 2021). Such datasets may be used to support the identification of emergent native pathogen strains, or provide valuable evidence to support claims of pathogen freedom. These datasets can also be evaluated to deepen understanding of host-pathogen-environment interactions and drivers of disease outbreak (Childress et al.,

2024), or be integrated with production data to provide insights into the subclinical impacts of infection on shrimp productivity. These outcomes will greatly improve future shrimp disease management. The potential of eNA sampling to provide significant immediate and future benefit has been directly recognised by the Australian shrimp farming industry. As a result, a subsequent research project ([Food Agility CRC](#)) has been developed and funded to further the work described in Chapter 5 of this thesis.

The success of contemporary disease management strategies are predicated on robust knowledge of pathogen presence, and the conditions and interactions between the host, pathogen(s) and environment that promote disease (i.e. ‘systems-based’), as first proposed by Snieszko (1974). The cumulative methodologies and understanding developed in Chapters 2 – 5 provide a robust foundation for improving pathogen detection within shrimp aquaculture. These advancements facilitate enhanced detection reliability and interpretation, and support progression towards ‘systems-based’ disease monitoring and research. Beyond these tools, pathogen challenge experiments (PCEs) provide a valuable mechanism for robustly elucidating host-pathogen-environmental interactions. Considerable global research efforts utilising PCEs for shrimp have been conducted to date, including for purposes of understanding pathogenesis (John et al., 2010; Marks et al., 2005a; Srisuvan et al., 2005), the impacts of environmental conditions on disease outcomes (Fouzi et al., 2010; Rahman et al., 2006; Rungrassamee et al., 2010), selective breeding for disease-related traits (Mohd Ghani and Bhassu, 2019; Noble et al., 2020a) and for assessment or validation of novel disease preventions and treatments (Babu et al., 2013; Srisapoom et al., 2018; Wongprasert et al., 2014). The progressive advancement of pathogen management within Australian shrimp aquaculture requires analogous applications of PCEs using Australian stocks. To ensure the Australian industry is best positioned to achieve such progressive advancements, Chapter 6 thoroughly reviewed, synthesised, and critically evaluated existing peer-reviewed research to identify and understand the host, pathogen and environmental factors which influence PCE outcomes. Extending this synthesis, the review additionally provides a clear and evidence-based guide for the design and reporting of future research utilising PCEs. The findings of this chapter strongly support holistic ‘systems-based’ approaches to disease management and research, and highlights key gaps in knowledge where further investigation or improvement in the field is needed. The review is a valuable educational resource, serving as a seminal study and key point of reference for both the research community and industry. Importantly, the review provides tangible support for the improved

delivery and translation of future research on shrimp health and disease to facilitate the progressive advancement of knowledge and application of this knowledge to benefit disease management across the industry.

The collective research works comprising this thesis, and their associated outcomes, represents a significant contribution to advancing understanding of the detection, dynamics, and management of viral pathogens in shrimp aquaculture systems. This understanding has already supported advancements in biosecurity and disease management within the Australian *P. monodon* farming industry and is poised to offer ongoing support in its future growth, and global shrimp aquaculture more broadly.

7.2 Future directions for shrimp pathogen detection and biosecurity

The research comprising this thesis has yielded significant advances in the field of pathogen detection and enables improved application of molecular detection technologies to benefit shrimp aquaculture. However, there are still key knowledge gaps that need to be addressed to realise the full benefit of these applications for achieving robust biosecurity and disease management.

While funding for conducting extensive pathogen surveillance among wild aquatic animal populations, in the absence of overt disease, can be difficult to attain (Krol et al., 2024), such analysis is conducted routinely by farms during the annual intake of wild-sourced broodstock. Until now, the benefits of such extensive pathogen testing have not been fully realised due to the lack of data sharing and aggregation amongst industry (Stephens, 2019). Results reported in this thesis, particularly those from Chapter 2, exemplify how such pathogen testing efforts can be aggregated and analysed to benefit the industry more broadly (Arbon et al., 2022). While the Australian farmed shrimp industry continues to rely on wild stocks and conduct associated pathogen screening, aggregation of de-identified detection data and provision of broad scale seasonal or yearly pathogen reports to farms would provide ongoing support to improve risk-averse collection strategy, and broader understanding of population-level epidemiological trends. The hesitancy of farms to share data due to their commercial interests, or disparate investment in data collection, is respectfully acknowledged. However, the extent to which aggregated data can improve broad-scale, industry-wide biosecurity awareness and strategy

provides strong support for this altruistic approach. Collaboration in this context would aid the industry's collective advancement towards industry-wide disease resilience.

The future of broodstock screening may also see a progressive shift towards extension of screening activities to include health indicators, such as markers associated with immune competency (Mohd Ghani and Bhassu, 2019), or the presence of endogenous viral elements (EVEs) (Flegel, 2020, 2009; Utari et al., 2017). EVEs are proposed to be non-retroviral fragments of extant viruses that are autonomously inserted into the genome of the host, and represent segments of genetic sequence with high homology between the host and viral species (Chiba et al., 2011; Flegel, 2009; Katzourakis and Gifford, 2010; Parrish and Tomonaga, 2016). When transcribed by the host, EVEs may produce antisense RNA which can hybridise specifically with viral mRNA during an infection. This hybridisation produces dsRNA. The presence of dsRNA stimulates the shrimp RNA interference (RNAi) response, resulting in cleavage of bound viral mRNA and preventing its translation into viral proteins (Su et al., 2008; Tijsterman and Plasterk, 2004). Thus, the presence of EVEs producing antisense RNA of an extant virus are theorised to enable the host and homologous virus to coexist without disease in the form of a harmless persistent infection, known as 'accommodation' (Flegel, 2020; Taengchaiyaphum et al., 2022, 2019). IHHNV and WSSV-like EVEs are well characterized within the genome of Australian *P. monodon* (de la Vega, 2005; Huerlimann et al., 2022; Tang and Lightner, 2006). Such EVEs are contained in chromosomal DNA and are consequently passed from broodstock to progeny through predictable patterns of mendelian inheritance (Brock et al., 2013; Taengchaiyaphum et al., 2019). While EVEs detected from other arthropod species, including mosquitos (Palatini et al., 2022; Suzuki et al., 2020), have been associated with improved host tolerance to their respective viruses, this has not yet been demonstrated in shrimp. However, the models to support screening of heritable and potentially protective EVE are developing (Blanco-Melo et al., 2024; Taengchaiyaphum et al., 2024, 2021a; Utari et al., 2017) and, at the time of writing this thesis, breeding trials are underway to test EVE efficacy for protection against WSSV in *L. vannamei* (Taengchaiyaphum et al., 2024). The outcomes of this research will be critical in directing future efforts using EVE screening within broodstock as a selection criteria to provide practical, heritable viral protection in breeding programs (Taengchaiyaphum et al., 2019).

The integration of viral sequences into the shrimp genome is also highly relevant to PCR-based diagnostics in cases where utilised assays cannot differentiate the origins of the two sequences. The occurrence of EVEs producing false positives for infectious virus, and the implications for disease management, are well recognised (Alday-Sanz et al., 2018; Saksmerprome et al., 2011; Tang and Lightner, 2006). Future work should aim to build capacity to accurately screen for, and differentiate between, the presence of EVEs and infectious viral sequence, taking into account the likelihood of distinct EVE presence between genetically distinct shrimp populations (Huerlimann et al., 2022). To ensure these detections provide tangible value and relevance to shrimp aquaculture, it is necessary to characterize the functionality and regulation of screened EVEs through controlled challenge experiments with the homologous virus (Taengchaiyaphum et al., 2024).

Advancements in nucleic acid extraction kits, qPCR master mixes, PCR technologies (e.g., ddPCR), primer and probe chemistries, and optimisation of region targeting offer significant potential for more accurate and reliable pathogen detection (Van Heuverswyn et al., 2016). These technological improvements will predictably lead to earlier and more precise identification of infections, enabling better management and control of disease outbreaks in shrimp aquaculture. The rapidly evolving capabilities of these new and developing technologies for pathogen detection necessitate active and ongoing revision of the standards which describe their optimised and appropriate application (Stärk et al., 2019). The work presented within this thesis provides robust evidence to support improved detection of IHHNV, GAV, and YHV-7 (Arbon et al., 2024a). However, extension of this work is necessary to similarly improve and guide the application of detection tools for other emerging or major pathogens of interest to the Australian and global industry (e.g. WSSV, AHPND, EHP). Applying contemporary detection technologies, further analysis is also required to elucidate the viral tissue tropism patterns at different stages of infection. Understanding these patterns is critical for refining diagnostic techniques and ensuring accurate detection and interpretation throughout the infection process.

Pleopods are consistently used for the detection of GAV (YHV-2) within industry practice, and yield comparable detection levels to other target tissues including gills (Arbon et al., 2024a; Noble et al., 2018). However, observation from two distinct cohorts, in both Chapters 3 (Arbon et al., 2024a) and 4, indicate that there is limited statistical correlation of GAV detection among

various tissues including pleopods from the same individual shrimp. The results of Chapters 3, 4 and 5 suggests that presence/absence determination of GAV from pleopods can be considered reliable, however, the relative quantitation from pleopod tissue may not be representative of overall shrimp GAV infection levels. This characteristic is not consistent between genotypes of YHV, for example, YHV-7 was demonstrated to produce highly linearly correlated detection across multiple tissue types of *P. monodon*, including pleopod (Arbon et al., 2024a). Unlike YHV-7 which is associated with mass mortality events (Cowley et al., 2019; Mohr et al., 2015), GAV infections are often chronic and asymptomatic and occur at high prevalence within Australian *P. monodon* stocks (Arbon et al., 2022; Cowley et al., 2002a; Mohr et al., 2015). The low correlation of GAV detection between tissue types, including from pleopod, may be related to a capacity for *P. monodon* to tolerate GAV, such that GAV infections are maintained at a sub-clinical level (Anantasomboon et al., 2008; Noble et al., 2020a). Patterns of tissue specific viral distribution or gene expression under viral tolerance may differ from those indicative of an acute infection, such as those triggered by additional stressors (De La Vega et al., 2004). Investigation into the expression dynamics of GAV during different infection stages would improve understanding of the biological relevance of detection using the current GAV assay (De La Vega et al., 2004), and may provide insights to guide assay refinement, or development of a modern assay. Further research is needed to understand the tissue-specific loading of GAV under different infection modes and throughout the progression of infection to determine the best approach for its accurate and sensitive detection and, critically, to enable biologically relevant interpretation of detection results. Likewise, the limited correlation of eNA-based GAV detection with tissue detection in Chapter 5 could also be related to these limitations in GAV quantitation. Leveraging the work within this thesis, future research may apply a hybrid experimental design of Chapter 3 (tissue tropism), Chapter 5 (eNA) and Chapter 6 (PCEs) to determine which tissue yields detection most correlated with environmental detection, to inform the utility, and appropriate, biologically relevant interpretation of GAV eNA detection results.

The field of environmental DNA and RNA (eNA) monitoring in aquatic systems is rapidly progressing, presenting opportunities for affordable, simple, non-invasive and sensitive screening (Bass et al., 2023; Rusch et al., 2018; Sieber et al., 2024; Vincent-Hubert et al., 2021; Wittwer et al., 2019). Based on the results described in this thesis, and those described more broadly, it is likely that future disease management will increasingly rely on results derived

from eNA analysis (Childress et al., 2024). Therefore, eNA analysis must be validated appropriately to ensure results are reliable, defensible and robust (Ip et al., 2024; WOA, 2022). This validation must be context-specific and provide statistically supported associations between eNA detections and confirmed host infections (Bass et al., 2023). While the work presented in this thesis takes significant steps towards this, further validation is required, and several knowledge gaps need to be addressed so that the potential of eNA can be realised and exploited in shrimp aquaculture. Foremost, determining the limit of detection from eNA samples in aquaculture systems is required (Childress et al., 2024). However, due to the sensitivity of eNA sampling for detecting viral presence within an environment, validation for estimating infection in the aquaculture host species will be complicated by the presence of non-target host taxa (e.g. cohabitating vector or reservoir species), which may confound host detection signal within the system. Understanding and determining how, or if, validation can be conducted *in situ* under such scenarios will be critical to progress eNA applications. Further, factors including pond biomass, moult-cycles, culture duration, pond water chemistry and climate conditions are each likely to impact eNA accumulation and degradation within the culture environment (Rusch, 2021; Wang et al., 2021). Elucidating these impacts is required to appropriately interpret eNA-based shrimp pathogen detections, and adequately understand data-associated caveats (Bowers et al., 2021). In doing so, it will likely be possible to infer host population pathogen loadings and prevalence based upon eNA detections. Future applications and validation of eNA in shrimp aquaculture biosecurity should leverage the significant body of work developing in broader fields including marine and terrestrial ecology and biosecurity (e.g. Bass et al., 2023; Bowers et al., 2021; Sieber et al., 2024, 2022; Thomsen and Willerslev, 2015), and human health (e.g. Farrell et al., 2021; Mtetwa et al., 2022; Nelson, 2022; Whitmore et al., 2023) to expedite and shape future advancements in this area.

The improved cost-efficiency of eNA-based detection, especially using passive sampling, will enable more frequent and comprehensive sampling within pathogen monitoring programs compared to traditional sampling approaches (Bowers et al., 2021; Sieber et al., 2024). The ability to more frequently collect passive eNA samples lends itself to integration with routine water quality monitoring and, when combined, could facilitate modelling and assessment of risk matrices for disease outbreak prediction across farms. Conversely, eNA methods may be developed to indicate disease resilience of production systems or stock (Bass et al., 2023). Such configurations could further leverage modern technologies including *in situ* monitoring, remote

sensing and artificial intelligence (AI) for modelling complex systems (Bass et al., 2023; Bowers et al., 2016; Yamahara et al., 2015), supporting wider ambitions towards 'precision aquaculture' (APFA, 2020; Cahill et al., 2022). In doing so, systems could achieve continuous assessment of production and pathogen metrics, significantly enhancing the capacity for early detection and the effectiveness of pro-active disease management (Farrell et al., 2021). Achieving truly insightful predictions and decision support tools from such monitoring systems will be contingent to the further development of understanding of the host, pathogen and environmental conditions that promote disease.

Effective and accurate pathogen detection systems are fundamental to disease mitigation and management (MacAulay et al., 2022; Stentiford et al., 2012). To ensure the information gained from such detection systems provides tangible value to end-users (i.e. for decision-making by farmers) a significantly improved understanding of the biological relevance of such detections is required (Lafferty et al., 2015; Stärk et al., 2019). Gaining this understanding will rely, in part, on pathogen challenge experiments (PCEs). Defined experimental hypotheses can be rigorously tested using well-constructed PCEs (Lambkin-Williams et al., 2018). However, the controlled and 'artificial' nature of PCEs imposes inherent limitations, creating a disconnect between the experiment and the highly complex conditions of aquaculture production systems (Cock et al., 2009; Noble et al., 2020b). To provide meaningful insight and translation to global shrimp production, the findings of PCEs will need to be better contextualised within a broader 'system' framework. Future studies utilising PCEs must consider the principles defined by the infectious disease triad, to ensure new research findings are applicable to real-world contexts, bridging the gap between controlled experiments and practical use. Leveraging the knowledge and resources developed in this thesis (Chapter 6; Arbon et al., 2024b), and developing upon them (e.g. as in Cox et al., 2024), will ensure progressive advancements are made in this field. Appropriate consideration and reporting of experimental metadata related to the disease triad will further facilitate comprehensive knowledge development through larger-scale comparative meta-analyses. This approach will extend the collective efforts of global research to advance our understanding and management of shrimp disease.

The complex interaction of components within aquatic production systems have posed significant challenges for the study of shrimp disease *in situ* (Munro et al., 2011; Valappil et al., 2021). However, our ability to capture and analyse this complexity is rapidly improving

with the advancement of technologies. For example, environmental analysis using eNA can facilitate simultaneous multi-pathogen monitoring, mapping and transmission tracing (Ip et al., 2024; Kawato et al., 2021; Sieber et al., 2024). Likewise, combining (meta)genomic/transcriptomic approaches in comparative studies of diseased and healthy stock will greatly facilitate our ability to profile diseased systems and the functional drivers of disease outbreak (Bass et al., 2023; Vayssier-Taussat et al., 2014). Such advancements will be particularly valuable to advance the study of diseases with syndromic aetiology underpinned by interactions of the pathobiome (see definition in Bass et al., 2019), where the ‘single pathogen - single disease paradigm’ has so far been inadequate (Bass et al., 2019; Infante-Villamil et al., 2021; Rajeev et al., 2021; Samsing and Barnes, 2024; Subasinghe et al., 2023). The rapidly increasing availability of genetic sequencing data also facilitates broader studies of viral phylodynamics (Grenfell et al., 2004). Viral phylodynamics uses phylogenetics to trace the genetic variability and evolutionary history of a pathogen within host populations across different spatial and temporal scales (Grenfell et al., 2004; Volz et al., 2009). In doing so, phylogenetic patterns can be used to infer key properties of viral disease epidemiology including transmission dynamics (Thompson et al., 2024; Walas et al., 2023), co-infection and reassortment dynamics (Lebarbenchon et al., 2012; Sun et al., 2022), emergence of lineages/ strains (Gupta et al., 2023), and identification of infectivity and virulence factors (Condon et al., 2022; Sun et al., 2022; Yamaguchi et al., 2019). The enhanced understanding phylodynamics can provide is critical for the design of surveillance systems and diagnostic tools, the development and maintenance of effective disease control measures, and for the ongoing evaluation of their efficacy (Attwood et al., 2022; Stärk et al., 2019).

While the tenet ‘prevention is better than cure’ has strong relevance to the value of effective pathogen detection for disease management in shrimp aquaculture, disease management is a broad multifaceted effort. Pathogen detection is a central pillar to disease management but must be part of a broader and comprehensive management plan. Effective disease management requires the coordinated efforts of industry, government, research, and the public (FAO, 2023; Peeler and Ernst, 2019; Subasinghe et al., 2023). Proactive and clear governance to ensure effective regulation and compliance with strong biosecurity standards will be critical to safeguarding Australian production, trade and environments from exotic incursions. Similarly, ongoing stakeholder engagement will remain essential to ensure the continuation and development of animal husbandry practices, strict biosecurity and sanitation, domestication

and selective breeding programs, and the adoption of effective novel technologies. Collaboration between researchers, industry, and government agencies will ensure that the latest advancements and insights are integrated into practical, real-world applications to safeguard the health and productivity of Australian shrimp aquaculture.

7.3 Conclusion

The research outlined in this PhD thesis has systematically advanced pathogen detection, and developed critical resources, for furthering our understanding of the complex interactions between host, pathogen(s), and environment within shrimp aquaculture. These developments lay a robust foundation for improved disease management strategies. Through the largest assessment of commercially important pathogens within Australia's wild sources of broodstock, the industry is now supported to reduce pathogen exposure via this pathway, using data-driven collection strategy. Furthermore, the evidence of tissue-specific viral distribution patterns developed within this thesis will improve detection reliability and interpretation of results, enhancing the value of pathogen screening in industry, and the rigour of related research. The viral tissue tropism analysis undertaken here highlights the necessity for active and ongoing review of detection methodologies and the standards which describe their optimised and appropriate application. Critically, a novel environmental sampling methodology was developed and validated. This tool will facilitate significant advancements towards implementation of large-scale surveillance programs across the industry through drastically improved cost-efficiency, user-friendliness, and scope of application. To support the translation of pathogen detection into biologically relevant and practical disease management approaches, further understanding of the impacts of pathogens, drivers of disease outbreak and the interactions described by the disease triad is needed. The development of this understanding will be facilitated by robust, 'system-based' challenge experiments, guided by the resources developed here. The breadth and depth of the studies presented in this thesis collectively provide the foundational knowledge, methodologies, and insights to make advancements across multiple aspects of shrimp pathogen detection and disease management. These advancements will improve the productivity and sustainability of Australian shrimp aquaculture, and shrimp aquaculture globally.

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APPENDICES

Appendix 1. Work published as part of this thesis

- Arbon, P.M., Condon, K., Andrade Martinez, M., Jerry, D.R., 2022. Molecular detection of six viral pathogens from Australian wild sourced giant black tiger shrimp (*Penaeus monodon*) broodstock. *Aquaculture* 548, 737651.
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- Arbon, P.M., Andrade Martinez, M., Jerry, D.R., Condon, K., 2024b. Towards a ‘systems’ approach for viral challenge experiments in shrimp: Reporting guidelines for publication. *Reviews in Aquaculture*. 16, 923–941.
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Appendix 2. Ancillary publications to this thesis. Additional publications generated in part by Phoebe Arbon during her PhD candidature which are relevant to the principal concepts presented in the thesis, but do not directly form part of it.

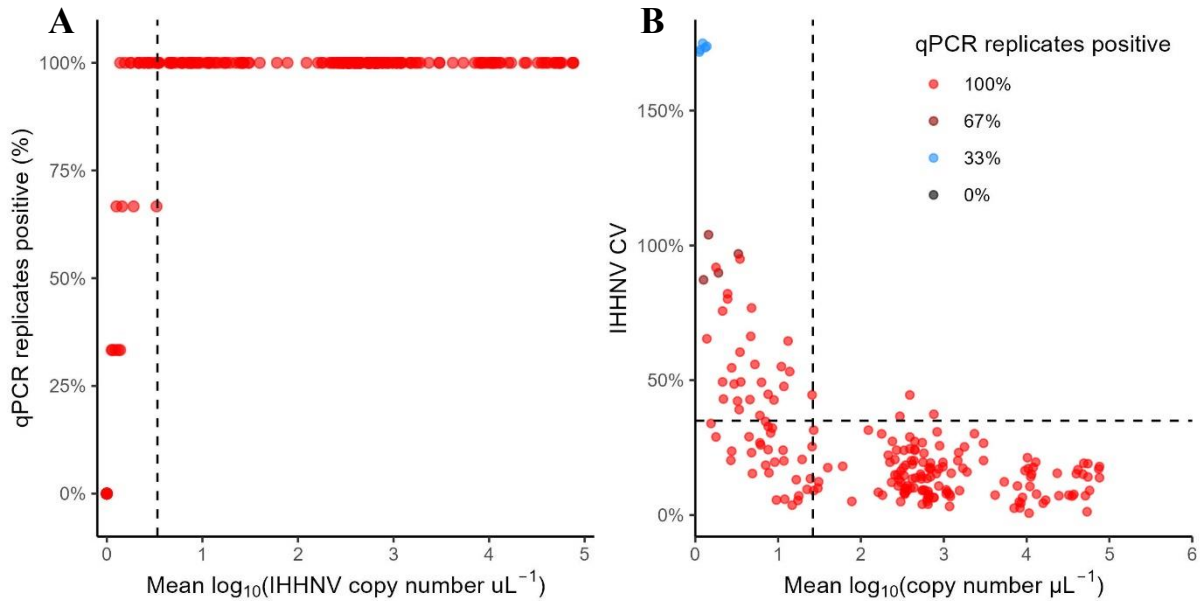
Condon, K., **Arbon, P.**, Huerlimann, R., Ariel, E., Jerry, D., 2020. Biosecurity in Northern Australian prawn aquaculture: Project Report A.1.1718113.

<https://crcna.com.au/resources/publications/biosecurity-northern-australian-prawn-aquaculture>

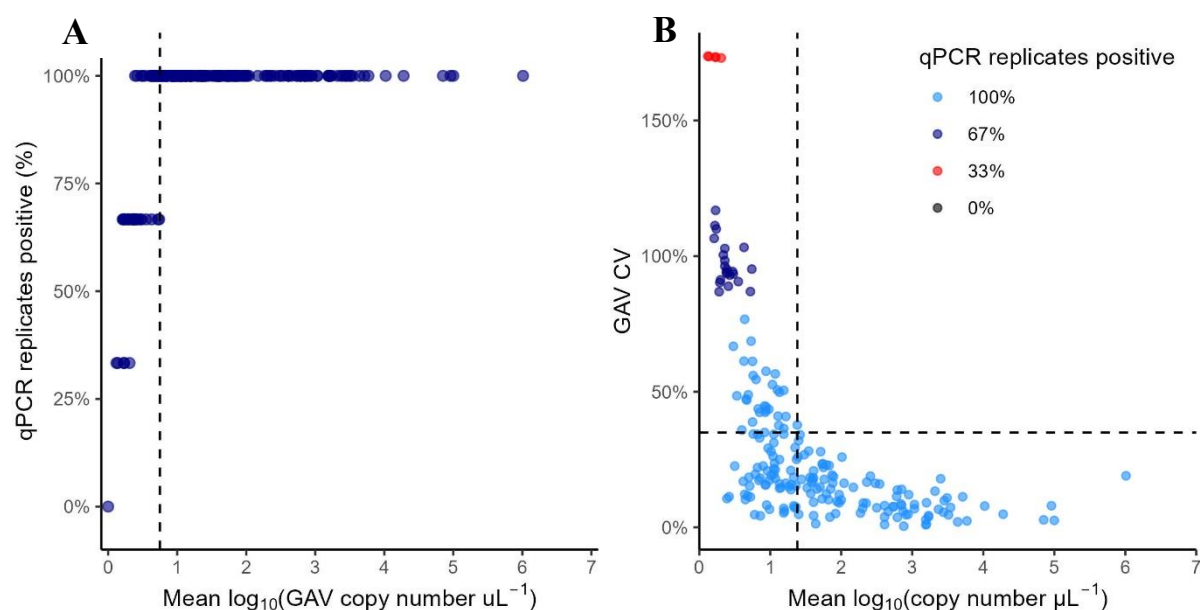
Condon, K., Huerlimann, R., Charles, T., Lowrey, R., **Arbon, P.**, Jerry, D.R., 2022. Using bacterial whole genome sequencing to identify toxin genes associated with disease outbreaks in black tiger shrimp (*Penaeus monodon*) aquaculture production.

Aquaculture 546, 737255. <https://doi.org/10.1016/j.aquaculture.2021.737255>

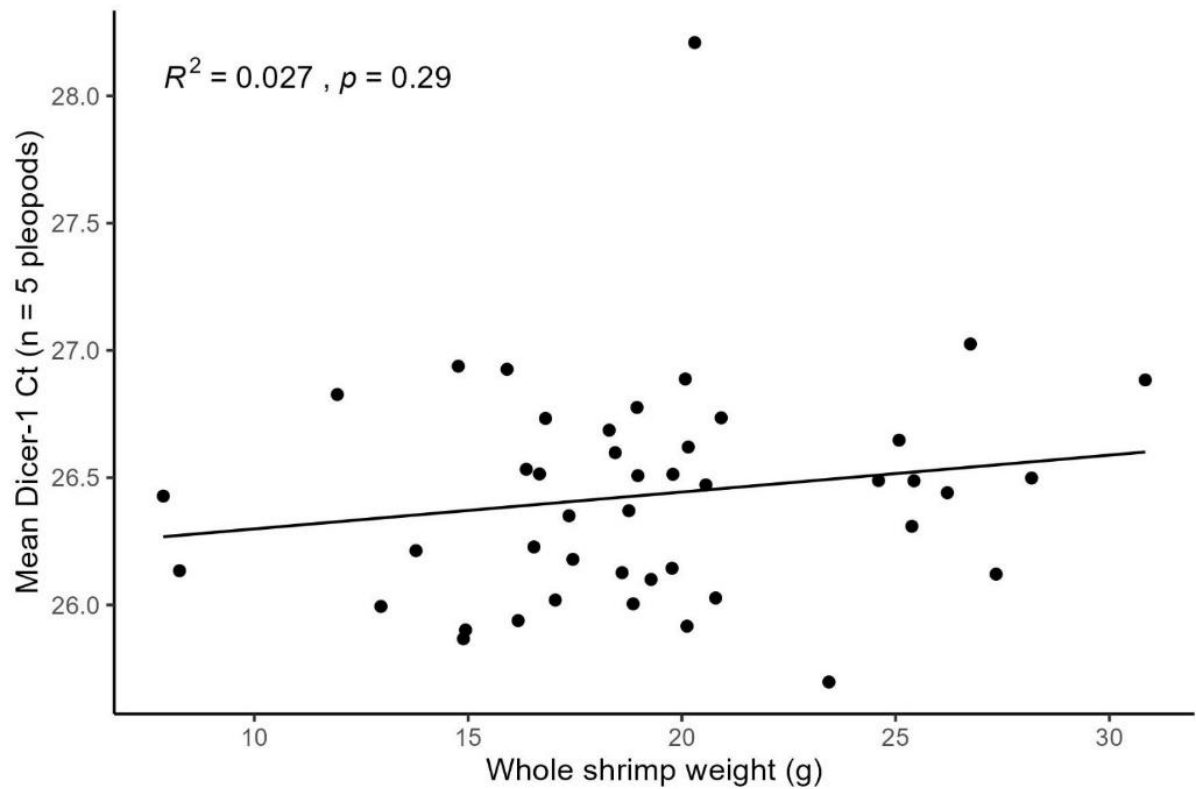
Chapter 4 Appendices



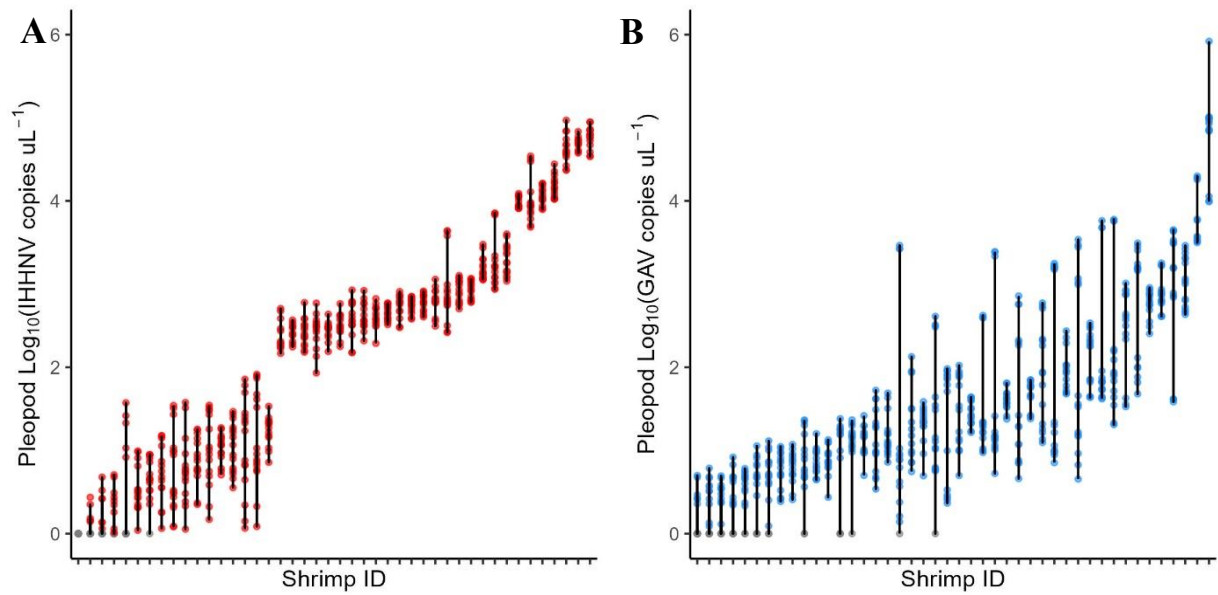
Appendix 3. (A) Proportion qPCR tests that returned IHNV detection > 0 copies μL^{-1} from triplicate TNA extract aliquots of pleopod tissue sampled from naturally infected *Penaeus monodon*. The estimated limit of detection, specified as the lowest amount of analyte in a sample that was detected at a positive call rate of above 95%, is shown as a dashed line ($10^{0.52}$; ~ 4 copies μL^{-1}). (B) Precision of IHNV qPCR detection from triplicate TNA aliquots extracted from single pleopod samples. Precision of replicate tests is represented by the coefficient of variation (IHNV CV) of untransformed IHNV copy number μL^{-1} detected from triplicate qPCR tests for each pleopod TNA extract and is compared to the mean log₁₀ transformed IHNV copy number μL^{-1} detected across all triplicate qPCR tests of each single pleopod TNA extract. The estimated limit of quantification, specified as the lowest amount of analyte in a sample where replicate qPCR tests consistently yielded a $\text{CV} \leq 35\%$ (horizontal dashed line) (Forootan et al., 2017), is shown as a vertical dashed line ($10^{1.41}$; ~ 26 copies μL^{-1}). Here the estimate for LoQ disregards three spurious pleopod detections at copy numbers beyond the CV threshold, and instead considers the overall trend of the data.



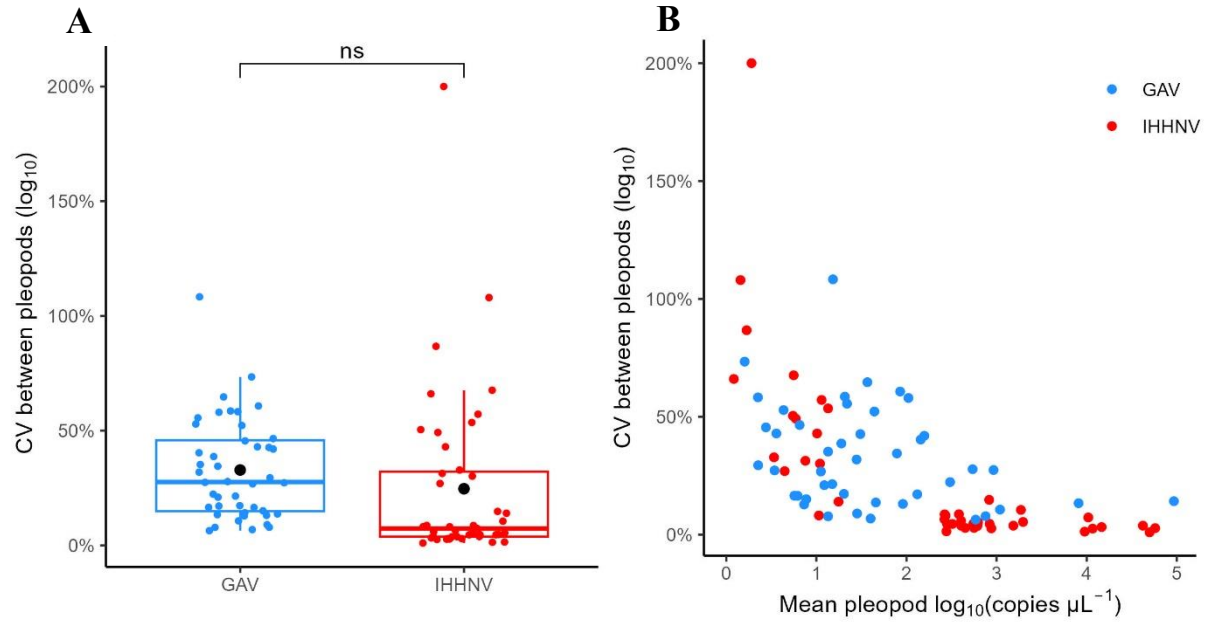
Appendix 4. (A) Proportion qPCR tests that returned GAV detection > 0 copies μL^{-1} from triplicate TNA extract aliquots of pleopod tissue sampled from naturally infected *Penaeus monodon*. The estimated limit of detection 'LoD', specified as the lowest amount of analyte in a sample that can be detected at a positive call rate of above 95%, is shown as a dashed line ($10^{0.75}$; ~ 6 copies μL^{-1}). (B) Precision of GAV qPCR detection from triplicate TNA aliquots extracted from single pleopod samples. Precision of replicate tests is represented by the coefficient of variation (GAV CV) of untransformed GAV copy number μL^{-1} detected from triplicate qPCR tests for each pleopod TNA extract and is compared to the mean log₁₀ transformed GAV copy number μL^{-1} detected across all triplicate qPCR tests of each single pleopod TNA extract. The estimated limit of quantification 'LoQ', specified as the lowest amount of analyte in a sample where replicate qPCR tests yield a $CV \leq 35\%$ (horizontal dashed line) (Forootan et al., 2017), is shown as a vertical dashed line ($10^{1.38}$; ~ 24 copies μL^{-1}).



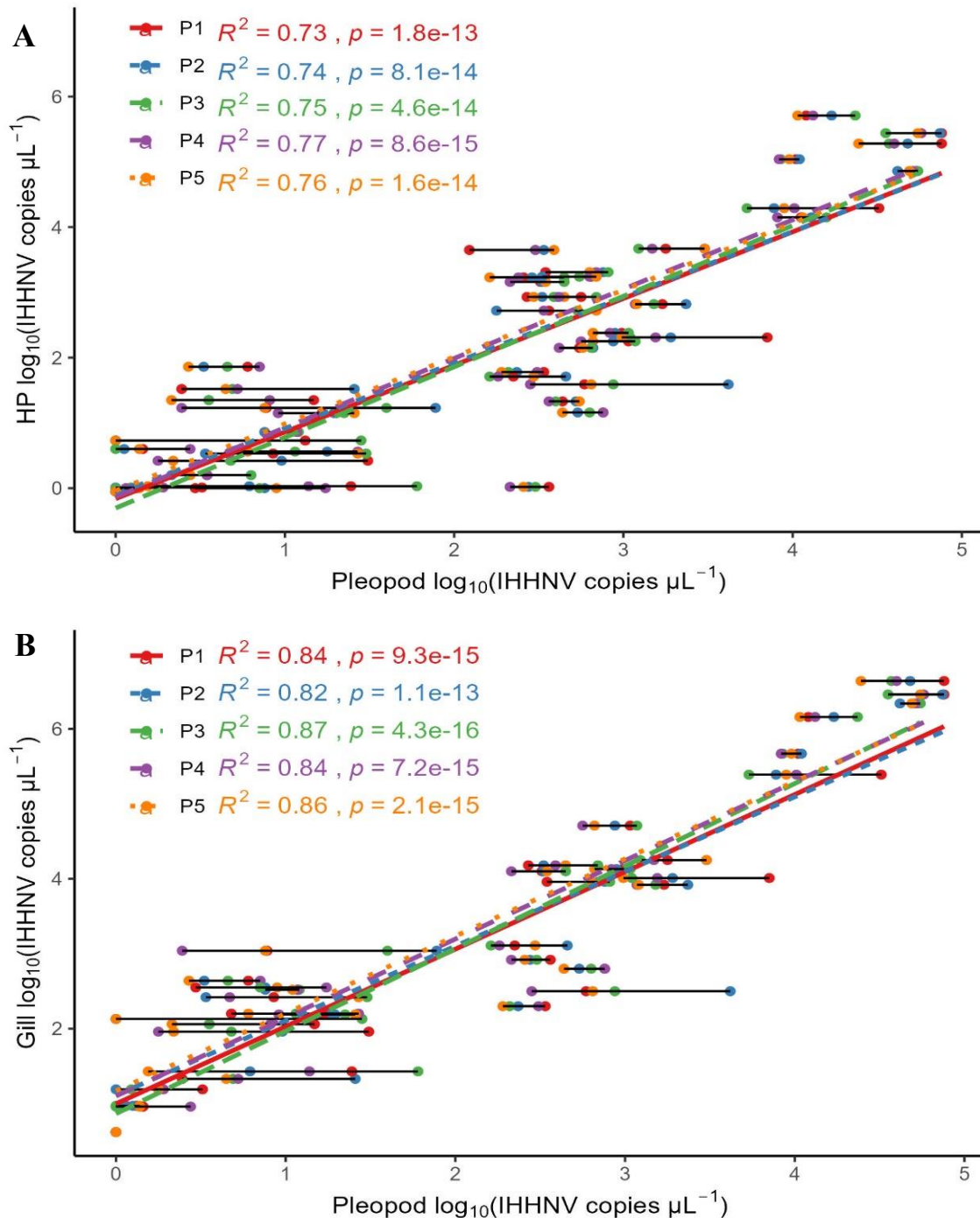
Appendix 5. Correlation between whole shrimp weight (g) and mean Dicer-1 cycle threshold (ct) value detected from pleopods ($n = 5$) sampled from individual *Penaeus monodon* shrimp ($n = 44$). The linear coefficient of determination (R^2) was determined using Pearson's correlation analysis.



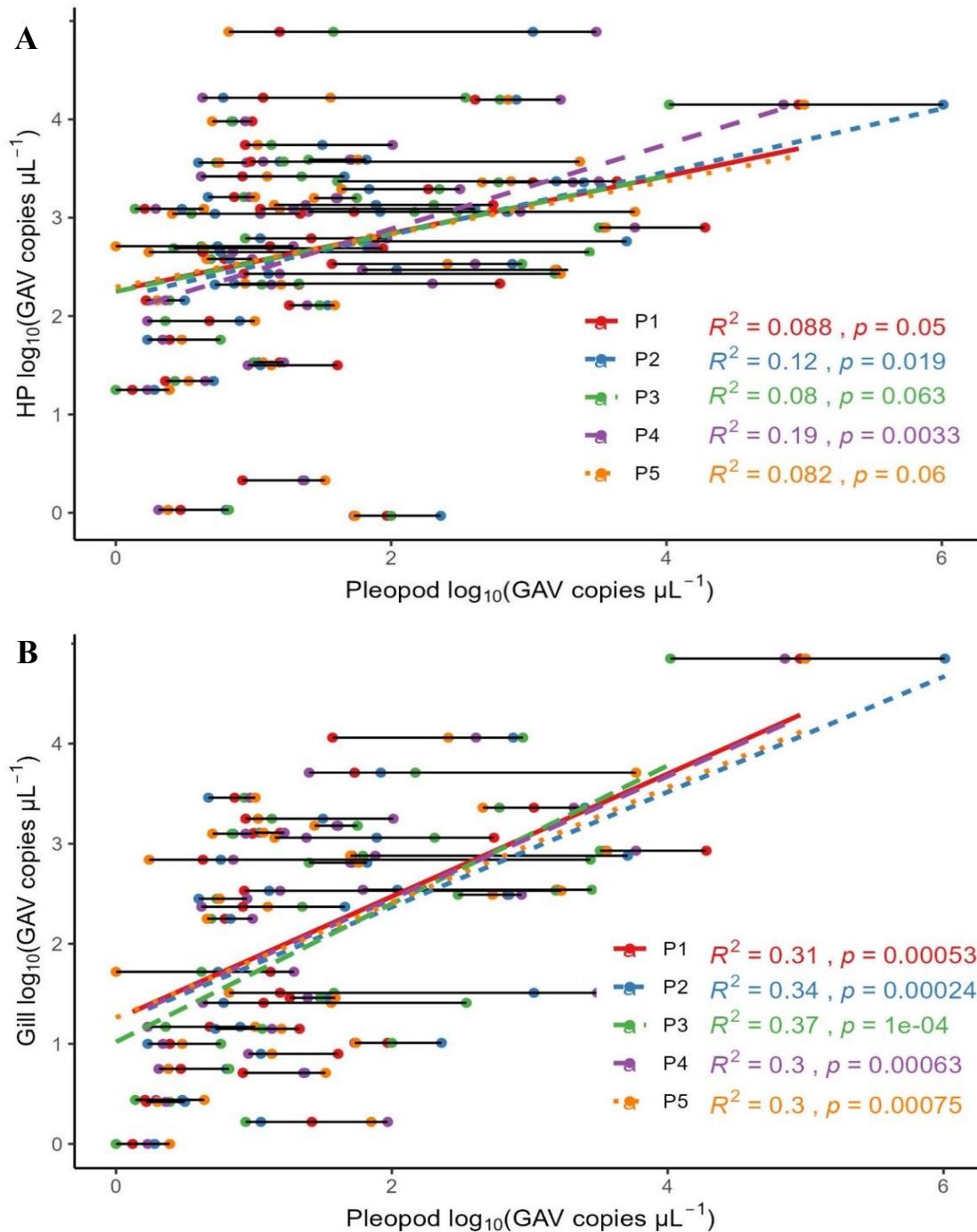
Appendix 6. Detected IHHNV (A) and GAV (B) log₁₀ copy number μL^{-1} from pleopod tissue of naturally infected *Penaeus monodon*. Individual points represent qPCR replicate detections from 5 pleopods sampled from each individual shrimp. Detection results for each individual shrimp are connected by a vertical black line. Shrimp IDs are sorted independently for IHHNV (A) and GAV (B) by ascending mean shrimp loading to facilitate the observation of variation in detection among pleopods of the same shrimp across the viral load range.



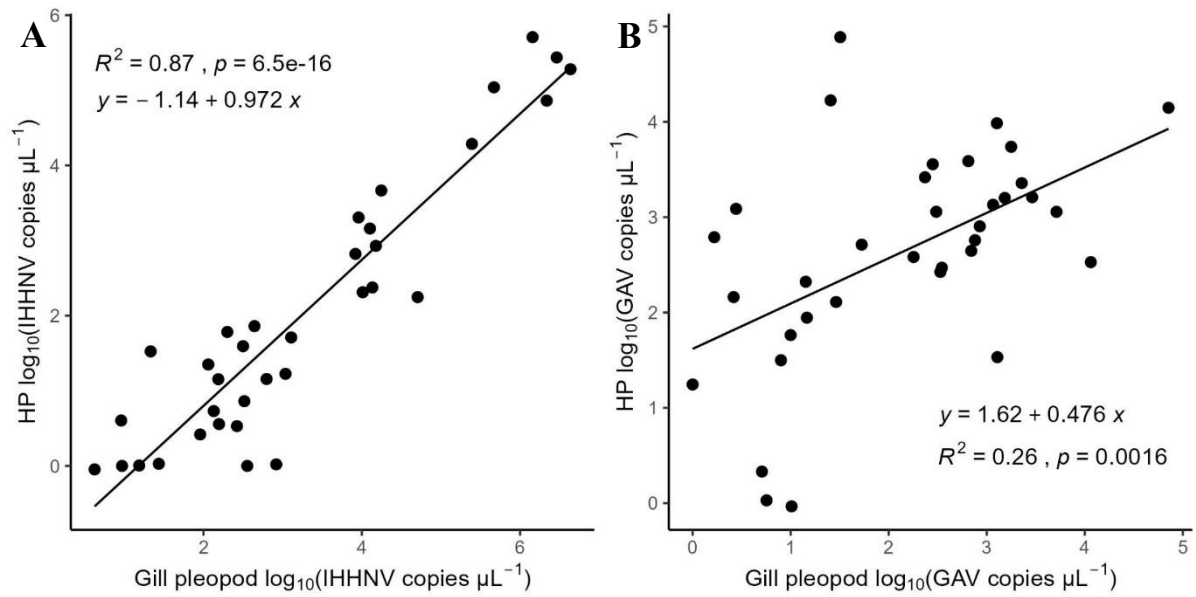
Appendix 7. Replication of Figure 4.6 using CV calculated from $\log_{10}$ transformed copy number μL^{-1} TNA extract ($CV_{\log_{10}}$). (A) Overall differences in $CV_{\log_{10}}$ for GAV (blue) and IHNV (red) detection and their significance as determined by a student's t -test ($\alpha = 0.05$). (B) $CV_{\log_{10}}$ of GAV (blue) and IHNV (red) detection as they relate to the $\log_{10}$ transformed mean detection from all 5 pleopods of an individual shrimp



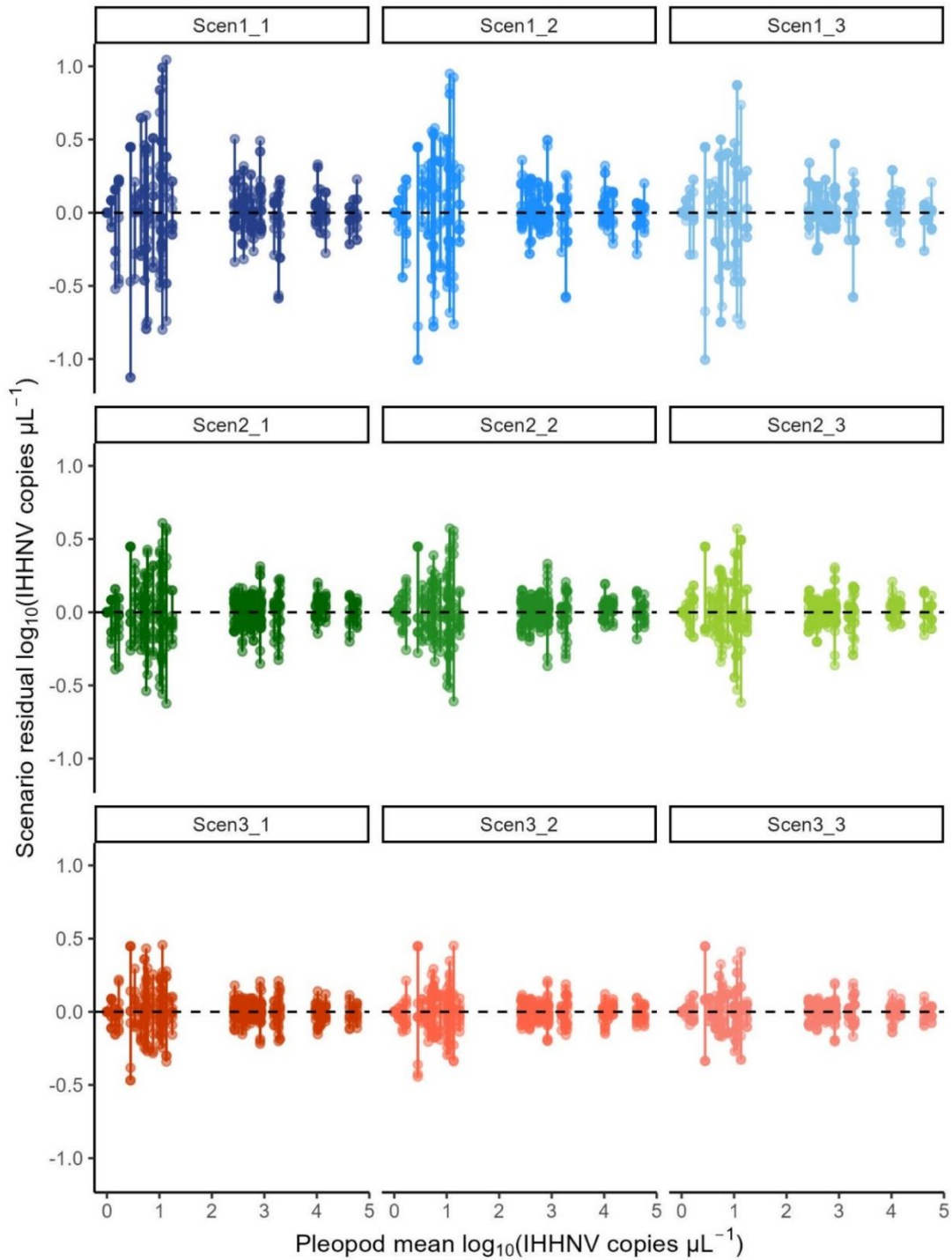
Appendix 8. Correlation of $\log_{10}$ IHHNV copy number μL^{-1} detected from a single pleopod and a (A) hepatopancreas (HP) or (B) gill tissue sample from individual *Penaeus monodon* shrimp. For each shrimp, five pleopods (P1-P5) were sampled and analysed. Each plot displays the correlation between (A) HP ($n = 44$) or (B) gill ($n = 35$) with detection from a single pleopod randomly selected from the five available (e.g., P1) ($N = 44$ out of 220). Analysis was repeated using random sampling of one of the five pleopods, without replacement, until correlation had been calculated for all five pleopods. Pleopods belonging to the same individual shrimp are connected by a horizontal black line. The linear coefficient of determination (R^2) is described for Pearson's correlation analysis conducted with each set of randomly selected pleopods (P1-P5).



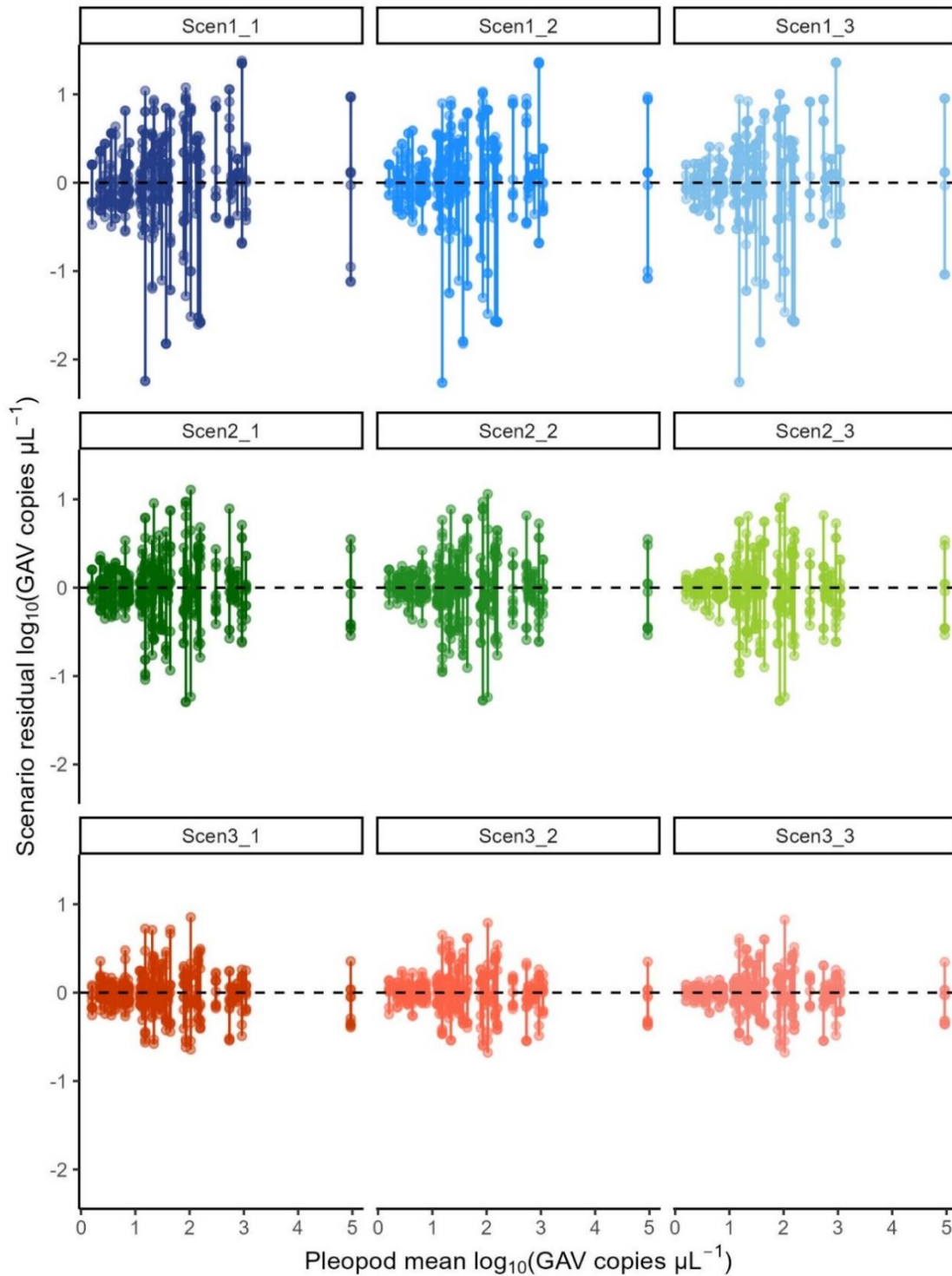
Appendix 9. Correlation of $\log_{10}$ GAV copy number μL^{-1} detected from a single pleopod and a (A) hepatopancreas (HP) or (B) gill tissue sample from individual *Penaeus monodon* shrimp. For each shrimp, five pleopods (P1-P5) were sampled and analysed. Each plot displays the correlation between (A) HP ($n = 44$) or (B) gill ($n = 35$) with detection from a single pleopod randomly selected from the five available (e.g., P1) ($N = 44$ out of 220). Analysis was repeated using random sampling of one of the five pleopods, without replacement, until correlation had been calculated for all five pleopods. Pleopods belonging to the same individual shrimp are connected by a horizontal black line. The linear coefficient of determination (R^2) is described for Pearson's correlation analysis conducted with each set of randomly selected pleopods (P1-P5).



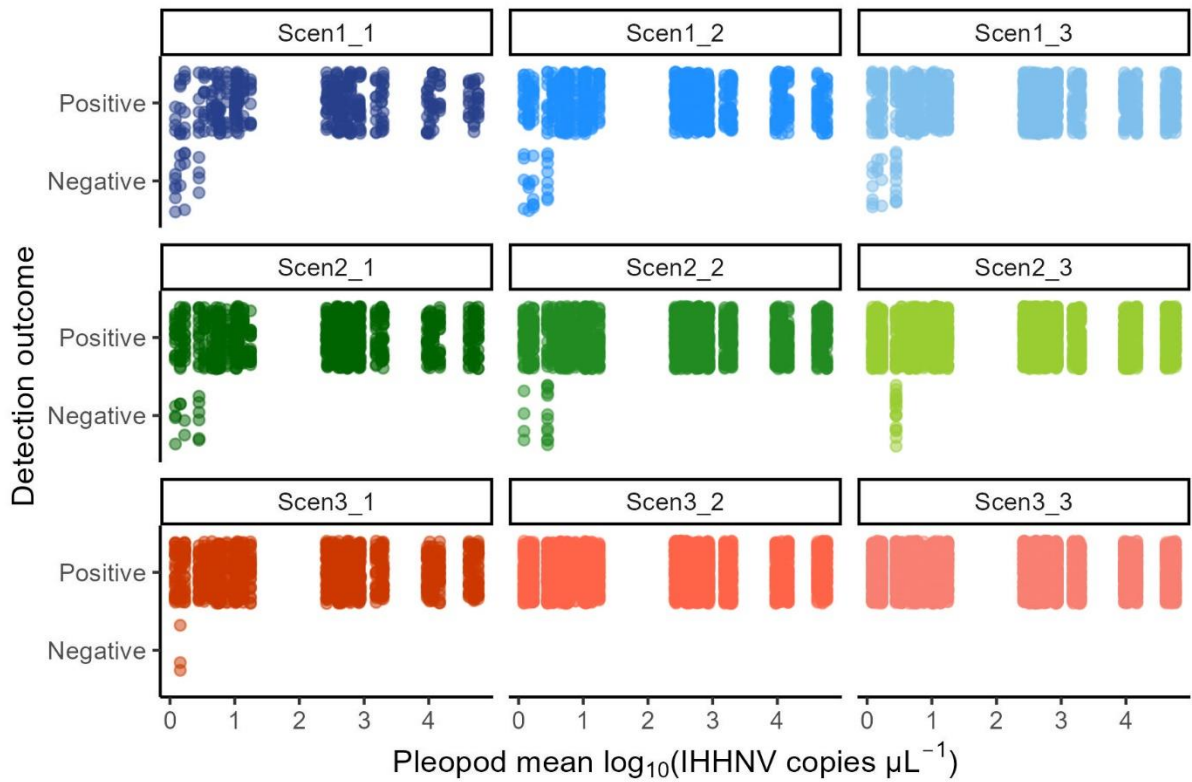
Appendix 10. (A) Correlation of IHHNV detection between gill and hepatopancreas samples from individual *Penaeus monodon* shrimp ($n = 35$). (B) Correlation of GAV detection between gill and hepatopancreas samples from individual *Penaeus monodon* shrimp ($n = 35$). The linear coefficients of determination (R^2) were determined using Pearson's correlation analysis.



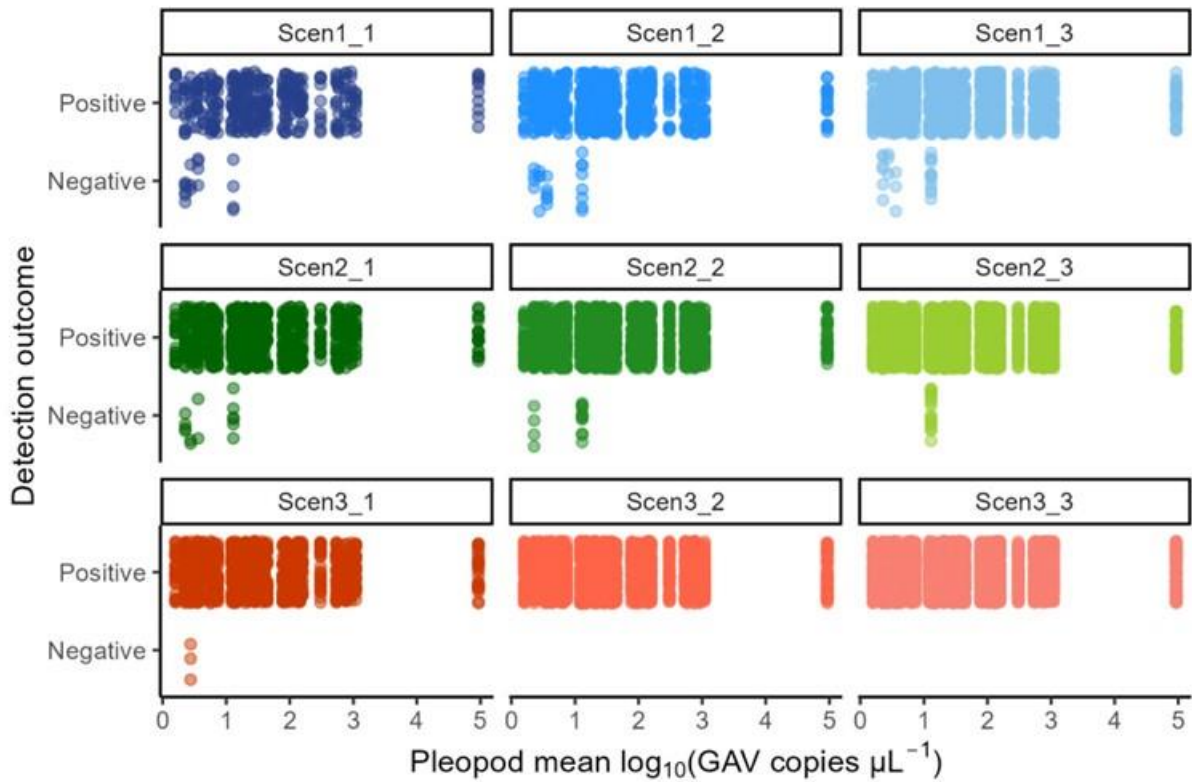
Appendix 11. Residuals between the 'indicative' averaged IHHNV detection [$\log_{10}(\text{IHHNV copies } \mu\text{L}^{-1} \text{ TNA extract})$] from pleopods randomly selected within simulation sampling scenarios and the 'ground truth' average IHHNV detection across all sampled pleopods ($n = 5$) for each individual *Penaeus monodon* shrimp. (ScenX_Y = sampling scenario X, where X is the number of pleopods 'sampled' for individual TNA extraction from each individual shrimp and, Y is the number of qPCR replicates run for each TNA extract, i.e. sampled pleopods).



Appendix 12. Residuals between the ‘indicative’ averaged GAV detection [$\log_{10}(\text{IHHNV copies } \mu\text{L}^{-1} \text{ TNA extract})$] from pleopods randomly selected within simulation sampling scenarios and the ‘ground truth’ average GAV detection across all sampled pleopods ($n = 5$) for each individual *Penaeus monodon* shrimp. (ScenX_Y = sampling scenario X, where X is the number of pleopods ‘sampled’ for individual TNA extraction from each individual shrimp and, Y is the number of qPCR replicates run for each TNA extract, i.e. sampled pleopods).



*Appendix 13. Detection outcomes of individual shrimp from simulated scenario sampling of pleopods with respect to mean pleopod detection of IHHNV. Where all *Penaeus monodon* shrimp ($n = 40$) within the experimental cohort were known to be positive for the detection of IHHNV. Individual shrimp were considered positive if any of the test replicates from any of the sampled pleopods within a sampling scenario had a positive IHHNV qPCR result. (ScenX_Y = sampling scenario X, where X is the number of pleopods ‘sampled’ for individual TNA extraction from each individual shrimp and, Y is the number of qPCR replicates run for each TNA extract, i.e. sampled pleopods).*



*Appendix 14. Detection outcomes of individual shrimp from simulated scenario sampling of pleopods with respect to mean pleopod detection of GAV. Where all *Penaeus monodon* shrimp ($n = 41$) within the experimental cohort were known to be positive for the detection of GAV. Individual shrimp were considered positive if any of the test replicates from any of the sampled pleopods within a sampling scenario had a positive GAV qPCR result. (ScenX_Y = sampling scenario X, where X is the number of pleopods 'sampled' for individual TNA extraction from each individual shrimp and, Y is the number of qPCR replicates run for each TNA extract, i.e. sampled pleopods).*

Appendix 15. Tissue specific (RT-)qPCR detection of IHHNV and GAV from naturally infected *Penaeus monodon*. Detection values in estimated copies μL^{-1} TNA extract are averages across triplicate qPCR technical replicates. Tissues include excised 5 mm pleopod tips ($n = 5$ per shrimp, 44 shrimp), 100 mg of the left hepatopancreas lobe ($n = 44$ shrimp) and 100 mg of gill tissue ($n = 35$ shrimp).

Shrimp ID	Tissue	Average IHHNV copies μL^{-1}	SD IHHNV copies μL^{-1}	Average GAV copies μL^{-1}	SD GAV copies μL^{-1}
1	Gill	22.73	8.82	179.62	12.02
1	Hepatopancreas	33.89	6.19	382.49	12.68
1	Pleopod 1	3.07	2.52	6.23	1.22
1	Pleopod 2	28.15	12.54	7.14	3.12
1	Pleopod 3	4.94	0.76	5.32	2.6
1	Pleopod 4	6.05	3.38	9.85	1.71
1	Pleopod 5	4.58	1.33	5.01	2.37
2	Gill	115.01	10.36	1775.93	79.56
2	Hepatopancreas	23	6.79	5508.94	796.37
2	Pleopod 1	14.73	0.54	8.71	1.37
2	Pleopod 2	3.58	0.01	31.71	0.42
2	Pleopod 3	3.93	1.94	14.74	7.34
2	Pleopod 4	8.44	2.57	103.53	26.82
2	Pleopod 5	2.59	1.96	11.93	6.28
3	Gill	28.13	8.28	15.04	4.4
3	Hepatopancreas	0.4	0.7	88.23	1.15
3	Pleopod 1	24.98	3.37	4.81	0.56
3	Pleopod 2	6.27	1.63	8	1.81
3	Pleopod 3	60.81	11	2.43	2.39
3	Pleopod 4	15.64	8.32	1.72	2.01
3	Pleopod 5	1.59	0.54	10.43	2.92
4	Gill	91.27	13.13	350.32	40.93
4	Hepatopancreas	2.88	2.62	302.74	83.91
4	Pleopod 1	31.39	3.87	1591.92	144.48
4	Pleopod 2	9.49	0.53	110	17.91
4	Pleopod 3	5.69	4.37	2844.83	280.72
4	Pleopod 4	1.83	0.53	63.43	14.58
4	Pleopod 5	2.37	1.02	1536.75	14.32
5	Gill	-	-	-	-
5	Hepatopancreas	1.67	0.56	22.17	3.59
5	Pleopod 1	3.84	2.32	2.5	2.57
5	Pleopod 2	2.39	1.18	5.18	0.79
5	Pleopod 3	7.05	3.47	2.98	2.77
5	Pleopod 4	4.58	4.35	4.49	0.46
5	Pleopod 5	2.83	0.67	3.67	1.78
6	Gill	134.54	3.49	1286.56	122.03
6	Hepatopancreas	5.43	1.03	34.89	9.15
6	Pleopod 1	15.06	9.72	16.95	8.56
6	Pleopod 2	0	0	11.3	2.96
6	Pleopod 3	29.45	11.34	9.93	0.55
6	Pleopod 4	0	0	16.68	2.72
6	Pleopod 5	0	0	13.89	7.86

Appendix 15. Continued

Shrimp ID	Tissue	Average IHHNV copies μL^{-1}	SD IHHNV copies μL^{-1}	Average GAV copies μL^{-1}	SD GAV copies μL^{-1}
7	Gill	15.69	4	5.69	0.36
7	Hepatopancreas	0.35	0.6	0.41	0.71
7	Pleopod 1	3.43	1.45	3.51	3.31
7	Pleopod 2	0	0	6.97	3.8
7	Pleopod 3	0.6	1.05	6.72	1.48
7	Pleopod 4	1.77	1.59	2.93	5.07
7	Pleopod 5	2.33	2.14	2.55	2.4
8	Gill	9106.86	446.68	2.86	0.91
8	Hepatopancreas	2062.24	442.21	1222.03	28.92
8	Pleopod 1	350.32	35.25	1.53	1.63
8	Pleopod 2	799.41	298.89	3.67	2.45
8	Pleopod 3	816.55	125.35	0.87	1.51
8	Pleopod 4	693.13	118.21	1.85	1.67
8	Pleopod 5	628.37	31.51	4.43	0.54
9	Gill	13803.74	2976.81	25.91	5.3
9	Hepatopancreas	242.16	56.94	16786.84	956.16
9	Pleopod 1	976.71	174.84	11.77	2.13
9	Pleopod 2	913.39	234.69	5.98	0.28
9	Pleopod 3	1071.56	84.44	346.59	55.42
9	Pleopod 4	853.92	263.46	6.23	6.43
9	Pleopod 5	660.62	50.6	36.52	6.72
10	Gill	8313.44	471.88	5219.51	1038.09
10	Hepatopancreas	672.59	124.72	1140.83	107.31
10	Pleopod 1	1713.7	297.34	54.71	12.71
10	Pleopod 2	2445.96	737.48	83.13	4.22
10	Pleopod 3	1555.87	360.51	147.97	21.82
10	Pleopod 4	1165.13	37.37	25.65	8.2
10	Pleopod 5	1207.98	83.27	5848.03	138.87
11	Gill	-	-	-	-
11	Hepatopancreas	1783.57	445.44	215.74	6.21
11	Pleopod 1	641.61	56.41	622.37	85.74
11	Pleopod 2	331.41	48.51	7.26	0.31
11	Pleopod 3	562.51	151.02	21.73	4.01
11	Pleopod 4	633.4	85.91	198.89	13.94
11	Pleopod 5	704.29	99.95	9.43	4.19
12	Gill	17969.74	4395.91	0	0
12	Hepatopancreas	4647.81	347.44	17.89	3.95
12	Pleopod 1	1823.2	460.93	0.76	1.32
12	Pleopod 2	3110.16	828.5	1.75	1.52
12	Pleopod 3	1243.37	192.87	0	0
12	Pleopod 4	1497.94	302.33	1.58	2.74
12	Pleopod 5	3071.23	621.34	2.65	2.52

Appendix 15. Continued

Shrimp ID	Tissue	Average IHHNV copies μL^{-1}	SD IHHNV copies μL^{-1}	Average GAV copies μL^{-1}	SD GAV copies μL^{-1}
13	Gill	-	-	-	-
13	Hepatopancreas	4465.04	390.13	1101.68	51.28
13	Pleopod 1	129.01	40.62	22.01	1.6
13	Pleopod 2	342.05	26.55	5.26	0.59
13	Pleopod 3	301.9	15.01	4.47	4.05
13	Pleopod 4	304.32	37.16	11.61	3.62
13	Pleopod 5	417.58	185.93	2.79	2.48
13	Gill	-	-	-	-
14	Gill	65656.98	59588.85	380.22	195.02
14	Hepatopancreas	181.25	47.92	268.1	38.83
14	Pleopod 1	1080.39	101.02	8.57	1.34
14	Pleopod 2	885.1	151.02	13.68	5.61
14	Pleopod 3	1182.5	91.37	1544.38	43.18
14	Pleopod 4	564.36	52.44	16.15	5.89
14	Pleopod 5	662.77	115.4	1693.92	73.53
15	Hepatopancreas	530.97	119.74	3784.49	738.06
15	Pleopod 1	362.85	39.34	10.27	4.47
15	Pleopod 2	182.12	54.83	15.37	1.08
15	Pleopod 3	536.47	76.85	17.64	7.21
15	Pleopod 4	337.65	27.14	11.94	2.54
15	Pleopod 5	693.5	133.96	2351.19	156.49
16	Gill	-	-	-	-
16	Hepatopancreas	880.3	279.84	495.61	22.36
16	Pleopod 1	565.6	110.78	88.3	8.43
16	Pleopod 2	412.39	83.08	68.36	15.6
16	Pleopod 3	395.13	114.44	2.65	0.3
16	Pleopod 4	422.08	79.33	75.45	14.56
16	Pleopod 5	308.26	112.93	5.28	4.05
17	Gill	16073.49	6596.52	32.27	4.55
17	Hepatopancreas	862.63	178.51	77329.26	3967.96
17	Pleopod 1	272.1	65.48	15.59	0.83
17	Pleopod 2	334.18	28.09	1068.74	89.96
17	Pleopod 3	702.06	123.53	38.36	6.32
17	Pleopod 4	393.89	96.73	3067.42	333.13
17	Pleopod 5	455.61	111.03	6.82	2.34
18	Gill	13227.29	4462.21	2278.03	192.25
18	Hepatopancreas	1472.69	332.45	2282.53	214.64
18	Pleopod 1	326.17	57.25	1083.6	74.26
18	Pleopod 2	335.84	80.62	2523.15	452.36
18	Pleopod 3	458.74	110.06	609.42	69.58
18	Pleopod 4	214.91	47.71	2079.43	277.26
18	Pleopod 5	347	64.31	460.35	26.87

Appendix 15. Continued

Shrimp ID	Tissue	Average IHHNV copies μL^{-1}	SD IHHNV copies μL^{-1}	Average GAV copies μL^{-1}	SD GAV copies μL^{-1}
19	Gill	-	-	-	-
19	Hepatopancreas	1855.58	796.88	1227.14	47.81
19	Pleopod 1	258.15	38.47	11.36	2.46
19	Pleopod 2	288.95	37.95	403.59	15.53
19	Pleopod 3	456.18	124.46	20	3.13
19	Pleopod 4	244.47	66.76	19.77	2.75
19	Pleopod 5	163.14	13.76	16.38	5.63
20	Gill	-	-	-	-
20	Hepatopancreas	22.14	6.29	15828.33	671.2
20	Pleopod 1	443.46	89.4	411.38	4.32
20	Pleopod 2	524.01	79.52	822.44	38.24
20	Pleopod 3	399.34	39.98	612.95	22.44
20	Pleopod 4	364.67	33.51	1715.29	66.51
20	Pleopod 5	552.19	50.13	710.92	57.39
21	Gill	626.31	21.34	14.91	5.23
21	Hepatopancreas	14.55	2.84	210.47	14.23
21	Pleopod 1	751.5	48.64	21.55	1.71
21	Pleopod 2	544.95	73.11	8.03	6.98
21	Pleopod 3	628.47	37.59	11.58	2.74
21	Pleopod 4	766.76	49.73	13.49	2.17
21	Pleopod 5	440.04	44.41	15.73	0.95
22	Gill	-	-	-	-
22	Hepatopancreas	143.01	23.94	2343.34	135.39
22	Pleopod 1	547.63	21.87	4404.55	88.66
22	Pleopod 2	661.16	44.22	1568.62	19.2
22	Pleopod 3	644.31	25.8	40.43	1.75
22	Pleopod 4	418.85	56.35	3205.39	147.44
22	Pleopod 5	579.34	132.86	734.29	43.81
23	Gill	317.32	42.7	1528.87	60.2
23	Hepatopancreas	39.27	0.85	1589.75	26.26
23	Pleopod 1	597.04	55.08	40.99	3.81
23	Pleopod 2	4181.53	309.27	41.41	7.56
23	Pleopod 3	880.51	94.71	57.04	12.59
23	Pleopod 4	282.13	30.41	40.56	6.96
23	Pleopod 5	642.48	48.14	27.63	4.89
24	Gill	10367.35	1773.81	8.02	1.19
24	Hepatopancreas	205.33	11.09	31.7	3.76
24	Pleopod 1	7008.8	178.58	41.45	8.64
24	Pleopod 2	1909.04	305.16	11.63	3.99
24	Pleopod 3	1091.94	94	13.52	1.95
24	Pleopod 4	1568.33	141.83	9.25	1.93
24	Pleopod 5	989.92	193.1	13.6	3.4

Appendix 15. Continued

Shrimp ID	Tissue	Average IHHNV copies μL^{-1}	SD IHHNV copies μL^{-1}	Average GAV copies μL^{-1}	SD GAV copies μL^{-1}
25	Gill	199.64	4.46	852.09	85.05
25	Hepatopancreas	61.13	8.84	802.01	28.13
25	Pleopod 1	338.75	33.11	18990.53	921.45
25	Pleopod 2	236.62	28.76	3458.54	254.13
25	Pleopod 3	209.48	46.9	3264.7	147.38
25	Pleopod 4	310.07	15.82	5952.57	137.03
25	Pleopod 5*	-	-	-	-
26	Gill	1298.35	262.19	53.51	8.96
26	Hepatopancreas	52.47	13.51	514.95	17.08
26	Pleopod 1	224.14	43.9	13.99	5.26
26	Pleopod 2	457.24	62.99	8.96	8.53
26	Pleopod 3	162.48	21.93	8.86	12.52
26	Pleopod 4	182.25	13.47	19.86	4.17
26	Pleopod 5*	-	-	-	-
27	Gill	846.1	212.31	658.37	133.56
27	Hepatopancreas	0.74	0.72	3872.04	189.26
27	Pleopod 1	371.11	89.59	51.5	11.18
27	Pleopod 2	280.69	41.79	66.53	6.79
27	Pleopod 3	304.14	49.44	25.38	1.22
27	Pleopod 4	228.19	103.77	50.9	8.39
27	Pleopod 5	258.69	53.23	57.95	10.26
28	Gill	9.56	2.62	2.64	0.4
28	Hepatopancreas	0	0	145.45	11.38
28	Pleopod 1	0.75	1.3	1.6	1.78
28	Pleopod 2	0.94	0.82	3.23	0.73
28	Pleopod 3	0	0	2.66	2.49
28	Pleopod 4	0.51	0.88	2.44	2.35
28	Pleopod 5	1.56	1.02	1.94	1.77
29	Gill	5.25	3.81	2905.59	101.79
29	Hepatopancreas	0.24	0.42	1630.03	243.11
29	Pleopod 1	0	0	7.38	1.34
29	Pleopod 2	0	0	5.03	2.36
29	Pleopod 3	0	0	9.05	3.84
29	Pleopod 4	0	0	9.59	2.8
29	Pleopod 5	0	0	10.27	1.97
30	Gill	10.25	5.36	10.4	3.48
30	Hepatopancreas	4.05	0.59	59.03	14.39
30	Pleopod 1	1.26	1.31	2.45	0.26
30	Pleopod 2	0.46	0.79	1.61	2.79
30	Pleopod 3	0	0	5.96	2.05
30	Pleopod 4	3.15	1.72	2.29	2.3
30	Pleopod 5	0.88	1.53	3.65	3.41

Appendix 15. Continued

Shrimp ID	Tissue	Average IHHNV copies μL^{-1}	SD IHHNV copies μL^{-1}	Average GAV copies μL^{-1}	SD GAV copies μL^{-1}
31	Gill	246496.52	11987.29	72875.23	18609.58
31	Hepatopancreas	19602.45	4047.82	14041.77	381.7
31	Pleopod 1	32220.06	2383.38	92274.36	7360.82
31	Pleopod 2	7781.74	839.08	1028257.98	195593.22
31	Pleopod 3	5340.45	657.21	10390.71	817.41
31	Pleopod 4	10343.14	2201.5	70923.53	1954.64
31	Pleopod 5	8938.47	585.04	100090.31	2566
32	Gill	4597230.25	1769400.7	759.43	14.45
32	Hepatopancreas	194252.87	44940.97	573.42	20.2
32	Pleopod 1	77325.98	13911.11	52.08	14.53
32	Pleopod 2	47880.22	7259.94	5130.6	578.38
32	Pleopod 3	37157.71	2841.58	62.59	7.7
32	Pleopod 4	39928.46	6114.39	76.96	12.61
32	Pleopod 5	24546.15	1755.59	50.33	7.47
33	Gill	1460953.17	306934.05	29.52	6.54
33	Hepatopancreas	510399.82	14794.28	129.04	6.75
33	Pleopod 1	12202.81	2156.34	18.4	2.77
33	Pleopod 2	17124.2	947.55	35.52	9.99
33	Pleopod 3	23684.22	3669.66	31.09	8.33
33	Pleopod 4	13318.97	1030.96	25.13	6.53
33	Pleopod 5	10596.6	72.66	39.61	7.15
34	Gill	2933529.34	585791.42	305.59	8.67
34	Hepatopancreas	276346.18	45177.2	1140.68	57.2
34	Pleopod 1	76648.75	10617.97	697.69	60.47
34	Pleopod 2	74963.24	12823.77	709.3	99.42
34	Pleopod 3	35918.88	2529.02	305.68	49.71
34	Pleopod 4	57897.89	5268.94	863.86	41.46
34	Pleopod 5	55908.47	7898.89	537.91	41.34
35	Gill	-	-	-	-
35	Hepatopancreas	14518.21	4171.73	2040.62	830.59
35	Pleopod 1	11375.95	1613.07	185.63	9.76
35	Pleopod 2	13050.99	2560.06	69.49	2.64
35	Pleopod 3	15820.32	693.96	222.36	19.85
35	Pleopod 4	8224.13	400.11	316.91	22.88
35	Pleopod 5	11696.68	1773.35	44.06	0.59
36	Gill	494110.1	182504.66	5.28	1.6
36	Hepatopancreas	111806.54	25290.7	2.22	2.22
36	Pleopod 1	10504.95	1809.29	9.05	4.05
36	Pleopod 2	11055.02	1173.21	24.1	6.04
36	Pleopod 3	8531.46	375.62	23.07	3.51
36	Pleopod 4	8336.53	219.53	22.83	1.72
36	Pleopod 5	9639.16	1588.23	33.62	4.91

Appendix 15. Continued

Shrimp ID	Tissue	Average IHHNV copies μL^{-1}	SD IHHNV copies μL^{-1}	Average GAV copies μL^{-1}	SD GAV copies μL^{-1}
37	Gill	2268433.55	857517.65	10.29	1.47
37	Hepatopancreas	73012.74	5635.73	0.26	0.46
37	Pleopod 1	53797.29	661.33	92.83	8.39
37	Pleopod 2	42535.48	7154.14	232.63	38.88
37	Pleopod 3	56035.78	10677.08	100.55	10.13
37	Pleopod 4	50975.54	3645.54	54.1	5.69
37	Pleopod 5	49501.43	9502.28	56.41	13.27
38	Gill	171.63	98.41	1.38	1.95
38	Hepatopancreas	4.18	3	616.35	26.79
38	Pleopod 1	4.84	1.12	27.28	9.31
38	Pleopod 2	17.65	1.25	11.29	2.21
38	Pleopod 3	11.64	2.8	9.31	4.02
38	Pleopod 4	27.06	2.45	92.86	11.23
38	Pleopod 5	6.23	1.67	72.08	10.21
39	Gill	156.1	33.78	1185.6	288.37
39	Hepatopancreas	15.76	8.86	1359.3	198.15
39	Pleopod 1	16.56	2.17	549.94	41.73
39	Pleopod 2	19.69	4.06	78.12	14.61
39	Pleopod 3	22.28	2.12	203.97	18.36
39	Pleopod 4	9.22	1.81	24.88	9.39
39	Pleopod 5	26.28	6.67	14.19	2.08
41	Gill	328.68	33.16	282.64	35.32
41	Hepatopancreas	7.28	0.46	3597.7	263.37
41	Pleopod 1	13.08	6.24	6	2.33
41	Pleopod 2	7.75	1.88	4.15	1.49
41	Pleopod 3	11.96	0.7	6.35	4.36
41	Pleopod 4	11.84	2.38	9.01	0.74
41	Pleopod 5	12.57	6.92	6.52	3.99
42	Gill	1088.46	91.99	1269.8	70.39
42	Hepatopancreas	18.6	10.01	9691.38	994.36
42	Pleopod 1	7.79	1.22	9.89	0.66
42	Pleopod 2	78.41	3.97	7.37	2.43
42	Pleopod 3	40.47	7.11	6.93	1.2
42	Pleopod 4	3.07	2.46	9.99	5.75
42	Pleopod 5	7.82	2.57	5.09	0.94
43	Gill	450.69	118.2	700.9	107.99
43	Hepatopancreas	73.16	10.88	443.72	23.48
43	Pleopod 1	6.23	2.3	5.19	3.18
43	Pleopod 2	4.17	4.04	6.7	3.75
43	Pleopod 3	4.88	2.09	2749.95	153.56
43	Pleopod 4	7.38	2.56	7.5	3.18
43	Pleopod 5	2.76	0.56	1.7	1.87

Appendix 15. Continued

Shrimp ID	Tissue	Average IHHNV copies μL^{-1}	SD IHHNV copies μL^{-1}	Average GAV copies μL^{-1}	SD GAV copies μL^{-1}
44	Gill	279.36	105.81	239.5	62.97
44	Hepatopancreas	3.52	1.26	2628.87	269.64
44	Pleopod 1	8.92	2.88	8.8	3.08
44	Pleopod 2	3.63	1.42	45.52	6.45
44	Pleopod 3	30.26	3.02	23.17	6.81
44	Pleopod 4	6.05	4.01	4.25	0.72
44	Pleopod 5	27.92	8.79	13.56	6.87
45	Gill	357.18	25.19	11519.93	185.55
45	Hepatopancreas	0	0	362.75	149.34
45	Pleopod 1	3.19	1.55	37.4	4.67
45	Pleopod 2	8.06	3.61	751.94	3.27
45	Pleopod 3	7.14	1.32	901.28	108.58
45	Pleopod 4	17.3	0.94	405.47	32.56
45	Pleopod 5	9.58	4.09	261.8	49.47

Chapter 5 Appendices

Appendix 16. Summarised water quality parameters collected from Penaeus monodon ponds within the farm-scale monitoring study. [†]Value indicative of the minimum reading from all 31 ponds at 05:00. [◊]Value indicative of the maximum reading from all 31 ponds at 13:00.

Water quality parameter	Minimum [†] - maximum [◊]	Mean ± SD
Temperature (°C)	25.1 - 31.1	28.1 ± 1.3
pH	7.1 - 8.1	7.5 ± 0.1
Dissolved oxygen (DO; ppm)	3.2 – 11.4	5.9 ± 0.6
Salinity (ppt)	-	30.5 ± 1.3

Appendix 17. Summarised target detection results from Penaeus monodon samples within the farm-scale eNA monitoring study.

Sample	Ponds GAV positive	Ponds IHHNV positive	Samples GAV positive (%)	Samples IHHNV positive (%)	Mean $\pm$ SD $\log_{10}$(GAV copies μL^{-1})	Mean $\pm$ SD $\log_{10}$(IHHNV copies μL^{-1})
Pooled pleopod tissue	31/31	31/31	381/403 (94.5%)	372/403 (92.3%)	$2.25 \times 10^2 \pm$ 8.99×10^2	$4.62 \times 10^6 \pm$ 1.09×10^7
eNA ₂₄	31/31	30/31	59/62 (95.2%)	58/62 (93.5%)	$2.20 \times 10^2 \pm$ 2.84×10^2	$1.65 \times 10^4 \pm$ 4.17×10^4

Appendix 18. Summary table of detection from submersion duration experiment. Superscript letters denote significant differences in detected target copy number between sample types, within each target.

Sample	Samples GAV positive (%)	Samples IHHNV positive (%)	Mean $\pm$ SD $\log_{10}(\text{GAV copies } \mu\text{L}^{-1})$	Mean $\pm$ SD $\log_{10}(\text{IHHNV copies } \mu\text{L}^{-1})$
Pleopod	48/50 (96%)	50/50 (100%)	$3.19 \times 10^3 \pm 1.98 \times 10^4$ ^a	$3.16 \times 10^7 \pm 5.93 \times 10^7$ ^a
Gill	49/50 (98%)	50/50 (100%)	$2.03 \times 10^2 \pm 2.55 \times 10^2$ ^{ab}	$1.82 \times 10^7 \pm 3.62 \times 10^7$ ^a
Hepatopancreas	48/50 (96%)	50/50 (100%)	$4.93 \times 10^3 \pm 1.58 \times 10^4$ ^{ab}	$8.87 \times 10^6 \pm 2.66 \times 10^7$ ^a
eNA ₍₁₋₂₄₎	50/50 (100%)	50/50 (100%)	$1.33 \times 10^2 \pm 1.99 \times 10^2$ ^b	$2.81 \times 10^2 \pm 3.54 \times 10^2$ ^b

Appendix 19. Pond level summary data of IHHNV and GAV detection from tissue and eNA samples collected from *Penaeus monodon* production ponds (N = 31) analysed in the farm-scale monitoring study.

IHHNV					GAV			
Pond	Positive samples		Mean $\pm$ SD copy number μL^{-1}		Positive samples		Mean $\pm$ SD copy number μL^{-1}	
	Pooled pleopod (n = 13)	eNA (n = 2)	Pooled pleopod	eNA	Pooled pleopod (n = 13)	eNA (n = 2)	Pooled pleopod	eNA
1	4	1	3.28 $\pm$ 5.63	1.80 $\times 10^{-1} \pm 2.60 \times 10^{-1}$	13	2	1.24 $\times 10^2 \pm 1.91 \times 10^2$	1.08 $\times 10^3 \pm 9.66 \times 10^2$
2	6	2	2.12 $\pm$ 2.88	2.32 $\pm$ 1.46	13	2	3.70 $\times 10^1 \pm 2.76 \times 10^1$	2.88 $\times 10^2 \pm 4.19 \times 10^1$
3	5	0	5.92 $\pm$ 9.80	Not detected	13	2	3.66 $\times 10^2 \pm 6.66 \times 10^2$	4.69 $\times 10^2 \pm 3.81 \times 10^2$
4	11	1	5.44 $\pm$ 6.83	4.90 $\times 10^{-1} \pm 6.90 \times 10^{-1}$	13	2	3.24 $\times 10^2 \pm 9.23 \times 10^2$	6.06 $\times 10^2 \pm 2.01 \times 10^2$
5	9	2	1.83 $\pm$ 1.57	8.60 $\times 10^{-1} \pm 4.00 \times 10^{-2}$	13	2	1.31 $\times 10^2 \pm 2.12 \times 10^2$	2.40 $\times 10^2 \pm 4.16 \times 10^1$
6	13	2	4.92 $\times 10^1 \pm 1.97 \times 10^1$	7.87 $\times 10^1 \pm 1.06$	13	2	1.51 $\times 10^2 \pm 4.30 \times 10^2$	9.49 $\times 10^1 \pm 2.36 \times 10^1$
7	13	2	5.26 $\times 10^1 \pm 2.07 \times 10^1$	6.20 $\times 10^1 \pm 1.40 \times 10^{-1}$	13	2	1.47 $\times 10^2 \pm 3.27 \times 10^2$	1.62 $\times 10^2 \pm 2.68 \times 10^1$
8	13	2	4.76 $\times 10^1 \pm 2.11 \times 10^1$	4.61 $\times 10^1 \pm 6.30$	13	2	1.08 $\times 10^2 \pm 1.85 \times 10^2$	1.91 $\times 10^2 \pm 1.05 \times 10^1$
9	13	2	7.15 $\times 10^2 \pm 1.27 \times 10^3$	2.82 $\times 10^1 \pm 4.59$	13	2	3.36 $\times 10^2 \pm 6.11 \times 10^2$	3.36 $\times 10^2 \pm 8.72 \times 10^1$
10	13	2	3.64 $\times 10^2 \pm 2.38 \times 10^2$	3.18 $\times 10^2 \pm 3.00 \times 10^1$	2	2	1.45 ± 4.83	2.80 $\times 10^2 \pm 5.10 \times 10^1$
11	13	2	1.27 $\times 10^7 \pm 1.82 \times 10^7$	1.46 $\times 10^4 \pm 1.13 \times 10^3$	13	2	2.81 $\times 10^2 \pm 4.47 \times 10^2$	7.92 $\times 10^2 \pm 3.75 \times 10^2$
12	13	2	1.69 $\times 10^5 \pm 5.90 \times 10^5$	2.96 $\times 10^2 \pm 7.74$	13	2	7.77 $\times 10^1 \pm 1.67 \times 10^2$	3.96 $\times 10^2 \pm 4.56 \times 10^1$
13	13	2	4.10 $\times 10^3 \pm 1.88 \times 10^3$	7.65 $\times 10^1 \pm 7.20$	13	2	1.42 $\times 10^2 \pm 3.96 \times 10^2$	3.27 $\times 10^2 \pm 6.11 \times 10^1$
14	13	2	1.77 $\times 10^7 \pm 1.73 \times 10^7$	9.23 $\times 10^3 \pm 8.93 \times 10^2$	13	2	4.02 $\times 10^1 \pm 3.67 \times 10^1$	3.77 $\times 10^1 \pm 6.18$
15	13	2	2.39 $\times 10^7 \pm 1.71 \times 10^7$	3.50 $\times 10^4 \pm 3.64 \times 10^4$	13	1	8.36 $\times 10^2 \pm 1.74 \times 10^3$	7.22 $\times 10^1 \pm 1.02 \times 10^2$
16	13	2	1.41 $\times 10^7 \pm 7.07 \times 10^6$	1.41 $\times 10^3 \pm 3.93 \times 10^2$	12	2	2.39 $\times 10^1 \pm 5.45 \times 10^1$	3.59 $\times 10^2 \pm 3.35$
17	13	2	3.90 $\times 10^6 \pm 7.05 \times 10^6$	3.12 $\times 10^3 \pm 2.01 \times 10^2$	13	2	7.31 $\times 10^1 \pm 9.29 \times 10^1$	8.55 $\times 10^1 \pm 3.97 \times 10^1$
18	12	2	4.55 $\times 10^5 \pm 1.09 \times 10^6$	2.21 $\times 10^3 \pm 3.48 \times 10^2$	13	1	1.20 $\times 10^2 \pm 3.41 \times 10^2$	4.43 $\times 10^1 \pm 6.27 \times 10^1$
19	13	2	7.25 $\times 10^3 \pm 8.22 \times 10^3$	6.26 $\times 10^2 \pm 1.02 \times 10^2$	13	2	1.85 $\times 10^2 \pm 4.52 \times 10^2$	9.89 $\times 10^1 \pm 6.10$

Appendix 19. Continued

IHHNV					GAV			
Pond	Positive samples		Mean $\pm$ SD copy number μL^{-1}		Positive samples		Mean $\pm$ SD copy number μL^{-1}	
	Pooled pleopod (n = 13)	eNA (n = 2)	Pooled pleopod	eNA	Pooled pleopod (n = 13)	eNA (n = 2)	Pooled pleopod	eNA
20	13	2	$2.70 \times 10^5 \pm 6.30 \times 10^5$	$2.49 \times 10^4 \pm 1.22 \times 10^4$	13	1	$3.02 \times 10^1 \pm 2.54 \times 10^1$	$3.17 \times 10^1 \pm 4.48 \times 10^1$
21	13	2	$2.23 \times 10^6 \pm 6.62 \times 10^6$	$6.57 \times 10^3 \pm 2.08 \times 10^3$	12	2	$6.67 \times 10^1 \pm 1.72 \times 10^2$	$6.21 \times 10^1 \pm 1.23 \times 10^1$
22	13	2	$1.52 \times 10^6 \pm 2.28 \times 10^6$	$2.05 \times 10^2 \pm 2.76 \times 10^1$	12	2	$1.05 \times 10^1 \pm 1.40 \times 10^1$	$1.66 \times 10^2 \pm 8.35 \times 10^1$
23	13	2	$1.87 \times 10^7 \pm 2.35 \times 10^7$	$3.22 \times 10^4 \pm 1.15 \times 10^4$	13	2	$6.98 \times 10^1 \pm 1.99 \times 10^1$	$4.46 \times 10^1 \pm 4.39$
24	13	2	$1.58 \times 10^7 \pm 1.52 \times 10^7$	$1.81 \times 10^5 \pm 1.32 \times 10^5$	12	2	$3.68 \times 10^2 \pm 1.01 \times 10^3$	$5.91 \times 10^1 \pm 2.36 \times 10^1$
25	13	2	$2.00 \times 10^6 \pm 2.90 \times 10^6$	$2.34 \times 10^3 \pm 1.07 \times 10^1$	13	2	$1.60 \times 10^3 \pm 3.92 \times 10^3$	$7.12 \times 10^1 \pm 5.74$
26	13	2	$1.34 \times 10^6 \pm 2.14 \times 10^6$	$3.60 \times 10^4 \pm 2.59 \times 10^4$	11	2	$1.93 \times 10^2 \pm 4.71 \times 10^2$	$4.59 \times 10^1 \pm 3.23 \times 10^1$
27	13	2	$6.98 \times 10^5 \pm 1.14 \times 10^6$	$6.31 \times 10^3 \pm 1.02 \times 10^3$	8	2	$2.74 \times 10^2 \pm 5.30 \times 10^2$	$2.20 \times 10^1 \pm 1.11$
28	13	2	$5.29 \times 10^5 \pm 1.56 \times 10^6$	$1.52 \times 10^4 \pm 7.25 \times 10^3$	13	2	$2.90 \times 10^2 \pm 7.38 \times 10^2$	$4.15 \times 10^1 \pm 1.64 \times 10^1$
29	13	2	$3.49 \times 10^6 \pm 7.13 \times 10^6$	$3.45 \times 10^3 \pm 1.80 \times 10^3$	13	2	$6.98 \times 10^1 \pm 5.06 \times 10^1$	$1.11 \times 10^2 \pm 9.42 \times 10^1$
30	13	2	$1.09 \times 10^7 \pm 1.31 \times 10^7$	$7.25 \times 10^4 \pm 7.06 \times 10^4$	13	2	$1.04 \times 10^2 \pm 2.45 \times 10^2$	$1.07 \times 10^2 \pm 9.99 \times 10^1$
31	13	2	$1.30 \times 10^7 \pm 1.53 \times 10^7$	$6.43 \times 10^4 \pm 6.77 \times 10^4$	13	2	$3.82 \times 10^2 \pm 8.04 \times 10^2$	$8.94 \times 10^1 \pm 2.73$

Appendix 20. IHHNV and GAV detection data from shrimp pleopod (PLEO), gill and hepatopancreas (HP) tissue, and passive eNA samples collected from *Penaeus monodon* production ponds (N = 5) analysed in the submersion duration experiment. Superscript letters indicate significant differences in a target's detection within a sample type, between ponds.

Target	Pond	Positive samples (out of 10)				Mean $\pm$ SD copy number			
		PLEO	Gill	HP	eNA	PLEO (mg ⁻¹)	Gill (mg ⁻¹)	HP (mg ⁻¹)	eNA (μ L ⁻¹)
<i>IHHNV</i>	<i>A</i>	10	10	10	10	$1.54 \times 10^3 \pm 1.39 \times 10^3$ ^a	$5.07 \times 10^2 \pm 3.89 \times 10^2$ ^a	$3.51 \times 10^2 \pm 9.39 \times 10^2$ ^a	$6.78 \times 10^1 \pm 1.40 \times 10^2$ ^a
	<i>B</i>	10	10	10	10	$7.96 \times 10^7 \pm 9.32 \times 10^7$ ^d	$5.00 \times 10^7 \pm 4.15 \times 10^7$ ^d	$2.10 \times 10^7 \pm 1.81 \times 10^7$ ^d	$6.44 \times 10^2 \pm 5.05 \times 10^2$ ^b
	<i>C</i>	10	10	10	10	$6.05 \times 10^6 \pm 1.89 \times 10^7$ ^b	$5.89 \times 10^5 \pm 1.75 \times 10^6$ ^b	$2.48 \times 10^5 \pm 7.34 \times 10^5$ ^b	$8.03 \times 10^1 \pm 7.54 \times 10^1$ ^{ab}
	<i>D</i>	10	10	10	10	$6.05 \times 10^7 \pm 6.04 \times 10^7$ ^d	$2.31 \times 10^7 \pm 3.63 \times 10^7$ ^{cd}	$2.12 \times 10^7 \pm 5.44 \times 10^7$ ^{cd}	$4.03 \times 10^2 \pm 3.79 \times 10^2$ ^b
	<i>E</i>	10	10	10	10	$1.20 \times 10^7 \pm 2.61 \times 10^7$ ^c	$1.75 \times 10^7 \pm 4.72 \times 10^7$ ^c	$1.84 \times 10^6 \pm 4.05 \times 10^6$ ^{bc}	$2.10 \times 10^2 \pm 1.70 \times 10^2$ ^b
<i>GAV</i>	<i>A</i>	8	9	9	10	$1.62 \times 10^2 \pm 2.57 \times 10^2$ ^a	$2.08 \times 10^2 \pm 4.00 \times 10^2$ ^a	$9.76 \times 10^3 \pm 2.23 \times 10^4$ ^a	$1.21 \times 10^2 \pm 8.75 \times 10^1$ ^a
	<i>B</i>	10	10	10	10	$2.93 \times 10^2 \pm 3.67 \times 10^2$ ^{ab}	$2.27 \times 10^2 \pm 3.49 \times 10^2$ ^a	$2.49 \times 10^3 \pm 4.33 \times 10^3$ ^a	$1.33 \times 10^2 \pm 1.29 \times 10^2$ ^a
	<i>C</i>	10	10	10	10	$1.96 \times 10^2 \pm 1.54 \times 10^2$ ^a	$2.06 \times 10^2 \pm 2.20 \times 10^2$ ^a	$7.85 \times 10^2 \pm 1.79 \times 10^3$ ^a	$7.93 \times 10^1 \pm 4.34 \times 10^1$ ^a
	<i>D</i>	10	10	9	10	$4.07 \times 10^2 \pm 5.21 \times 10^2$ ^{ab}	$2.21 \times 10^2 \pm 1.26 \times 10^2$ ^a	$1.12 \times 10^4 \pm 2.68 \times 10^4$ ^a	$7.57 \times 10^1 \pm 1.55 \times 10^1$ ^a
	<i>E</i>	10	10	10	10	$1.49 \times 10^4 \pm 4.41 \times 10^4$ ^b	$1.51 \times 10^2 \pm 7.03 \times 10^1$ ^a	$4.04 \times 10^2 \pm 6.78 \times 10^2$ ^a	$2.56 \times 10^2 \pm 4.31 \times 10^2$ ^a

Appendix 21. Individual pond data collected at the time of passive eNA and shrimp sampling, capturing all ponds analysed within the farm-scale surveillance study and submersion experiment study (N = 33). Pond data includes days of culture (DOC), pond water pH, temperature, dissolves oxygen (DO) and salinity.

Pond No.	DOC	pH (mean $\pm$ SD)	Temperature (mean $\pm$ SD $^{\circ}$C)	DO (mean $\pm$ SD)	Salinity (ppt)
1	128	7.37 $\pm$ 0.34	28.85 $\pm$ 0.64	5.95 $\pm$ 3.18	31.0
2	128	7.44 $\pm$ 0.32	28.75 $\pm$ 0.64	5.75 $\pm$ 3.04	31.1
3	128	7.5 $\pm$ 0.33	29 $\pm$ 0.71	6.4 $\pm$ 4.1	31.7
4	126	7.46 $\pm$ 0.3	28.85 $\pm$ 0.49	5.8 $\pm$ 2.97	31.6
5	126	7.44 $\pm$ 0.3	28.75 $\pm$ 0.78	5.6 $\pm$ 2.69	28.6
6	129	7.58 $\pm$ 0.21	26.4 $\pm$ 1.41	5.85 $\pm$ 2.62	29.8
7	129	7.61 $\pm$ 0.26	26.2 $\pm$ 1.41	6.25 $\pm$ 3.18	28.7
8	128	7.6 $\pm$ 0.22	26.25 $\pm$ 1.48	6.2 $\pm$ 2.83	28.6
9	128	7.59 $\pm$ 0.23	26.15 $\pm$ 1.34	6.15 $\pm$ 2.47	28.8
10 (A)	169	7.32 $\pm$ 0.09	26.95 $\pm$ 1.2	5.4 $\pm$ 1.98	31.3
11	126	7.68 $\pm$ 0.23	26.2 $\pm$ 1.56	6.2 $\pm$ 2.55	30.0
12	126	7.75 $\pm$ 0.4	26.3 $\pm$ 1.56	7.55 $\pm$ 4.45	29.6
13	125	7.65 $\pm$ 0.34	26.4 $\pm$ 1.56	7.6 $\pm$ 5.37	29.4
14	129	7.68 $\pm$ 0.32	28.7 $\pm$ 0.99	5.75 $\pm$ 3.61	29.8
15	129	7.69 $\pm$ 0.37	28.45 $\pm$ 1.2	6.35 $\pm$ 3.89	29.1
16 (B)	163	7.36 $\pm$ 0.38	27 $\pm$ 1.13	5.85 $\pm$ 3.46	31.7
17	128	7.46 $\pm$ 0.14	28.75 $\pm$ 1.2	5 $\pm$ 2.12	29.7
18	128	7.56 $\pm$ 0.34	28.55 $\pm$ 1.2	5.8 $\pm$ 3.25	29.5
19	128	7.59 $\pm$ 0.45	28.45 $\pm$ 1.2	6.35 $\pm$ 4.17	29.4
20	127	7.52 $\pm$ 0.23	28.4 $\pm$ 1.13	5.6 $\pm$ 2.83	30.0
21	125	7.61 $\pm$ 0.29	28.35 $\pm$ 1.06	5.75 $\pm$ 3.18	30.0
22 (C)	159	7.4 $\pm$ 0.3	26.85 $\pm$ 1.06	5.8 $\pm$ 2.83	32.1
23	125	7.89 $\pm$ 0.29	28.6 $\pm$ 1.13	6.8 $\pm$ 4.81	29.0
24	129	7.42 $\pm$ 0.3	29 $\pm$ 0.57	5.8 $\pm$ 3.39	31.4
25	129	7.41 $\pm$ 0.21	28.9 $\pm$ 0.42	5.45 $\pm$ 2.19	32.0
26	128	7.45 $\pm$ 0.21	28.95 $\pm$ 0.64	5.75 $\pm$ 2.62	32.0
27	128	7.46 $\pm$ 0.22	29.05 $\pm$ 0.64	5.6 $\pm$ 2.83	31.7
28	127	7.45 $\pm$ 0.14	29 $\pm$ 0.42	5.2 $\pm$ 2.26	31.8
29	127	7.46 $\pm$ 0.15	30.25 $\pm$ 1.06	5.4 $\pm$ 2.69	32.1
30	127	7.47 $\pm$ 0.08	30.3 $\pm$ 1.13	5.1 $\pm$ 2.4	32.3
31	125	7.39 $\pm$ 0.04	30.3 $\pm$ 1.13	4.95 $\pm$ 2.05	32.2
D	163	7.41 $\pm$ 0.33	26.9 $\pm$ 0.99	6.1 $\pm$ 3.39	32.0
E	161	7.44 $\pm$ 0.11	26.8 $\pm$ 0.99	5.75 $\pm$ 2.19	31.9

Chapter 6 Appendices

Appendix 22. Reported experimental detail in published PCE studies of viral diseases in the giant black tiger shrimp, Penaeus monodon, published between 1997 and 2023. Experimental details related to the host, environment, and pathogen components of the disease triad were identified, given their relevance to a 'systems' approach to disease management in shrimp aquaculture. This non-exhaustive list of studies was identified via a literature search of PubMed, conducted on 1 August, 2023, using the search terms 'virus' AND 'monodon'.

Excel 2007 spreadsheet, 75.9 KB available at:

https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1111%2Fraq.12877&file=raq12877-sup-0001-Supplementary_Table_1.xlsx