Management of Betanodavirus infection in Queensland giant grouper, *Epinephelus lanceolatus* (Bloch)

Juvenile *Epinephelus lanceolatus*

Image courtesy Dr Richard Knuckey

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DECLARATION OF ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the National Statement on Ethics Conduct in Research Involving Human (1999), The Joint NHMCR/AVCC Statement and Guidelines on Research Practice (1997), the James Cook University Policy on Experimentation Ethics, Standard Practices and Guidelines (2001), and the James Cook University Statement and Guidelines on Research Practice (2001). The research methodology received clearance from the James Cook University Experimentation Ethics Review (Approval numbers A2256 and A2370).

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	Performed the bioinformatics analysis of the genome
	Prepared manuscript drafts
Shaun Bochow	Assisted with sample collection and field trip
	Assisted with TNA extraction and PCR
	Assisted with cloning
	Assisted with manuscript preparation
Ellen Ariel	Manuscript preparation
Terrance Miller	Assisted with funding proposal preparation
	Manuscript revision

STATEMENT OF THE CONTRIBUTION OF OTHERS

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This manuscript is the culmination of three years of investigation intertwined with an additional year while I tried to navigate towards my place in the world. When I commenced this project, I was under no illusion that I was studying "Science". I did not consider myself a student in "Philosophy". In fact, I think I would have been at a loss to accurately define philosophy. In preparing the first page of this manuscript, as I wrote the words "For the degree of Doctor of Philosophy in Natural and Physical Sciences", I stopped to consider if I could legitimately make such a claim. (Following "PhD write up digression path # 1353", calculated as a conservative estimate of once day during the period of this study). Wikipedia defines philosophy as "the study of general and fundamental questions about existence, knowledge, values, reason, mind and language." (Does anyone go beyond Google or Wikipedia anymore? #1354). Those listed in my acknowledgments have contributed towards my scientific studies. Each has also richly contributed to lessons in existence, values, reason, mind and, at times, language. You have taught me answers to questions I had not even asked. With sincere gratitude, I thank you for your presence in my days, short or long, during this work.

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To all of the Aquaculture farmers in regional QLD that battle through the rigors of farming. Thank you for the seafood. Wishing you a time in the future with less challenge from disease. *You've got a friend in me.*

I once explained to my youngest daughter that scientists are like the detectives of nature. We spend time trying to investigate problems and looking for evidence. I now realise that the study of natural science is more like interviewing a difficult suspect. Mother Nature will always answer a question with another question. To that end, to quote another, that is older and therefore wiser than I:

"The scientist is not a person who gives the right answers, they're the one who asks the right questions"-Claude Levi-Strauss.

And so, we begin...

ABSTRACT

Australia was among the first countries to report the emergence of viral encephalopathy and retinopathy (VER), observed as mass mortality of larval fish from marine aquaculture during 1987-1990. The viral aetiology of the disease was not identified until 1997 by which time nervous necrosis virus (NNV) became one of the first aquatic pathogens considered significant by the OIE. Unfortunately, the disease emerged during a period when transboundary biosecurity controls governing the transport of live aquatic organisms was poor. Today, the almost global distribution of the virus, led to its exclusion as a notifiable disease within the modern OIE framework in 2003/2004. In Australia, VER remains a notifiable disease of finfish and is the major impediment to the expansion and development of grouper aquaculture. Gaps in bodies of knowledge that are critical to understanding the disease hinder the management of VER in grouper aquaculture in Australia. A review of the literature is discussed in Chapter 1 and formed the platform for defining the aims of this thesis. This project aimed to improve knowledge about NNV in North Queensland and develop strategies to prevent the severe economic losses NNV causes grouper aquaculture in Australia.

At the commencement of this project, there were no complete genome sequences of NNV collected from grouper in Australia. The National Centre for Biotechnology Information (NCBI) database contained only two complete sequences from VER outbreaks in Australia. Neither strain originated from Queensland, which is the region of Australia with sufficient infrastructure and environmental parameters to support a grouper aquaculture industry. Only one sequence was from a tropical species.

This project has improved the knowledge of Betanodavirus strains present in Northern Queensland. Complete mRNA sequences of NNV were collected from three naturally occurring VER outbreaks in marine aquaculture farms of barramundi *Lates calcarifer*, gold-spotted grouper *Epinephelus coioides* and giant Queensland grouper, *Epinephelus lanceolatus* (Chapter 3) herein referred to collectively as the North Queensland Australia (NQAus) NNV strains. Phylogenetic comparison of the NQAus NNV genome sequences to reference strains from the four recognised Betanodavirus species determined all three strains were members of the Redspotted grouper nervous necrosis virus species (RGNNV) (Chapter 3). With this finding, RGNNV continues to be the only Betanodavirus species known to be associated with VER in Australian fish species. Comparative analysis of the NQAus NNV strains with other strains sourced from Australian fish species indicated remarkable conservation of the RGNNV genome both temporally and geographically. The RNA 1 and RNA 2 segments of the NQAus NNV strains collected in this study retained more than 97% homology to other NNV strains collected from

tropical species in Australia and 97-98% homology to the genome of the original RGNNV strain isolated from Japan (SGwak97) in 1997. The significant genetic conservation of the RNA 2 segment across all of the Australian strains of NNV, provided confidence that a vaccine that targeted the capsid protein would be applicable across a broad geographic range in Australia. The high level of conservation of capsid protein sequence in the RGNNV species also suggested that a vaccine effective against any of the three strains studied in this project could have a potential global market. The low variance of RNA 2 temporally, indicates there is potentially low risk of viral mutation and vaccine escape over time.

A comprehensive review of the literature discussing the functional motifs of the Betanodavirus was conducted (Chapter 4). Positions of the functional motifs to Protein A and the capsid protein were mapped on schematic diagrams. Review and identification of the multiple motifs across an entire genome have not been reported previously from any strain of Betanodavirus. The motifs that have been identified as critical for viral replication and associated with virulence were retained by the NQAus NNV strains. Confirmation of the motifs ensured the strains used in this study retained the virulence factors reported in the literature and were suitable strains for future studies within a context of ensuring that any successful outcomes from this study should be translatable to industry.

Examination of the genome sequences of the NQAus NNV strains collected in this study also indicated the RT-qPCRs developed by Hick & Whittington, (2010) would theoretically be acceptable to monitor the viral genome copy number throughout this study. PCR amplicons produced from RNA 1 and RNA 2 segments of the viral extracts were cloned to produce standard control plasmids for the qR1T and qR2T RT-qPCR assays described by Hick & Whittington (2010). The RT-qPCR assays of Hick & Whittington (2010) were implemented within the laboratory to support the subsequent project activities (Chapter 5).

Two prophylactics, namely a vaccine and a dsRNA construct were prepared to target and prevent disease caused by the NNV strain obtained from a VER outbreak in farmed gold spot grouper, *Epinephelus coioides* (Ec2NQAus) (Chapter 6). The vaccine was based on expressed capsid protein produced from the recombinant insertion of the mRNA of RNA 2 of Ec2NQAus NNV into a bacterial expression system. The dsRNA targeted nucleotide (nt) region 722 to 738 of the RNA 2 segment.

An experimental exposure model for application to test the efficacy of the prophylactic measures was tested (Chapter 7). Exposure models that are representative of the natural infection route are preferred to test the efficacy of prophylactic measures. In a novel study, waterborne challenge via co-circulation with diseased fish along with co-infection with the marine leech, *Zeylanicobdella arugamensis* was tested as an infection model. During a 40-day

trial, despite habitation within a shared recirculation system containing ten fish that displayed VER following exposure to Ec2NQAus RGNNV via IM challenge, none of the juvenile groupers *E.lanceolatus* exposed by co-circulation succumbed to VER. Furthermore, the viral genome was not detected by RT-qPCR from leeches collected from any tank or from tissues collected from *E.lanceolatus* that were exposed by co-circulation. The inability to induce VER via waterborne challenge despite the addition of leech infestation, lead to the adoption of intramuscular injection of viral extract as a challenge model for subsequent studies.

The prophylactic measures, including vaccine and dsRNA constructs, were tested for efficacy to prevent VER in juvenile *E.lanceolatus* (~18 g body mass) following IM challenge with Ec2NQAus NNV viral extract (Chapter 8). In an initial trial, the dsRNA appeared to have no impact in preventing the severity of disease following challenge. The initial trial indicated that improvement in survival with vaccination was modest. The vaccinated groups of fish displayed between 43-53% cumulative morbidity compared to 88% morbidity in dsRNA exposed groups. This modest improvement of 35-45% reduction in morbidity indicated the vaccine formulation presented some potential as a preventative measure. Also, the RT-qPCRs qR1T and qR2T were applied to trace the viral copy number of RGNNV during the progression of disease following experimental challenge (Chapter 8). Both RT-qPCR assays detected viral genome before the onset of clinical signs at a cycle threshold value range of 31.8-36.8 (qR1T) and 29.9-45 (qR2T). Both RT-qPCR assays detected viral genome with a cycle threshold range of 12.9 to 19.5 (qR1T) and 11.1 to 19.0 (qR2T) during the peak period of morbidity. Fish that did not display signs of disease were positive for the detection of viral genome indicating the vaccine may improve tolerance to the infection rather than preventing infection. However robust conclusions regarding fish resistance or tolerance cannot be determined based on RT-qPCR analysis. There was relatively few vaccinated fish over 50g that succumbed to VER.

A mass spawning event with eight potential parents produced the cohort of fish used in the experimental challenge trials. Multilocus sequence analysis (MLSA) using microsatellite markers was applied to vaccinated fish to investigate the influence of parentage on survival/mortality (Chapter 9). Parentage did not coincide with improved survival or increased mortality within the studies conducted.

Assessment of vaccine efficacy on juvenile fish can be flawed if conducted before the development of essential components of the fish immune system, therefore *E.lanceolatus* larger than 50g body weight were used to reassess the efficacy of vaccination (Chapter 10). An additional vaccine based on heat killed cell culture Ec2NQAus NNV was also evaluated. The refined strategy indicated improved protection against VER in the slightly larger fish. Only 20%-

23% of the *E.lanceolatus* that were vaccinated displayed signs of VER which was a marked improvement compared to the placebo vaccinated groupers (93% morbidity). Unfortunately, an investigation into the mechanism of improved protection is beyond the scope of this study. RT-qPCR analysis detected NNV genome in all three groups of groupers challenged with viral extract. NNV genome was detected in groupers that did and did not display signs of VER. Because detection by RT-qPCR does not indicate viability of virus further conclusions regarding the protective effect of the vaccines are not proposed.

This work has filled significant gaps in understanding the management of Betanodavirus in aquaculture of grouper in Australia, namely:

- the acquisition of the complete mRNA of three strains of NNV collected from VER outbreaks in aquaculture systems in North Queensland;
- compiling the collection of works that describe the functional motifs of the Betanodavirus genome;
- the preparation of a vaccine that reduced expression of VER to 20%-23% following experimental challenge with Ec2NQAus NNV strain;
- noting there is a mechanism that relates to body weight that affects vaccine efficacy and
- extending the fit for purpose application of two RT-qPCR assays developed by Hick & Whittington (2010)to track the pathogenesis of NNV in grouper.

Legacy outcomes from this project are the continued contribution to research into the management of disease in tropical aquaculture systems. Specifically, towards FRDC project 2018:098 which is field trials to test the efficacy of expressed recombinant capsid protein vaccine to prevent VNN in the grow-out aquaculture of *Epinephelus lanceolatus.* In addition, the application of the RT-qPCRs of Hick & Whittington (2010) in this project extended the fit for purpose of those assays beyond those initially described on tissues from barramundi and Australian bass and was applied to support NATA accreditation of a laboratory, JCU AquaPATH, to ISO17025 in the field of animal health. The establishment of the AquaPATH laboratory ensures that aquaculture industries in Northern Queensland have access to quality assured, rapid, high throughput, and quantitative molecular detection assays to help manage the risk posed by pathogens of aquaculture species.

"From little things, big things grow" (Kelly and Carmody, 1991).

Table of Contents

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

aa	Amino Acid
AHNNV	Atlantic Halibut Nervous Necrosis Virus (Betanodavirus)
ATP	Adenosine Tri-phosphate
avSFV	Avirulent Semliki Forest Virus
B1	Protein B1 of Nodavirus
B2	Protein B2 of Nodavirus
BLAST	Basic local alignment search tool
bp	Base pair of nucleotides
BSL	Biosecurity Sciences Laboratory, Queensland Government
cDNA	Complementary DNA
CNS	Central nervous system
CPE	Cytopathic effect
DI-RNA	Differential interfering RNA
DGNNV	Dragon Grouper Nervous Necrosis Virus
DNA	Deoxyribonucleic acid
Dpc	Days post challenge
dph	Days post hatch
dpi	Days post infection
DSCAM	Down syndrome cell adhesion molecule
dsRNA	Double stranded RNA
EDTA	Ethylenediaminetetraacetic acid
EIPA	5-(N-ethyl-N-isopropyl) amiloride
eEF	Eukaryotic elongation factor
ER	Endoplasmic Reticulum
FAT	Fluorescent antibody test
FHV	Flock house Virus (Alphanodavirus)
GF-1	Grouper fin cell line
GGNNV	Greasy grouper nervous necrosis virus
GL-av	Grouper liver cell line
HK	Heat-Killed
HIV	Human Immunodeficiency Virus
hpi	Hours post infection

Hsp	Heat shock Protein (40 or 70 or 90 as different proteins)
ICTV	International Committee for Taxonomy of Viruses
IFN	Interferon
IM	Intramuscular, referring to site of injection into muscle
IP	Intraperitoneal, referring to site of injection into peritoneal cavity
IRF	Interferon regulatory factor
Kb	Kilobase (kbp = 1,000 base pairs)
LB	Lysogeny broth
MDA-5	Melanoma Differentiation-association protein-5
MLS	Mitochondrial Location/Localisation signal
MLSA	Multi-loci sequence analysis
MTase-GTase	Methyltransferase-guanylyl transferase
MTD	Mitochondrial Transmembrane domain
mRNA	Messenger RNA
Mx	Myxovirus resistance protein
NCBI	National Centre for Biotechnology Information
NES	Nuclear Export Signal
NF-κB	Nuclear factor-kappa light chain enhancer of activated B cells
NLS	Nuclear Location/Localisation signal
NNV	Nervous Necrosis Virus
NoV	Nodamura Virus (Alphanodavirus)
NS	Non-structural protein
nt	Nucleotide
OIE	Office Internationales des Epizootics
ORFs	Open reading frames
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
pi	Post injection/post infection
ppt	Parts per thousand
qPCR	Quantitative/real-time polymerase chain reaction
RAC	Ribosome-associating complex
rCP	Recombinant Capsid Protein
RdRp	RNA dependant RNA polymerase

RGNNV	Red-spotted grouper Nervous Necrosis Virus (Betanodavirus)
RIG	Retinoic acid inducible gene
RNA	Ribonucleic acid
RNAi	RNA interference
RT-	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SE	Spongiform Encephalopathy
SGIV	Singapore Grouper Iridovirus
siRNA	Short interfering RNA
ssDNA	Single stranded DNA
SJNNV	Striped Jack Nervous Necrosis Virus (Betanodavirus)
STAT	Signal transducer activator of transcription
SSN	Striped Snakehead (cell line)
ssRNA	Single stranded RNA
Tat	Transactivator of transcription
TCID	Tissue culture infectious dose
TLR	Toll-like receptor
TMD	Transmembrane Domain
TNT	Terminal nucleotransferase
TBSV	Tomato Bushy Stunt Virus
TOM	The outer mitochondrial membrane
TPNNV	Tiger Puffer Nervous Necrosis Virus (Betanodavirus)
TRIM	Tripartite Motif-containing protein
Tris	Trisaminomethane
UTR	Untranslated region
VER	Viral Encephalopathy and Retinopathy
V-miRNA	Viral encoded micro RNA
VNN	Viral Nervous Necrosis
VLP	Viral like particles
VRC	Viral Replication Complex
WHO	World Health Organisation
WhNV	Wuhan Nodavirus
ZLE	Zebrafish cell line

LIST OF APPENDICES

CHAPTER 1. LITERATURE REVIEW

Background

- In Cairns, North Queensland, The Company One is one of the most efficient commercial grouper hatcheries in the world, with annual production of several million fingerlings.

- Commercial production of grouper in grow-out aquaculture production in Australia is restricted by the severe economic losses caused by Viral Encephalopathy and Retinopathy (VER) syn., Viral Nervous Necrosis (VNN) disease outbreaks.

- Groupers do not have the restricted period of susceptibility and VER outbreaks have been reported in fish up to 3 years old.

- In recent years, as the industry has attempted to expand, the rapid mass mortality of grouper in grow-out pond production have occurred with sufficient frequency to threaten the economic viability of the industry in Australia.

- Vaccines that protect against virulent strains of viruses are widely adopted as an effective strategy to prevent losses due to viral diseases in many fish aquaculture systems.

- Despite reported success in experimental systems, there are no commercially available vaccines to prevent VER in Australia.

Aims of this Chapter

- Provide current state of knowledge of Betanodavirus
- Discuss aspects of host biology that have an association with VER
- Discuss options for the management of Betanodavirus infections in aquaculture
- Define the research questions and aims of this thesis

1.1 Introduction

Grouper, *Epinephelus* spp., are an important marine fish aquaculture species in many countries, particularly Asia (Rimmer and Glamuzia 2017). In 2017 the global aquaculture production of grouper was 147 379 tonnes (value 0.7 US$ Billion) (FAO, 2018). The majority of the world's aquaculture production of grouper is from China and Indonesia (Rimmer and Glamuzia 2017). However, due to their rapid growth rates and strong market value, grouper have potential to be a profitable aquaculture species in Australia (Knuckey, 2015). In Cairns, North Queensland, The Company One is one of the most efficient commercial grouper hatcheries in the world, with annual production of several million fingerlings. However, the majority of the fingerlings produced are exported into Asia for grow out aquaculture production (Knuckey pers. comm. 2019).

In recent years, as the industry has attempted to expand into grow out production, the rapid mass mortality of grouper due to Viral Encephalopathy and Retinopathy (VER) syn., Viral Nervous Necrosis (VNN) have occurred with sufficient frequency to threaten the economic viability of the industry in Australia. Commercial production of grouper in grow-out aquaculture production in Australia is restricted by the severe economic losses caused by VER disease outbreaks (Knuckey pers. comm. 2019). Management of VER in grouper hatchery systems can be achieved through the implementation of strict biosecurity protocols that prevent the entry of Betanodavirus into the culture system. The strategies impose increased costs to production but are not practical to prevent VER outbreaks in grouper grow-out farming systems such as sea cage or pond cultures.

Other management strategies must be developed for grouper grow out and pond systems. Forty years of research into VER has resulted in the development of cell culture replication systems, serological and molecular detection techniques and vaccination and novel anti-VER preventatives (Hick et al., 2010; Hick et al., 2011; Tanaka et al., 2001) . However, in Australia, there is no commercially available preventative or treatment to manage VER outbreaks. Pharmaq™ and HIPRA recently commenced commercial sale of a VNN vaccine in the Mediterranean to protect European sea bass against NNV. However, neither vaccines are approved for import into Australia and the suitability of the vaccine to prevent VER in grouper is untested.

Vaccines that protect against virulent strains of viruses are widely adopted as an effective strategy to prevent losses due to viral diseases in many fish aquaculture systems. However, despite the long history of VER in Australia, knowledge of the strains of Betanodavirus that cause VER in Northern Australia is limited. The paucity of knowledge of Betanodavirus in

Australia along with gaps in the knowledge about critical aspects of grouper biology, including antiviral immunity, creates a situation whereby evidence-based management of VER is very difficult. This review will discuss the emergence of VER; characteristics of viral taxonomy and replication; and host/environmental factors that are believed to associate with VER outbreaks. Knowledge of such aspects can be applied to develop evidence-based strategies to manage VER in grouper grow out systems in Australia.

1.2 Emergence of VER in Australia

Members of the genus *Betanodavirus* cause the disease Viral Encephalopathy and Retinopathy (VER) syn. Viral Nervous Necrosis (VNN) (OIE, 2018). The disease emerged in Australasia, Europe and North America during 1985 to 1989 and has been reported from wild and cultured freshwater and marine fish in all continents except South America and Antarctica. The National Centre for Biotechnology Information (NCBI) database contains 1200+ nucleotide accessions of Betanodavirus sourced from over 220 fish species and 30+ countries (Condon et al., 2019). Ten gene sequences of Betanodavirus have been published in NCBI from Australian fish (www.ncbi.nlm.nih.gov accessed 12.2.2019). In Queensland, VER outbreaks occur in commercial larval barramundi, *Lates calcarifer* (Bloch, 1790) and giant grouper, *Epinephelus lanceolatus* (Bloch, 1790) hatcheries. The World Organisation for Animal Health, Office International des Epizooties (OIE) delisted VER in 2004, as notifiable disease, due to the worldwide distribution failing to meet one of the defining criteria of a restricted geographical host range (OIE, 2004). Despite the de-listing, VER is notifiable in Australia and an impediment to successful culture of a number of highly susceptible fish species worldwide.

1.3 Viral Taxonomy

The Nodaviridae consists of the genera, *Betanodavirus*, which infect fish and *Alphanodavirus*, which infect insects. Members of the Nodaviridae also infect crustaceans but taxonomic divisions recognising the crustacean-infecting species have not occurred. Four species of Betanodavirus are officially recognised by the International Committee for the Taxonomy of Viruses (ICTV) namely Striped jack nervous necrosis virus (SJNNV), Barfin flounder nervous necrosis virus (BFNNV), Tiger puffer nervous necrosis virus (TPNNV) and Red spotted grouper nervous necrosis virus (RGNNV) (ICTV//www.ictvonline.org./virustaxonomy.asp). The species names represent the host species of the original viral isolate and are supported by variation in the viral genomic sequence. An additional viral strain isolated from Turbot, proposed as a new Betanodavirus species, Turbot Nervous Necrosis Virus (TNNV), displays variation in genomic

sequence from the four recognised species however formal recognition of TNNV as another species has not occurred (Johansen et al., 2004,ICTV//www.ictvonline.org./virustaxonomy.asp) The viral species were originally proposed to have strong host specificity. However, excluding TPNNV, the different genotypes can infect a variety of fish species (Thiery et al., 2004). The viral species were also originally observed to have species-specific temperature dependency (Iwamoto et al., 1999) however, variation from the original temperature distributions are known to occur and RGNNV exhibits the greatest temperature tolerance (Panzarin et al., 2016). The taxonomic distribution of the 1400+ sequences published in NCBI nucleotide database includes SJNNV (182), RGNNV (389) BFNNV (76), TPNNV (5) and other strains which have not been formerly classified into species divisions (www.ncbi.nlm.nih.gov. accessed 18.8.19).

1.4 Viral genome characteristics

Nodaviruses possess a small linear single stranded bi-segmented positive sense RNA (+ss RNA) genome contained within an approximate 25 to 35 nm un-enveloped capsid of icosahedral symmetry (Venter and Schneemann 2008). Nodaviruses replicate exclusively in the cytoplasm. Virions are stable between pH 2 to 9 and resistant to heating at 56 °C for 30 min (Frerichs et al., 2000). The Nodaviridae possess one of the smallest animal infecting viral genomes. Genomes are approximately 4.5 kb nucleotides (nt) consisting of a Segment 1 (RNA 1) of 3.1kb nt and Segment 2 (RNA 2) 1.4kb nt. Both segments possess a 5’ end methylated cap that assists in recruiting the eukaryotic translation machinery to translate viral proteins (Mori et al., 1992). Both segments lack a 3’ poly a tail but the RNAs are protected by an unknown moiety (Venter & Schneemann 2008).

The RNA 1 (3.1kb nt) contains the mRNA for the ~ 1000 amino acid (aa) Protein A encoded by nt 79 to 3027( ). A sub-genomic RNA, termed RNA3, not packaged into virions, is synthesised from the 3’ end of the RNA 1 segment. RNA 3 consists of ~387 nt and encodes the B1 (111 aa) and B2 (72 aa) proteins (Venter & Schneemann 2008; Toffolo et al., 2007). The B1 protein is translated in the same reading frame as Protein A and is encoded by the 336nt of the 3’ terminus of the RNA 1(nt 2688 to 3027). The B2 protein requires a +1-reading frame shift for translation compared to B1 and is encoded by 227 nt on the RNA 1 (nt 2753 to 2980) (Biacchesi 2011; Venter & Schneemann 2008). RNA 3 also acts as a transactivator in the replication of RNA 2. Paradoxically, the replication of RNA 2 results is the cessation of replication of RNA 3 (Venter & Schneemann 2008). RNA 2 (1.4kb nt) contains the mRNA for the capsid protein. In the Alphaviruses the capsid protein is ~ 430 aa compared to 338 aa of the Betanodaviruses (Venter & Schneemann 2008). How the nodavirus Viral RNA interacts to

infect and cause disease in vertebrates is not completely understood. Knowledge of viral replication processes will aid in developing novel anti-viral therapies to limit disease.

1.5 Phylogenetic comparison of *Betanodavirus*

Phylogenetic studies are useful to identify virulence factors and produce epidemiological models to understand viral transmission pathways. Initial phylogenetic studies of the Betanodaviruses were based on the viral capsid protein or RNA 2 and indicated strong nt and aa distinction between the species (Nishizawa et al., 1997). However, the presence of reassortments between RGNNV, SJNNV and BFNNV suggests the phylogenetic studies should consider both the RNA 1 and RNA 2 (Toffan et al., 2017 and Oliveira et al., 2009).

Phylogenetic comparisons between the Betanodavirus strains initially occurred through analysis of the nt sequence of RNA 2 or its translated capsid protein sequence. The RNA 2 of TPNNV and SJNNV regions are identical in nt length. BFNNV and RGNNV lack 6 bases at position 713 to 718 nt of the RNA 2 strand. Comparative similarity of a T2 region (nt 155 to 1030) within the RNA 2 strand between the different species was 75.8 % or more at nt and 80.9 % or more at aa level. Within T2 a highly conserved (>93 %) 134 aa region and highly variable T4 (62 %) 81 aa region was identified (Nishizawa et al., 1995). The T4 (nt 604 to 1030), T2 and base insertion or deletion at nt 713 to 718 were proposed as a site for species differentiation of the Betanodaviruses. The conclusions of that analysis supported the division of the 4 species originally defined by host species (Nishizawa et al., 1995).

Comparison of the T4 region in a phylogenetic analysis of 25 Betanodavirus isolates collected in Japan resulted in the divergence of the Japanese isolated Betanodaviruses into 4 clusters containing 95 % or greater nt sequence similarity (Nishizawa et al., 1997). The clusters were defined as TPNNV, SJNNV, BFNNV and RGNNV. The majority of NNV isolates from Japanese flounder aligned within the RGNNV species and only a single isolate (JF95Hok) aligned with the BFNNV species (Nishizawa et al., 1997). Japan remains the only country to report VER isolates from all four of the recognised Betanodavirus species. Phylogenetic analysis of the region consisting of RNA 2 nt 169 to 987 was conducted on Betanodaviruses isolated from cultured fish in Korea (Cha et al., 2007). The classification supported the 4 species previously identified and proposed an additional 5th group consisting of a single isolate from a Turbot from Norway (TNNV-Norway AJ608266) (Johansen et al., 2004).

Using deduced aa analysis encoded by the T4 region of 44 Betanodavirus isolates from various countries in Europe, Asia and the Mediterranean, a different classification nomenclature was proposed (Thiery et al., 2004). The classification consisted of 4 clusters and 5 subtypes namely Ia and Ib (RGNNV); IIa, IIb, IIc (BFNNV); III (TPNNV) and IV (SJNNV). The clustering within each

group was more related to the geographical source of the isolate than the host species (Thiery et al., 2004). Cherif et al. (2009) applied the phylogenetic clusters of Thiery et al. (2004) to investigate a number of NNV isolates in *D. labrax* and sea bream *Sparus aurata* from Tunisia. All isolates clustered within the RGNNV genotype. Four of the isolates were obtained from temperatures 15 to 19 °C, which is outside that typically observed for the RGNNV. In a novel report, nine different sequences were observed within a single farm. In contrast to previous observations, geno-grouping of the fish Betanodaviruses appeared to reflect an adaptation to a range of temperatures rather than geographic location or host specificity (Cherif et al., 2009). Recognising some discrepancy in the classification of Betanodavirus strains based on T4, some researchers report phylogenetic analysis including both RNA 1 and RNA 2 of the Betanodavirus genome (Toffolo et al., 2007). Analysis of the RNA 1 nt 121 to 1050 of SJNNV identified 25 unique RNA 1. Analysis comparing RNA 2 from nt 388 to 894 of SJNNV that contained the species-specific 6 nt insert region identified 22 unique sequences. Phylogenetic division of the Betanodaviruses was possible using either RNA 1 or RNA 2 however, the divisions strongly contrasted (Toffolo et al., 2007). Using the RNA 1 strand the SJNNV, BFNNV and RGNNV or clades IV, II and I phylogenetic groupings were statistically well-supported (Toffolo et al., 2007). A sister group relationship was proposed for the TPNNV, BFNNV and SJNNV or clades III, II and IV. Two of the Iberian isolates clustered within the SJNNV/IV when grouped by analysis of RNA 1. The same two were positioned in the RGNNV/I cluster when grouped by analysis of RNA 2. There was an absence of any evidence of recombination between different RNA 1 segments however; recombination in RNA 2 between different isolates was observed. Toffolo et al. (2007) proposed both RNA 1 and RNA 2 must be considered for phylogenetic purposes with RNA 1 possibly being a better marker to assess the origin of a single isolate. Phylogenetic relationships between VER strains detected in the Iberian Peninsula, collectively termed IBNNV, indicated the presence of re-assortment between Betanodavirus species (Olveira et al., 2009). Comparing RNA 1, all IBNNV isolates clustered within 97 % nt sequence homology to the RGNNV species (Olveira et al., 2009). Comparison of RNA 2 indicated divergence with only 1 of the IBNNV strains aligning within the RGNNV isolates and the remaining 6 displaying stronger similarity to the SJNNV species. Genomic analysis of Betanodaviruses from cultured fish species in Malaysia also considered RNA 1 and RNA 2 (Ransangan & Manin, 2012). All of the studied Betanodaviruses from Malaysia presented as nine clusters within the RGNNV species. Unlike Toffolo et al. (2007) the clustering was consistent between RNA 1 and RNA 2 analysis, which is expected in the absence of reassortment between multiple NNV species.

The limitations of the T4 region as a classification tool were not recognised until after 2004. Many of the sequences contained in the NCBI (1999 to 2004) are not complete and make retrospective phylogenetic studies with newly detected NNV strains difficult. A recent phylogenetic study was conducted comparing 189 RNA 1 Betanodavirus sequences (32 RGNNV, 154 BFNNV, 1 TPNNV and 2 SJNNV) and 73 RNA 2 Betanodavirus sequences (54 RGNNV, 8 BFNNV, 1 TPNNV and 10 SJNNV) (He & Teng, 2015). During the period the isolates were collected, the RNA 1 had a mean nt substitution rate of 3.60 per 10 000 nt per year compared to RNA 2 of 3.69. Within the RGNNV types substitution rates of 4.28 and 3.79 per 10 000 per year were calculated for the RNA 1 and 2 respectively (He & Teng, 2015). Taxonomic divisions proposed by Nishizawa were supported by the analysis with the identification of subclades within the genotypes. Using the RNA 1, the RGNNV could be divided into 3 subclades compared to 6 subclades using RNA 2 (He & Teng, 2015).

To develop more effective means to understand the epidemiology of VER a greater quantity of genomic sequence information of the isolates is required. An understanding of the contributions the genomic sequences make in the progression of disease (pathogenesis) of VER could assist in developing strategies to overcome the disease.

1.6 Viral Nervous Necrosis: The Disease

1.6.1 Emergence of VER:

VER, originally termed Whirling disease or Summer disease, emerged as a disease causing up to 88 % mortality in intensive larval fish hatcheries during 1983 to 1989 (Gallet de Saint Aurin, Raymond & Vianas, 1989). Clinical signs of VER in larval fish include loss of appetite, darkened colour, erratic and spiral swimming and hyperinflation of the swim bladder. In larval rearing systems, clinical signs are usually followed by mass mortality over 2 to 14 days (Moody & Crane, 2012).

Histopathology of VER disease involves extensive vacuolation of the brain and retinal tissues. Viral particles were reported in association with VER histopathology in larval production systems of, *Dicentrarchus labrax* in the French Caribbean Islands (Gallet de Saint Aurin et al., 1989), turbot, *Scopthalmus maximus* in Norway (Bloch et al., 1991), Japanese parrotfish, *Oplegnathus fasciatus* in Japan (Yoshikoshi & Inoue 1990), and barramundi, *Lates calcarifer* in Australia and Tahiti (Glazebrook et al., 1990) (Renault et al., 1991).

Purification and partial characterisation of viral particles from striped jack *Pseudocaranx dentex* suffering VER was reported in 1992 (Mori et al., 1992). Based on the viral characteristics VER was proposed to be caused by new member of the Nodaviridae designated

Striped Jack Nervous Necrosis Virus (SJNNV) (Mori et al., 1992). SJNNV was the first nodavirus to be purified from a vertebrate during a time when no member of the Nodaviridae had been isolated from outside Australasia (Johnson et al., 2000). Experimental infection trials confirmed SJNNV as the aetiological agent of VER in *P.dentex* (Arimoto et al., 1993). Nishizawa (1995) provided viral genome and aa analysis to support the classification of NNV as a member of the Nodaviridae. Betanodavirus was officially accepted as a new genus in the family Nodaviridae in 1997 (ICTVonline.org/proposals/Ratification_1997.pdf). SJNNV became the type species of the Betanodavirus genus. Other species include Barfin Flounder Nervous Necrosis Virus (BFNNV), Redspotted Grouper Nervous Necrosis Virus (RGNNV) and Tiger Puffer Nervous Necrosis Virus (TPNNV).

1.6.2 Host range:

Betanodavirus isolates have been collected from larval and juvenile stages of over 157 marine and freshwater fish species in tropical and temperate climates (www.ncbi.nih.gov/). VER disease outbreaks have been reported from farmed cultures and wild fisheries (Koohkan et al., 2012) (Breuil et al., 2000). In experimental infection trials, susceptibility of fish species has been classified as highly susceptible, less susceptible or resistant to NNV (Furusawa et al., 2007). A similar pattern occurs in wild fisheries. The highly susceptible species display mortalities of 80 to 100 % in either larval or larval and later life stages. Most of the highly susceptible species are only susceptible to VVN during early larval stages. Except, the groupers, *Epinephelus* sp. and European seabass, *Dicentrarchus labrax*, are susceptible beyond the larval stages. Groupers up to 3 years old have been recorded with VER (Tanaka et al., 1998). Notably, there have been no reports of VER in Atlantic salmon, *Salmo salar*, despite the geographical presence of NVV where they are cultured and the fish being reportedly susceptible to experimental infection with BFNNV via intraperitoneal injection (Korsnes et al., 2005). Also notable in the Northern Australian aquaculture context is the absence of reported disease outbreaks in cobia, *Rachycentron canadum* which are being experimentally grown within marine systems in the same geographic range as barramundi and grouper. Chu et al. (2013) reported a case study of mortality exceeding 80% in cobia from 3 farms in Malaysia during 2007. Fish displayed signs of VER which included the mass mortality and abnormal spiral swimming behaviour and “flashing” and were positive for the detection of NNV by RT-PCR (Chu et al., 2013). No similar reports have occurred from cobia aquaculture despite, in some circumstances in Australia, cobia being cultured in pond cages immediately adjacent to grouper suffering a VER outbreak (*unpublished data*).

Betanodaviruses have also been detected from a number of non-piscine hosts including the spiny lobster *Panulirus versicolor* (Gomez et al., 2006), Artemia sp, Copepod *Tigriopus japonicas*, shrimp *Acetesinte medius*, Charybdid crab *Charybdis bimaculata*, Southern humpback shrimp *Pandalus hypsinotus* , Mediterranean mussel *Mytilus galloprovincialis* (Gomez et al., 2008) and Japanese common squid *Todarodes pacificus* (Gomez et al., 2010). The detections were not associated with any clinical signs of disease and the role these alternate hosts play in the transmission of Betanodaviruses to fish species has not been demonstrated. There is increased awareness of the biosecurity threats posed by the bioaccumulation of NNV in molluscs as a reservoir for infection to cultured fish (Volpe et al., 2017). Re-isolation of RGNNV from the hepatopancreas and detection of virus in surrounding water following experimental exposure to RGNNV was recently demonstrated from the Manila clam *Ruditapes philippinarum* (Volpe et al., 2017).

1.6.3 Transmission of Betanodavirus

VER has been experimentally induced in susceptible fish species through exposure to viral extracts via multiple routes including intramuscular injection (IM), intraperitoneal injection (IP), intranasal inoculation (IN), water bath exposure and co-habitation with infected individuals. Betanodavirus RNA was detected in developing embryos of *E. coioides* by PCR and in-situ RT-PCR suggesting a vertical transmission pathway within the hatchery system (Kuo et al., 2012). Likewise, BFNNV with a copy number of 10^3 per sample was detected from the gonads, eggs and fertilized eggs of Pacific cod *Gadus macrocephalus* (Mao et al., 2015). In the same study, food sources including *Artemia*, *Chlorella* and enhancer limacinum were negative for detection of Betanodavirus by PCR. In contrast, Whittington (2012) reported NNV was transmitted horizontally, from unknown source but not vertically in a larval barramundi hatchery system. Individual fish from susceptible species which survive Betanodavirus infection have been demonstrated to carry the virus. The sub-clinically infected fish are believed to act as a reservoir source for horizontal transmission of the virus to other fish. Within hatchery systems, VER has been effectively managed through strict management protocols which involve the quarantining larval fish production by disinfection of incoming water, fertilised eggs, hatchery equipment and workers. In the fish species that are only susceptible during larval stages, this strategy is very effective. For species, including groupers and European sea bass, which have a longer period of susceptibility that are cultured in outdoor pond or cage systems, the management of VER continues to be an issue.

1.6.4 Progression of Betanodavirus infection

In natural infections, the pathways involved in the spread of Betanodavirus from the site of infection to the nervous tissues have not been determined. Brain-infecting viruses can spread to the brain by either axonal transport from the skin or muscle to dorsal root ganglion or anterior horn cells and then to neurons and the CNS or can spread to the brain via the blood (Lu et al., 2008). Betanodavirus has been detected in the epithelial cells of skin and in the intestinal epithelium with concurrent detection in the nerve cells of the brain in the early stage of NNV infection (Chi, 2011). Infection via intranasal swab was reported to proceed through the nasal epithelium, pass through the olfactory nerve and olfactory bulb and invade the olfactory lobe (Chi, 2011). The pathway of Betanodavirus spread in zebrafish is proposed to occur via the blood (Lu et al., 2008). In contrast, no RGNNV or SJNNV was detected in blood samples in experimentally infected fish despite positive detection in the brain, spinal cord and eye of *E. moora* and Striped Jack, *P. dentax* (Banu et al., 2007). Ikenaga et al. (2002) observed SGNNV injected into the retinal ganglion cells was transported in the optic nerve to the axon terminal of the brain, where the virus proceeded to spread various regions of the brain. The virus can be consistently isolated from the eye and brain and with varying success from fin tissue, spleen, liver, stomach and heart (Chi, 2011). Brain and eye are the target tissues for virus isolation procedures in disease investigations (Moody & Crane, 2012). Histopathological changes in fish displaying clinical disease symptoms involve vacuolation of the nervous tissues with the absence of any notable inflammatory immune response (Tanaka et al., 2004). For specific descriptions of histopathology refer to the descriptions by Tanaka et al. (2004).

1.6.5 Pathogenesis of Betanodavirus Infection and VER disease

A number of factors reportedly modulate the expression of VER disease. Age of fish, salinity, water temperature and water sediment load are proposed to influence the development of disease. How the factors influence progression to disease is unknown. Environmental conditions are proposed to play a play a key role in switch form a carrier-state to acute phase mortality (Kara et al., 2014).

1.6.5.1 Age of fish

Larval fish are particularly susceptible to VER (Moody & Crane, 2012). In larval stages of highly susceptible marine species, mortalities of 80 to 100 % occur over 4 to 6 days from the onset of clinical signs (OIE, 2019). The period of larval susceptibility varies between species. Although other stressful age-dependent developmental events may contribute, presumably an immature immune system in the fish contributes to the mass deaths in the presence of Betanodavirus. Jamarillo et al. (2017) reported an age susceptibility pattern in juvenile

barramundi whereby only larval stages are highly susceptible to NNV. Juvenile barramundi, older than 5 weeks of age, develop a subclinical infection, whereas younger cohorts suffer clinical disease and mass mortality (Jaramillo et al., 2017). The immune pathway that may confer this change is susceptibility is unknown. The report of Jaramillo et al. (2017) is the only study to demonstrate the age-related conversion from susceptibility to a tolerance of RGNNV. Few studies encompass the development of immune function and Betanodavirus infection and VER disease. Some studies have detected and monitored changes in specific immune factors. In grouper, *E. coioides*, Down syndrome cell adhesion molecule (DSCAM) A and B and Myxovirus resistance protein (Mx) were monitored by qPCR for 27 days post-hatch (dph). DSCAM A and B displayed a modulating 7-day cycle peaking at 4- and 11-days post-hatch (Yew et al., 2012). Mx was detected at minimal levels until 6 dph whereby it continued to increase until 9 to 12 dph before reaching relatively stable levels for the next 14 days (Yew et al., 2012). Interferon-α was not detected in larval zebrafish during the time they display high susceptibility to VER disease (Lu et al., 2008). In *Epinephelus bruneus,* although primary lymphoid organs are present from days 1 to 12, the development of the immune system in is incomplete until 30 dph (Huang & Han, 2015). "Complete" immune system development was determined by histology of tissues rather than demonstration of functional capability (Huang & Han, 2015). The age-related factors which favour VER disease outbreaks require further investigation.

1.6.5.2 Water Temperature

1.6.5.2.1 Temperatures associated with natural disease outbreaks:

In vitro the four Betanodavirus species display temperature range specificity.

Disease outbreaks in the wild and experimental infections with strains of Betanodavirus indicate temperature is a modulating factor in VER disease (Ciulli et al., 2006). The modulating effects of temperature occur at ranges which were beyond those reported for *in vitro* growth. In the wild, a BFNNV strain has caused disease at temperatures between 4 to 15 °C (Tanaka et al., 1998). A SJNNV strain induced disease in striped jack larvae in water temperatures ranging from 18 to 27 °C (Tanaka et al., 1998). Natural outbreaks of disease caused by RGNNV disease tends to occur at both increased and decreased temperatures. In grouper species, increased mortalities from VER have been reported to occur with temperature decreases below 22 °C even though the RGNNV strain does not replicate well at this temperature range *in vitro* (Kara et al., 2014). A sudden drop in temperature induced VER in *D. labrax* which displayed no clinical signs when held at higher temperatures (Thiery et al., 1999). Vertical transmission of VER can occur in *D. labrax* at temperature below 15 °C (Breuil et al., 2002). *In vitro* growth of a

RGNNV strain isolated from the Adriatic Sea displayed growth over a temperature range of 15 to 30 °C (Ciulli et al., 2006). Conversely in the Asian continent, grouper mortalities due to VER were noted in Japan and Taiwan when water temperatures exceeded 25 °C and 29 °C respectively (Kara et al., 2014). In an outbreak of VER caused by a RGNNV strain in Italy, the highest mortalities were observed in fish held at highest temperatures (28 to 30 °C). Lower mortalities were observed and ceased when the farmer lowered the temperature below 23 °C (Bovo et al., 2011).

1.6.5.2.2 Temperatures associated with experimental challenge:

In experimental infections some variation from temperatures has occurred in SJNNV and RGNNV and recombinants of these strains (Souto et al., 2015b). Immersion exposure of Senegalese sole to a RGNNV RNA 1/SJNNV RNA 2 natural re-assortment at a dose of 10^5 $TCID_{50}$ had reduced mortality and clinical signs at 16 °C compared to fish held in 18 °C and 22 °C (Souto et al., 2015b). Only 8.3 % mortality was recorded in infected fish held at 16 °C compared with >80 % at the other temperatures (Souto et al., 2015b). The detection of RNA 1 indicated significantly greater copy number at 22 °C and 18 °C (10^{10}) compared at 16 °C (10^6 to 10^8) over the 30-day trial. Groupers infected at 16 °C and subsequently exposed to temperature increased to 22 °C displayed mortalities approaching 100 % (Souto et al., 2015b). Hyperactivity increased in fish 4 to 5 days after a temperature increase and earlier for fish which had been acclimated to 16 °C for longer than 15 days. Viral copies were detected in all fish infected at 16 °C however, viral load displayed changes over time and decreased from 2.7 x 10^9 at 45 to 66 days post-challenge (dpc). Viral copy number in fish at 22 °C was in the 10^9 to 10^{10} range depending on the period of time which the fish had originally been held at 16 °C (Souto et al., 2015b). Viral loads of 10^7 to 10^8 were observed in fish which died at 16 °C indicating that increased viral load did not cause the mortality.

Challenge of larval fish with RGNNV at 28 °C induced 100 % mortality at 50 to 80 hpi (Lio-Po & de la Pena 2004). An experimental challenge of *E. akaara* juveniles with RGNNV caused 100 % mortality at 24 to 28 °C. At 16 to 20 °C mortality was reduced to 57 to 61 % and the time to onset of clinical signs was delayed. In addition, viral antigen was detected among survivors 50-dpc (Lio-Po & de la Pena 2004). Similar results were reported when juvenile sevenband grouper, *E. septemfasciatus*, were experimentally exposed to a RGNNV extract at different temperatures (Tanaka et al., 1998). In the experiment of Tanaka et al. (1998) none of the fish which survived RGNNV and were later held at 20 or 24 °C displayed a positive result using fluorescent antibody testing (FAT) against the capsid protein at 50 days post-challenge (dpc). In contrast, fish held at both the higher or lower temperatures were positive for VER by FAT.

Experimental exposure to a greasy grouper NNV (GGNNV) strain induced 100% mortality in larval grouper at temperatures ranging from 24 to 32 °C (Chi et al., 1999). Fish held at a constant temperature of 28 °C displayed 100 % mortality by 50 hours post-challenge (hpc). In contrast, fish held at ambient temperature which ranged from 28 to 24 °C displayed 100 % mortality after 80 hpc (Chi et al., 1999). An RGNNV strain caused increased mortality in larval Australian bass *M.novemaculata* reared at 22 °C compared to 17 °C (Jaramillo et al., 2015). Testing on older resistant fish revealed no change in mortality occurred with increased temperature (Jaramillo et al., 2015).

Temperature is a major factor for stimulating asymptomatic carriers to become symptomatic fish (Kara et al., 2014). VER was induced in sub-clinically infected zebrafish by increased water temperature (25 to 32°C) and crowding (10 to 40 fish/L) (Binesh 2014). The changes in mortality rate could be due to an alteration in fish anti-viral immunity induced by temperature rather than temperature modulated alterations on Betanodavirus replication (Tanaka et al., 1998). Breuil et al. (2000) reported the detection of antibodies to nodavirus in the serum of adult *D.labrax* for 2 years post infection with antibody levels declining over winter. The effect of age and temperature on antiviral immunity was investigated in zebrafish (Dios et al., 2010). In larval Zebrafish, known to be susceptible to VER, anti-viral immune response genes including Mx, MDA-5, Interferon- (IFN-1), Toll-like Receptor 3 (TLR-3), Interferon regulatory factor 3 (IRF3) and Interferon- ϒ (IFN-ϒ), were very sensitive to low temperature (Dios et al., 2010). Further investigation is required to elucidate the relationship between temperature and VER disease events.

1.6.5.3 Salinity:

VER occurs in freshwater, brackish and marine environments indicating a large salinity tolerance of the Betanodaviruses. Two strains of VER isolated from *D. labrax* in the Mediterranean lost infectivity after 6 months storage at 15 °C in freshwater compared with a $TCID_{50}$ of $10^{4 \text{ to } 4.5}$ when stored in seawater of 20 or 37 ppt (Frerichs et al., 2000). An RGNNV strain caused varying levels of disease when injected into freshwater fish (Furuzawa et al., 2007). According to some grouper farmers, mortality due to VER can be downregulated by decreasing the salinity of the sea water (Chi, 2011). In an experimental infection with a grouper NNV strain, mortality was reduced in fish acclimated at 15 ppt compared to those held at 30 ppt (Chen, 2014).

1.6.5.4 Organic load and Sediment

Verbal reports from hatchery workers suggest VER outbreaks are preceded by periods of high sediment in inlet water (*unpublished pers comms numerous aquaculturists*). The association has not been investigated but could involve a number of mechanisms. Firstly, Betanodavirus particles shed by wild fish may directly attach to estuarine sediments which then enter the hatchery system during periods of high turbidity. The adsorption and detachment of enteric viruses in estuarine sediment is influenced by salinity and organic matter (LaBelle & Gerba, 1979). No similar studies have been conducted on Betanodaviruses. In addition, higher sediment loads may induce stress in larval fish which could downregulate components of the antiviral immune system which protect against infection. High sediment loads negatively affect the efficacy of ozone and UV sterilisation protocols used to prevent VER. Levels of pesticides or other environmental contaminants are known to have adverse effects on the immune functioning of fish (Bols et al., 2001). The pesticides diuron, atrazine, hexazinone, tebuthiuron and ametryn have been detected in the Great Barrier Reef waters with greatest concentration in estuarine waters which are the intake waters for the vast majority of tropical finfish aquaculture systems in Northern Queensland (http://reefrescueresearch.com.au/news/183-pesticide-dynamics-in-the-gbr.html).

1.7 Managing VER in grow out fish aquaculture

A major impediment to the successful management of VER in the grow-out culture of some fish species, groupers and *D. labrax*, is caused by the lack of understanding into the initiation of the disease. It is unknown if VER disease outbreaks on grow-out farms are caused by exposure of naïve individuals to *Betanodavirus* or a result of changes in the sub-clinical fish-virus dynamic to favour progression of disease. The noting of factors associated with VER disease suggests some form of immune modulation must be involved. Regardless, considering the wide host range, presence of sub-clinical carriers and the wide environmental tolerance of the virus, if the first scenario is the cause of VER outbreaks on grow out farms it is unlikely exposure to *Betanodaviruses* can be prevented in open pond systems. Hence, managing the culture conditions to favour fish survival in the presence of Betanodavirus may be a more appropriate strategy. Attempts to manage Betanodavirus, and generally fish viral infections, in grow out aquaculture systems can be broadly categorised as either the production of:

- anti-viral compounds which target virus replication or
- Immuno-modulators which target fish immune function.

1.7.1.1 Anti-Viral Compounds

The study of anti-viral compounds for application to fish aquaculture is very limited. With the exception of ribavirin, the anti-viral compounds which have been reported have only been investigated *in vitro* and none have been approved for use as commercially available and registered for use in aquaculture in Australia. Considering the lengthy process involved in registering compounds for aquaculture applications and the negative regard consumers have for chemical compounds in food, it is unlikely an anti-viral therapy will be available to aquaculture in the near future. In addition, considering the long culture period (6-10 months) of some grow out systems, it is likely multiple doses of any effective compound would be required. Nonetheless, research into such therapies may contribute towards improved understanding of the host-virus dynamic.

Some compounds which inhibit endosomal acidification have been reported to inhibit RGNNV-induced CPE e.g. NH_4Cl, chloroquine and bafilomycin A1 (Adachi et al., 2007). Neuraminidase and tunicamycin blocked the attachment of DGNNV to SSN-1 cells (Liu et al., 2005).

Ribavirin is a known inhibitor of RNA viruses and used as an anti-viral drug to combat human viral infections. Ribavirin (25 µM) inhibited Betanodavirus induced CPE by 54 % in GF-1 cells (Huang et al., 2015). Expression of RNA 1 and RNA 2, detected by RT-PCR, were decreased by approximately 80 % (Huang et al., 2015). The interaction between ribavirin and Betanodavirus was hypothesised to occur with Protein A at the region between AA 585 to 744. Based on comparisons of ribavirin binding with foot and mouth disease virus Protein A specific RGNNV-ribavirin binding residues were proposed to be aa K586, F589, D590, N655, S646 and G647. All of the residues except N655 are located on the conserved motifs of the viral RNA dependant RNA polymerase motifs (Huang et al., 2015). Ribavirin was demonstrated to reduce mortality in *Salmo salar* caused by infectious salmon anaemia virus (ISAV) by 90% (Rivas-Aravena et al., 2011). However, the drug is not approved for use in aquaculture.

The development of a cell-based screening platform to assess anti-Betanodavirus compounds allowed the detection of forty-three compounds which reduced RGNNV–induced CPE by at least 50% (Huang & Han 2015). Only proadifen hydrochloride, which is a known inhibitor of neuronal nitric oxide synthase (NOS1) and cytochrome P-450, was further investigated. Effective compounds were listed and broadly categorised as antibacterial, antifungal, antimalarial, enzyme inhibitors, hormones, membrane transport modulators, neurotransmitter agents, phytochemicals, sterols, peripheral nervous system agents and the colouring agent erythrosine sodium. Interestingly, erythrosine sodium, *syn*. erythrosine B (FD&C Red No. 3) has been demonstrated to be a non-discriminant potent inhibitor of the protein-protein

interactions between: Tumor Necrosis Factor (TNF) -Receptor-TNFα, epidermal growth factor receptor (EGF-R–E) and cluster of differentiation (CD) CD40-CD154 (Ganesan et al., 2011). The mode of action of erythrosine sodium on Betanodavirus replication has not been determined. Considering erythrosine sodium is approved for use as a food dye this compound may be a promising anti-viral compound for aquaculture.

Although the expense and unfavourable acceptance by consumers may limit the application of anti-virals to aquaculture applications, the knowledge gained from studies using anti-virals contribute to a greater understanding of the host-Betanodavirus dynamic.

1.7.1.2 Immuno-modulation of the Fish host

In open pond or cage aquaculture, options that modulate the fish immune system will be more achievable than attempts to directly managing the virus to prevent VER. Unfortunately, this study is being attempted when there is limited knowledge about the grouper anti-viral immune system. Nonetheless, there is some reported success in preventing VER via modulation of the fish immune system.

1.7.1.2.1 Stimulation of the Non-specific (Cellular) immune pathway

Early attempts to improve survival of fish following Betanodavirus challenge using immune stimulating compounds reported success. The compounds were essentially “mimics” of pathogens which stimulated the non-specific immune pathways recognition by pathogen recognition receptors (PRRs). The intramuscular (IM) delivery of poly I:C and interferon have been reported to prevent VER if delivered during “appropriate time frames” (Kuo et al., 2016). Unfortunately, the “appropriate time frames” were a short period prior to Betanodavirus exposure or the appearance of clinical signs which could be unknown in a fish culture situation. Additionally, the protective period provided by such compounds was a short lived, 24 to 48 hours (Kuo et al., 2016).

1.7.1.2.2 Stimulation of the specific (Humoral) Immune pathway

Many report the successful reduction in mortalities due to NNV infection with an experimental vaccine. Vaccination is discussed in greater detail in Chapter 6. However, it is important to note that despite a long history of reported success in experimental systems, there are no vaccines commercially available in Australia or Asia. Pharmaq™ and HIPRA commenced the sale of the only vaccine against NNV available to Europe in 2018. Considering the demand and sizeable commercial value, the absence of a commercially available vaccine against NNV may indicate that the management of VER is more complicated than vaccination alone.

1.8 VER in Grow-Out Aquaculture systems. What determines Betanodavirus infection vs VER disease?

An increasing body of data indicates changes or differences in immune function in the fish host contribute to the expression of clinical VER disease. Reports noting histopathological differences in carrier and diseased fish suggest a role of immunity. Vacuolation of the nervous tissue is invariably associated with fish displaying disease symptoms. The abundance of vacuolation of the nervous tissues and the absence of notable inflammatory response in the neural tissues is associated with other vertebrate diseases. Spongiform encephalopathy (SE), caused by prions PrPsc, is also characterised by histopathology by the vacuolation of neurons and adjacent glial cells, and the absence of any inflammation or immune response to the agent (Murray et al., p.599). One of the earliest descriptions of VER pathology noted the similarity with the histopathology induced during SE (Gallet de Saint Aurin et al., 1989). The observation that vacuolation, associated with an absence of any inflammation or immune response, leads to death is also reported in Semliki Forest Virus (SFV) infections (Fazakerley et al., 2006). Experimental inoculation of immuno-competent mice with an avirulent strain of SFV (avSFV) results in a strong inflammatory immune response (Fazakerley et al., 2006). Early necrotic cell death of oligodendrocytes, followed by apoptotic cell death of uninfected cells lead to the clearance of the virus within 18 days pi and the absence of vacuolation of the nervous tissues (Fazakerley et al., 2006). However, athymic nu/nu mice, which are deficient in the production of cytotoxic-T cells, display vacuolation of the nervous tissue and die when injected with AvSFV (Fazakerley et al., 2006). In such cases, priming of the immune system by IP injection of poly (I:C) abrogates viral infection in the neural tissues. Experimental exposure of mice to Herpes Simplex Virus (HSV) also involves immune v non-immune histopathology, which reflects death vs survival (Grubor-Bauk et al., 2008). Mice which are deficient in the expression of natural killer T-cells (NKT) display vacuolation of neurons (Grubor-Bauk et al., 2008). The brains of immune-competent mice display a mononuclear inflammatory infiltrate (Grubor-Bauk et al., 2008). Treatment with activated CD8+T cells abrogated the infection in immune-incompetent mice (Grubor-Bauk et al., 2008).

Similarly, with VER, there are a small number of reports in which “healthy fish” are verified to be VER positive but not displaying vacuolation in the brain. Gjessing et al. (2009) reported histopathological changes in the nervous tissues from “healthy” Atlantic cod that included a “diffuse cellular reaction”, few vacuoles, and VER immune-positively staining macrophage-like cells near nervous tissue. *E. septemfasciatus* which survived experimental VER challenge when sampled 2 months post-infection did not display vacuolation in the brain or retina, but the

tissues reacted positively by FAT (Lopez-Jimena et al., 2011). Similar results were reported from an RGNNV outbreak in Italy (Bovo et al., 2011). Hybrid bass (*Morone saxatilis x Morone chrysops*), held on a farm that experienced VER displayed low-level mortality (20 %) and inconsistent results for the detection of the virus. Whittington (2012) detected VER in subclinical carriers by qPCR and stated "histopathology can no longer be used as the sole screening test" for VER due to the absence of histopathological changes in subclinical carrier fish. However, this conclusion is challengeable because the detection of NNV by qPCR does not necessarily indicate the presence of a viable virus.

Comparative analysis of the expression of immune factors also demonstrates a correlation between the development of immune competence and survival from Betanodavirus infection. Differential immune responses to VER in zebrafish juveniles compared to adults has been reported (Lu et al., 2008). Acute VER disease in larvae and juveniles is reportedly due to inactive interferon response in contrast to an active innate immune response during persistent infection in the adult stages (Lu et al., 2008). Interferon-α (IFN-α) and Mx were expressed in adult zebrafish brains within 4 to 20 hpi following injection with VER (Lu et al., 2008). In contrast, neither gene was expressed in larval fish with the same exposure. Adult fish were refractory to VER infection, and larval fish displayed 100 % mortality within 72 to 96 hpi. Injection of 1000U of IFN-α2a (Roche) into larval zebrafish 48 hours before VER injection protected against VER infection for 48 hpi (Lu et al., 2008). In developing human embryos, neuronal differentiation is associated with improved cellular responses to IFN1 and also an increase in type 1 interferon pathway components namely interferon regulatory factor (IRF-9) and interferon-α/β receptor βchain IFNAR2 (Farmer et al., 2013). The overexpression of signal transducer activator of transcription-2 (STAT 2) and interferon-α/β receptor βchain (IFNAR2) recapitulates the neuronal differentiation-dependant changes involving IFN-1 (Farmer et al., 2013). Farmer et al. (2013) also noted that these developmental events might contribute to the age-dependent encephalitis caused by SFV. Regarding Betanodavirus, Huang et al. (2015) and Lu et al. (2008) noted changes in the expression of STAT-1/3 but neither measured STAT-2.

Additional studies report transcriptome analysis of the immune responses of carrier vs susceptible fish. Poisa-Beiro et al. (2008) investigated the interferon response to VER infected *D. labrax* and *S.aurata*. In *S.aurata*, which are not highly susceptible to VER, NNV was detected at high levels by RT-PCR in the blood but declined 3 dpi. High levels of NNV were detected in the brain 3 dpi. Up-regulation in the expression of TNF-α and Interleukin 1 (IL-1)

were detected in the brain of sea bream 3 dpi once the virus reached the target organ. Expression of Mx was 1300-fold in brain 3 days pi. Measurements from *D. labrax* exhibited a similar pattern; however, a lower expression of Mx (10x) was noted compared to controls. Thanasaksiri et al. (2014) investigated the effect of temperature on the expression of Mx and survival of sevenband grouper *E.septemfasciatus* to experimental infection with RGNNV. Rearing fish at temperatures between 15 to 30 °C for 2 weeks before experimental infection did not affect RGNNV (JN662462) RNA 2 copy number. However, there were differences in the relative levels of Mx mRNA expressed in the head kidney with rearing temperature and also in survival of fish for 14 days post-exposure to RGNNV. How the Mx expression levels in the head kidney compare to those in the brain, which is the target organ for NNV replication is unknown. Survival of fish was not improved at any temperature other than 25 °C (compared to 15, 20 and 30°C) even though RGNNV copy numbers were 10 to 100-fold lower at the other temperatures.

Nie et al. (2015) measured the involvement of Retinoic Acid Inducible Gene (RIG)-1 in Nuclear factor-kappa light chain enhancer of activated B cells (NF-κB) and IFN signalling in antiviral signalling. A RIG-1 homolog from zebrafish larva (DrRIG-1) was stimulated by poly I:C resulting in the significant activation of NF-kappaB, TNF-α, IL-8, Mx, Interferon Stimulate Gene -15 (ISG-15) and viperin. However, the knockdown of Tripartite motif-containing protein-25 (TRIM 25) which is a pivotal activator for RIG-1 receptors suppressed the induced activation of the IFN signalling. (Nie et al., 2015). TRIM 25 has 3-N terminal domains, a coiled-coil domain (CCD) and a C-terminal domain (SPRY). To avoid IFN production, the NS1 of influenza virus interacts with the CCD domain of TRIM 25 to block RIG-1 ubiquitination. Another TRIM, TRIM 21 also stimulates the transcription factor pathways of NF-kappaB, AP-1, IRF3, IRF5 and IRF7. Antibody-coated viral particles activate TRIM 21, which binds to the Fc receptor and targets virions for degradation. The targeting of TRIMs by Betanodavirus would successfully mitigate both a humoral (antibody) and innate immune response. However, there are no published reports relating to TRIM and Betanodavirus infection.

1.9 Modulation of the fish immune system. What other mechanisms could Betanodaviruses employ to combat the host immune response?

A review of the immune response to Betanodavirus infections of marine fish was recently published (Chen et al., 2014). NNV is initially recognised by the innate immune system which through the detection of pathogen-associated molecular patterns (PAMPS), activates a range of intracellular signals to stimulate both an innate and cell-mediated immune response. The detection of antibodies with specificity against RGNNV in healthy fish indicates the humoral immune pathway is also activated. A neutralising antibody, RG-M18 mAB, required aa 195 to 202 (VNVSVLCR) of the capsid protein as the minimal epitope for NNV recognition (Chen et al., 2015). In the case of groupers and *D. labrax*, the immune responses, although ensuring survival, are insufficient to remove the virus, suggesting Betanodaviruses possess additional unknown mechanisms to avoid a functional immune response. The B1 and B2 proteins have been demonstrated to manipulate some components of immune pathways. However, mechanisms which allow Betanodaviruses, within the limitations of a small viral genome and the absence of other translated viral proteins, to avoid all of the immune pathways and persist in sub-clinical carrier fish have not been determined.

The importance of viral encoded microRNA (V-miRNA) is recognised as a potent mechanism used by viruses to achieve viral replication, persistence, immune evasion and cellular transformation (Cullen 2009; Grundhoff & Sullivan 2011; Tycowski et al., 2015). V-miRNAs represent a genomically efficient way for viruses to regulate host immune responses. A V-miRNA could target multiple genes in the same host or a highly conserved gene in multiple hosts (Weber et al., 2004). Additionally, V-miRNAs are known to target the expression of viral replication process and are involved in the Singapore grouper Iridovirus which encodes at least 16 V-miRNAs the functions of which are being studied (Guo et al., 2013). In addition, nine v-miRNAs have been detected during replication of the fish-infecting Megalocytivirus (Zhang et al., 2014).

Nodavirus V-miRNAs have not been reported. However, the replication of short incomplete copies of Differential interfering-RNA (DI-RNA) by the RdRp has been detected in the early phases of nodavirus replication *in vitro*. During RNA replication, Flock house virus (FHV) produces, ~ 400bp dsRNA (DI-RNA) from the 5' terminus of RNA 1 which serves as a Dicer-2 substrate (Aliyari et al., 2008). Pyrosequencing of the dsRNA formed 4 days post-FHV infection detected 4371 small RNAs the majority of which have strong homology and presumably target

a region comprising the first 400nt of RNA 1 (Aliyari 2008). No functional roles for the DI-RNAs have been demonstrated.

I propose the mass replication of the DI-RNAs could be an intermediate step in the production of V-miRNA which could serve to downregulate specific host genes such as those which regulate immune function or to regulate nodavirus replication. The lower expression of Protein A from RNA 1 compared to the capsid protein of RNA 2 despite both strands being transcribed in equal amounts supports the concept the RNA 1 transcripts could have functions beyond translation to protein A.

1.10 Concluding comments

The management VNN infections in grouper grow out culture is difficult. There are critical gaps in knowledge in many areas of the Betanodavirus-fish-environment dynamic that hamper progress towards preventing VER. Nonetheless, many fish diseases have been effectively managed by vaccination prior to the attainment of understanding of the mechanics of a specific pathogen-host dynamic (Gravningen et al., 2008). However, with the data presently available, there is poor understanding of the variation of strains of NNV that cause VER in tropical fish species in Australia. Insufficient knowledge of NNV strains represents a risk to the successful development of vaccines to prevent VER. Also, the paucity of information about the development of the grouper immune system creates a degree of uncertainty around the scheduled delivery of vaccines or other prophylactic measures. Finally, there has been no assessment to determine if the current RT-qPCRs that have been used to study NNV in barramundi and Australian Bass in Australia are fit for purpose to study NNV in groupers.

This project aims to improve knowledge about the VNN strains that cause VER in North Queensland and apply this knowledge to the development of therapies to limit the impact of VER on grouper aquaculture. The project also aims to determine if the RT-qPCR (qR2T) (Hick & Whittington 2010) that is the current Australian New Zealand Standard Protocol for the detection of NNV, and an additional assay RT-qPCR (qR1T) (Hick & Whittington, 2010) are fit for purpose to study NNV in grouper. Finally, the project aims to test the efficacy of vaccination and dsRNA as treatments to prevent VER outbreaks in the Queensland giant grouper *Epinephelus lanceolatus*.

The research questions posed by this study:

1. **What strains of Betanodavirus are associated with VER outbreaks of tropical marine fish species in Northern Queensland?**
2. **Is RT-qPCR a useful tool to assist in the management of VER in giant Queensland grouper, *Epinephelus lanceolatus* in Australia?**
3. **How effective is vaccination or dsRNA designed against the endemic strains of NNV in preventing disease?**

How the project aims were achieved:

1. **Positioning this research, an overview of VER disease in finfish.**
   Achieved in Chapter 1: Literature Review
2. **Develop the laboratory skills required to complete project activities.**
   Achieved in Chapter 2: General Material and Methods
3. **Obtain full sequence of the Betanodavirus strains and determine the level of variation in strains that cause VER in Northern Queensland.**
   Achieved in Chapter 3: Phylogenetic analysis of NNV strains in Australia.
4. **Investigate the genome sequences of the Betanodavirus strains and identify the presence of motifs that are critical for the development of prophylactic measures.**
   Achieved in Chapter 4: Review of functional motifs of the Betanodavirus genome
5. **Implement qPCR analysis as a tool for the quantitative detection of Betanodavirus.**
   Achieved in Chapter 5: Development of qPCR Standard controls.
6. **Develop prophylactic measures of experimental vaccine and dsRNA to confer protection against Betanodavirus that is present in NQ**.
   Achieved in Chapter 6. Development of prophylactic measures against NNV
7. **Determine if exposure to RGNNV by cohabitation with/without co-exposure to a marine leech, *Zeylanicobdella arugamensis* leads to VER**
   Achieved in Chapter 7: Experimental challenge via co-habitation with marine leech
8. **Measure the efficacy of the therapies to prevent VER via experimental challenge.**
   Achieved in Chapter 8: Measuring efficacy of prophylactic measures against NNV
9. **Determine if survival to experimental challenge is related to parentage assignment**
   Achieved Chapter 9: Role of family in survival of VER in E.lanceolatus
10. **Refine the vaccination strategy to prevent VER via experimental challenge**
    Achieved in Chapter 10: Refinement of experimental vaccination against NNV

CHAPTER 2. GENERAL MATERIALS AND METHODS

Background

- This thesis is a culmination of three years of investigation.
- Some of the procedures were conducted as a single task that align with multiple chapters of work.
- Some procedures that were repeated many times were standardised as an operational procedure.

Aims of this Chapter

- Describe the laboratory protocols that align with multiple chapters of work.
- Describe the protocols that were conducted as standard operational procedures throughout this thesis.

2.1 Introduction

This thesis is a compilation of three years of research. Throughout the project there were a number of protocols that were repeated many times. Standard operational protocols and laboratory worksheets were prepared for tasks that were repeated many times. In addition, one procedure, namely the preparation of viral extract, was completed once, yet utilised many times. The aim of this chapter is to describe the protocols that were performed as standard operational procedures and preparation of the viral extract used throughout this study.

2.2 Molecular biology

2.2.1 Collection of Betanodavirus positive material

Betanodavirus extract was prepared from 3 separate, natural VER outbreaks that occurred in North Queensland fish farms during 2015-2016. Whole fish including gold spot grouper, *Epinephelus coioides* (Hamilton, 1822), juvenile barramundi, *Lates calcarifer*, and sub-adult giant grouper *Epinephelus lanceolatus* were supplied from commercial fish farms suffering VER outbreaks.

Moribund fish were euthanized by lethal overdose of AQUI-S® and frozen at -20˚C. The Biosecurity Sciences Laboratory (BSL), Queensland Government Veterinary Laboratory, subsequently diagnosed VER. Eye tissue, up to a maximum of 500mg, was aseptically removed from fish displaying clinical signs and subjected to total viral nucleic acid extraction using the High Pure™ Viral Nucleic Acid kit (Roche) according to the manufacturer's instructions. Remaining tissue was stored at -20˚C.

2.2.2 Reverse transcription reaction/ cDNA synthesis

Complementary DNA (cDNA) was reverse transcribed (RT) from RNA 1 and RNA 2 segments in nucleic acid extracts. cDNA was synthesized using a Maxygene thermal cycler (Axygen) in a 20 µL reaction using random hexamers with the Tetro™ cDNA synthesis kit (Bioline) according to the manufacturer's instructions. The reverse transcriptase (RT) thermal cycle consisted of incubation of 10 min at 25^{o}C; 30 min at 45 ^{o}C and an RT deactivation of 5 min at 85^{o}C.

2.2.3 Nucleic acid extraction

Nucleic acid purification was conducted on 400 µL of viral extracts or dissected eye and brain tissue (maximum of 400mg) from clinically diseased fish. Extraction was completed using the High Pure™ Viral Nucleic Acid kit (Roche 11858874001) according to the manufacturer's instructions. Purified nucleic acid was eluted into 100 µL RNAse free water and stored at -20°C.

2.2.4 Polymerase chain reaction

Multiple PCRs were performed using a Maxygene thermal cycler (Axygen). PCRs were performed as a 20 µL reaction with 2.5 µL of cDNA and 100 nM primer (Hick & Whittington 2010) (see Table 1) in a MyFi™ DNA Polymerase Mix according to the manufacturer's instructions (BIO-21117 Bioline, NSW). The thermal cycle profile consisted of a 3 min incubation at 95^{o}C followed by 45 cycles of 15 s denaturation at 95^{o}C, 30 s of annealing temperature (Table 2-1 Tm primer), a 30 s extension at 72^{o}C; and a final extension step of 5 min at 72^{o}C.

Table 2-1: Description of the primer and probe sequences used in this thesis. Including PCR assay type, primer name, primer nt sequence, RNA segment target, nt binding position the and melt temperature of primer and probe.

Source of all primer and probe sequences Hick &Whittington (2010). * indicates amplicon product was used to prepare positive control standards for RT-qPCR. ~ Position is with reference to NCBI records GQ904198 (RNA 1) or GQ904199 (RNA 2).

PCR format	Primer Name	Sequence (5'-3')	RNA segment Target	Position~	Tm Primer (°C)
RT-qPCR	qR1T-F	GCTACCGCCTGTTGACCTC	1	140	61
	qR1T-R	TTGTTTCTTCTCAGCGATGATGC	1	219	64
	qR1T-Probe	TGGCGAATCCTCAACACGTCC	1	171	
RT-PCR	R1F1*	CACTTACGCAAGGTTACCG	1	48	60
	R1 F2	CACGGGTCACGTCAGTTCTA	1	419	63
	R1R4	TCAACTCATGCATGTCCAC	1	523	60
	R1F3	CACGGGTCACGTCAGTTCTA	1	935	63
	R1R3	CTCAGAGATGTAAGTGACTG	1	998	52
	R1R5*	TCTGCTGCTCCTCGACATAC	1	1527	63
	R1F5	AGCAGACCAAGCCGTTACAG	1	1541	64
	R1F4	CGTGCAGTCGCCATTAAG	1	2295	63
	R1R2	AATCTCTGACTGGGTATCAC	1	2339	55
	R1R1	GAAGCGTAGGACAGCATAAAGC	1	3022	60
RT-qPCR	qR2T-F	CTTCCTGCCTGATCCAACTG	2	401	62
	qR2T-R	GTTCTGCTTTCCCACCATTTG	2	476	61
	qR2T-Probe	CAACGACTGCACCACGAGTGG	2	454	
RT-PCR	R2F1*	CATATGGTACGCAARGGTGA	2	3	59
	R2R1*	CTCGAGTTAGTTTTCCGAGTCA	2	1023	59

2.2.5 Gel Electrophoresis

PCR amplicons were visualized by agarose gel electrophoresis. Agarose (Cat. 9010B, Scientifix, Victoria) gel was prepared and run in 1x TAE (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) buffer was stained with 0.05 µL ml-1 Gel Red (Cat. 41003, Biotium, Fisher Biotec, Australia). The gels were loaded with 20 µL of PCR product and subjected to 60 min at 100 volts with a Hyper Ladder 1 kb DNA ladder (BIO-33025, Bioline, NSW) (1 % gel). The gels were visualised on a UV transilluminator.

2.2.6 Preparation of viral extract from natural VER outbreaks.

Viral extract for experimental challenge was prepared from the *E.coioides* VER outbreak. Figure 2.1 displays image of pond culture and fish collected during the VER outbreak. The fish, of average body mass of ~600 g were cultured in cages in commercial earth ponds in North Queensland, Australia. Viral extract was prepared by separately pooling the brain and eyes from several fish into sterile phosphate buffered saline (PBS) (Sigma Aldrich®). Brain tissue (8.3 g) was suspended in 57 mL and eye (18.9 g) was suspended in 100 mL of sterile PBS. Tissue in PBS was frozen and thawed thrice from -25˚C to 4˚C, and homogenized using an Ultra-Turrax T 25 (IKA works) at 20,000 rpm for 5 min. Tubes of tissue were held in crushed ice throughout the homogenization. Tissue homogenate was clarified in a Sorval RC 6+ centrifuge (Thermo Scientific) using a F12s-6 x 500 LEX rotor for 10 min at 610 g at 4˚C. The supernatant was removed and further clarified by centrifugation for 10 min at 3 803 g at 4 ˚C. The supernatant was filtered through 0.45 µm and 0.22 µm filters (Sartorius). The extract was confirmed free of culturable bacteria by inoculation onto sheep blood agar and overnight incubation at 28 ˚C. Separate filtered supernatants from each tissue, herein termed viral extract, was stored in sterile 50 mL centrifuge tubes at -20˚C.

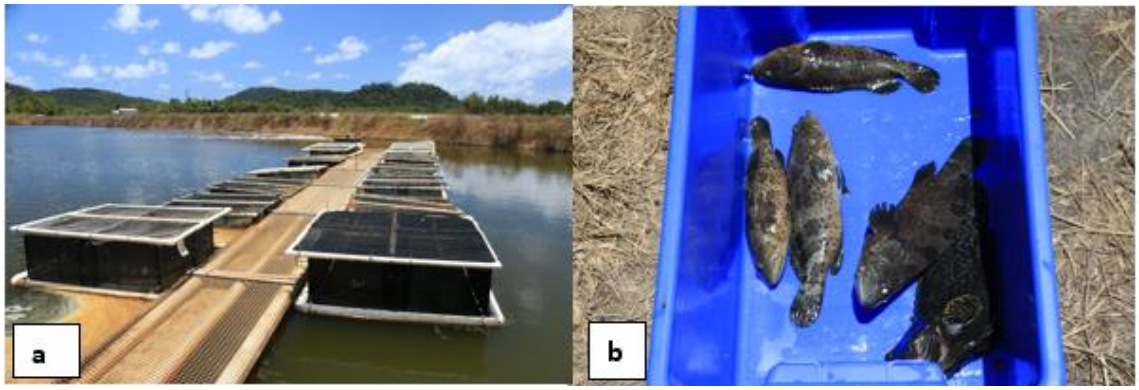

Figure 2-1: Pond culture and *E.coioides* collected from a natural outbreak of VER used to prepare viral extract. (a) Fish were held in cages within 1-hectare earthen ponds containing estuarine water. (b). Euthanased gold-spotted grouper, *E.coioides*, that exhibited signs of VER. Fish displayed erratic swimming and hyperinflation of swim bladder but appeared otherwise healthy.

2.2.7 Cloning and sequencing of PCR products

PCR amplicons were excised from gels using individual scalpel blades (SBLDCL, Livingstone, NSW) and purified using an Agarose Gel DNA Extraction Kit (Bioline). Purified amplicons were cloned into competent *Escherichia coli* JM109 cells (A1360, Promega, NSW) using the pGEM-T Easy Vector System (A1380, Promega, NSW), or One shot TOP10 chemically competent *E. coli* using the pCR4-TOPO TA vector (K4575-01, Life Technologies, VIC) as per manufacturer's instructions. Transformed E. coli were grown for 16 - 18 hours at 37 °C on lysogeny broth (LB) (Bertani, 1951) agar (Appendix 1) supplemented with 100 μg mL^{-1} or 50 μg mL^{-1} ampicillin (Cat. A9393-5G Sigma-Aldrich, NSW) for pGEM™ and TOPO™ clones respectively. Three white colonies were selected from each agar plate and cultured at 150 rpm (Bioline incubator shaker 8500 Edwards Instruments, NSW) for 16 - 18 hours in lysogeny broth (Bertani, 1951) (Appendix 1) supplemented with 100 μg mL^{-1} or 50 μg mL^{-1} ampicillin (A9393-5G, Sigma-Aldrich, NSW) for pGEM™ and TOPO™ cells respectively. Plasmid DNA was extracted using a High Pure™ Plasmid Isolation kit (11754777001 Roche, NSW) as per manufacturer's instruction. Plasmid extracts were submitted for Sanger sequencing to Macrogen Inc. (Seoul, Korea) or the Australian Genome Research Facility (Brisbane, Australia) for sequencing analysis. Bioinformatic analysis was conducted to confirm Betanodavirus genome sequence and assess suitability of the plasmids for application in subsequent qPCR analysis.

2.2.8 Bioinformatics analysis

Sequencing data was analysed using Geneious 9.1 or Geneious Prime (created by Biomatters, available from http://www.geneious.com), and the tools available at National Centre for Biotechnology Information (NCBI). These included Basic Local Alignment Search Tool (BLASTn and BLASTx) and Open Reading Frame (ORF) finder tool using the default parameters of all of the programs (http://www.ncbi.nlm.nih.gov).

2.2.9 Real-time PCR of viral extract

All cDNA reactions were performed as previously described in 2.1.3. For quantification of viral copy number, real-time PCR was performed using a Rotor Gene 6000 system (Qiagen®). All qPCRs were performed as separate assays in 20 μL reactions using SensiFAST™ Probe No ROX master mix (Bioline) according to the manufacturer's instructions including 2.5 μL of cDNA the primer (200 nM) and probe (5 nM) sequences (Hick & Whittington, 2010; see Table 1). The thermal cycle profile consisted of a 3 min incubation at 95 ^{o}C followed by 45 cycles of 15 s denaturation at 95 ^{o}C, 30 s annealing and extension at 60 ^{o}C.

2.3 Husbandry and handling of groupers

2.3.1 Husbandry of groupers

For experimental challenges, juvenile giant Queensland grouper, *Epinephelus lanceolatus*, were provided by a commercial hatchery, The Company One (Cairns, Australia). Fish were held under the conditions approved within the JCU institutional animal ethics permits outlined in the declaration of ethics (page iv). Experimental animals were held in recirculation systems that consisted of 10 replicate systems of 2 x 300 L tanks with aeration, water circulation and connected to a biological filter containing 200L of filter material and bio-wheels. The entire system was housed in an experimental room with 12/12h controlled light/dark cycle and water temperature was maintained between 24-28˚C. Saltwater was obtained from the Australian Institute of Marine Science. Water was collected from the ocean and subjected to 5 treatments which included 5mm screen within a high-density polyethylene basket; a timex hydroclone that removed particles down to 120 µm; Arkal spin filter 120 µm discs; fractionation and a final ultrafiltration to 0.04 µm. Seawater concentration ranged from 26 to 35 ppt. Fish were fed twice daily *ad lib* on commercial fish feed pellet appropriate to the size of the fish (Ridley AgriProducts Pty Ltd). Fish were acclimated for two weeks prior to commencement of any experimental procedures.

2.3.2 Anaesthesia of groupers

2.3.2.1 Sedation of groupers

Fish were sedated to Stage 2 of fish anaesthesia (Coyle et al., 2004) using AQUI-S® anaesthetic (AQUI-S® New Zealand Ltd).

Briefly fish were placed in a 40L Nally®Bin containing 30ppt seawater containing a working solution of 15-20mg/L of AQUI-S®. For sedation, fish were held for sedation until they reached a state of reduced swimming and breathing and partial loss of equilibrium. Fish retained reaction to touch stimuli. Fish were sedated with AQUI-S® (AQUI-S) during all procedures that were assessed as causing minor distress, which were principally all processes involving injection.

For recovery fish were placed into a 40L Nally®Bin containing 30ppt seawater and gently assisted to swim in a figure 8 direction ensuring they were always moving in a forward-facing motion. When fish escape behaviour progressed beyond gentle, they were released into the secondary container in the recovery tank. The secondary container consisted of a 20 L plastic container with numerous small holes drilled into the container to allow water flow. When an entire tank consignment had been subject to procedures, they were transferred to their

experimental holding tank using the secondary container. Aeration of water was provided throughout the procedure.

2.3.2.2 Euthanasia of groupers

Fish were sedated to Stage 4 of fish anaesthesia (Coyle et al., 2004) using AQUI-S®. Briefly fish were placed in a 10L Bucket containing 30ppt seawater containing a working solution of 15-20mg/L of AQUI-S. For euthanasia, fish were held until death. Aeration of water was provided throughout the procedure.

2.3.3 Intramuscular injection of groupers

All injection procedures, including vaccination and challenge with viral extract, were conducted as intramuscular (IM) injection on sedated fish. Fish were injected into the dorsal musculature using a sterile 25g x 5/8" Microlance™ needle (Becton Dickinson Ireland) attached to a 1mL Terumo® syringe (Terumo Philippines). The site of injection was at the posterior dorsal junction between the white and black marking on the juvenile *E.lanceolatus* (Figure 2.2). To prevent possible skin infection, a drop of Betadine® solution (10% povidone-iodine) was placed on the injection site immediately prior to and following injection. To reduce irritation to skin, fish were handled with clean wet hands and placed on a clean wet cotton clot during injection. The approximate length from the anterior end of the bottom jaw to the end of the tail was measured with a ruler. (Figure 2.2). Approximate weight of fish was measured with a portable 2kg digital kitchen scale (Propert).

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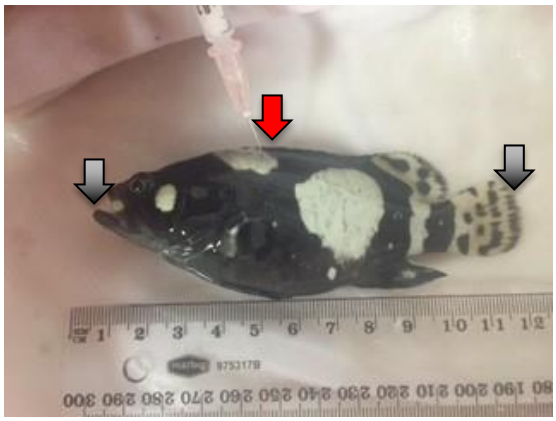


Figure 2-2: Sedated juvenile *E.lanceolatus* with location of IM vaccine injection site indicated (Red arrow) and reference points of body positions (jaw to tail) used to determine approximate body length. Image from fish used in experimental trial described in Chapter 8.

2.3.4 Pre-trial experiments

Prior to the commencement of each experimental challenge discussed in Chapters 7, 8 and 10, two experimental procedures were conducted namely:

1. Confirmation of VNN free status of experimental fish.
2. Confirmation of pathogenicity of the viral extract.

Confirmation of VNN free status was determined by conducting RT-qPCR (as discussed in 2.1.9) analysis on two pools of brain and eye tissue from ten fish using qR2T i.e. four RT-qPCR samples including two samples each of pooled brain from five individual fish and pooled eye from five individual fish.

Confirmation of pathogenicity of the viral extract was confirmed by challenging ten fish from each experimental cohort with 100µl IM injection of RGNNV extract prepared as described in 2.1.6. Viral extract was injected in the approximate position of the vaccine injection indicated in Figure 2.2. The ten fish were held in the same experimental circulation systems under the conditions described in 2.2.1. Fish were monitored twice daily for signs of adverse health and euthanized by overdose to Aqui-S when more than one sign of VER, including erratic swimming, hyperinflation of swim bladder, disorientated floating, absence of feeding or severe lethargy became evident. Analysis by RT-qPCR was conducted on pooled brain and eye tissue as described in 2.1.9 to confirm detection of high copy number of RGNNV genome.

2.4 Results

2.4.1 Cloning and sequencing of PCR products

Results of cloning and sequencing of PCR products are discussed in Chapter 3: Phylogenetic comparison of Betanodavirus genomes collected from North Queensland.

2.4.2 Real-time PCR of viral extract

Analysis of viral extract by RT-qPCR detected RGNNV with a Ct value of 13.95 (qR1T) and 10.64 (qR2T) which equates to an estimated calculated viral copy number of 1.99×10^7 and 1.07×10^8 copies mL^{-1} respectively. The average between each of the two copy number calculations, 6.36×10^7 copies mL^{-1} was accepted in subsequent calculations for preparing viral extract for challenge studies. Viral extract was diluted to an approximate copy number of 6.36×10^4 mL^{-1} in sterile PBS for challenge studies. Further discussion of RT-qPCR analysis is provided in Chapters 7, 8 and 10.

2.4.3 *Pre-trial experiments*

All cohorts of experimental fish were negative for the detection of RGNNV by RT-qPCR prior to the commencement of experiments. In all three pre-experimental pilot trials, IM injection with the viral extract lead to 100% morbidity in the challenged fish within 14 days post injection. The clinical signs of VER appeared suddenly at days 7-10 post challenge, followed by rapid, mass morbidity during the subsequent 1 to 3 days (Figure 2-3).

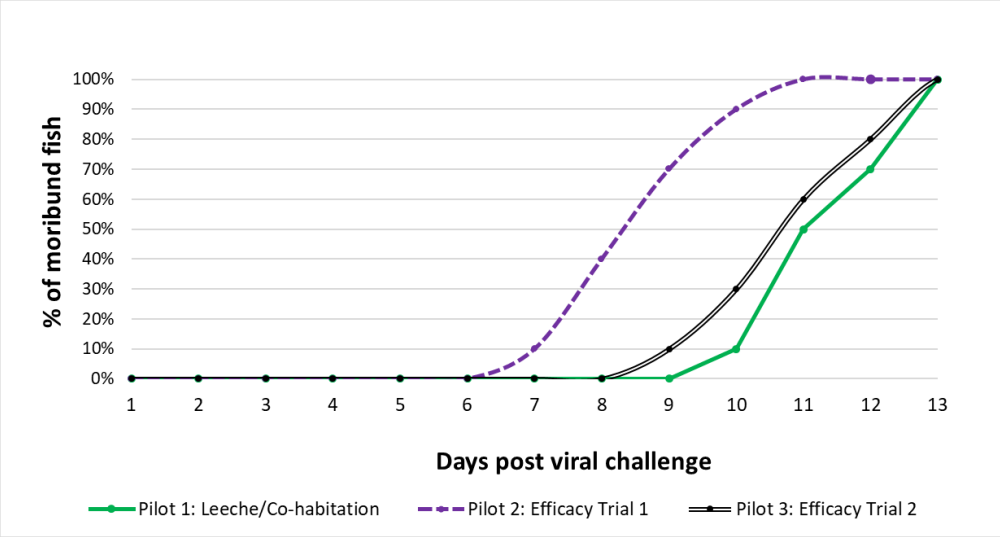


Figure 2-3: Cumulative Morbidity (%) of *E.lanceolatus* v days post challenge during the three pilot studies conducted prior to challenge trials to confirm viability of viral extract.

2.5 Conclusion

The aims of this chapter were met in the following manner:

- The methods that were used as standard operational procedures namely husbandry and handling of fish, pre-trial experiments and a range of molecular biology protocols were described.
- The protocols that were completed once but apply to multiple chapters of this thesis were described.

CHAPTER 3. PHYLOGENETIC COMPARISON OF BETANODAVIRUS GENOMES COLLECTED FROM VIRAL ENCEPHALOPATY AND RETINOPATHY OUTBREAKS IN NORTH QUEENSLAND

Background

- At the commencement of this study there were 2 complete sequences of *Betanodavirus* RNA 1 and RNA 2 from Australian species, neither of which originated from North Queensland.
- The capsid protein, which is encoded by RNA 2, has been demonstrated as the antigenic target of grouper antibodies to protect against VER.
- There is variation in the capsid protein across the Betanodaviruses.
- Knowledge of the genome sequences and level of variation in the RNA 2 of Betanodavirus strains that cause VER in Northern Queensland is required to guide decisions of vaccine development and design of dsRNA.
- Knowledge of the genome sequences of RNA 1 is required to guide decisions for dsRNA design to attempt NNV knockdown.
- PCR amplicons from RNA 1 and RNA 2 are required to prepare synthetic positive controls for the RT-qPCRs.

Aims of this Chapter

- Obtain a viable viral extract from a natural VER outbreak in Northern Queensland.
- Obtain RT-qPCR amplicons from the NNV strains associated with VER outbreaks in North Queensland.
- Determine the complete sequence of both mRNA segments of *Betanodavirus* associated with natural VER outbreaks in marine finfish grow-out systems in Northern Queensland.
- Compare the complete sequences of mRNA 1 to other sequences in the NCBI database to determine the species of Betanodavirus associated with VER in North Queensland.
- Compare the complete sequences of RNA 2 to other sequences in the NCBI database to determine conservation of the capsid protein temporally and geographically.

3.1 Introduction

Knowledge of the RNA 2 segment sequence that encodes the capsid protein of strains causing VER in Australian grouper is critical to multiple decisions regarding vaccine formulation. In addition to ensuring targeted protection against Betanodavirus strains that are associated with VER outbreaks, herein referred to as NNV strains, knowledge of capsid protein sequence assists in determining:

- the level of variation in NNV strains that cause VER;
- the potential geographic market of an effective vaccine and
- the duration for which the vaccine could be expected to be effective considering the typically rapid mutation rate of RNA viruses.

Although North Queensland was among the first regions in the world to report VER outbreaks, there are few genome sequences of Australian NNV strains. At the commencement of this study, there were only two complete genome sequences of Betanodavirus from Australian species within the NCBI database, neither of which originated from Queensland (Hick & Whittington, 2010). There are a further eight partial sequences of RNA 2 segments collected from Australian species (Moody et al., 2009).

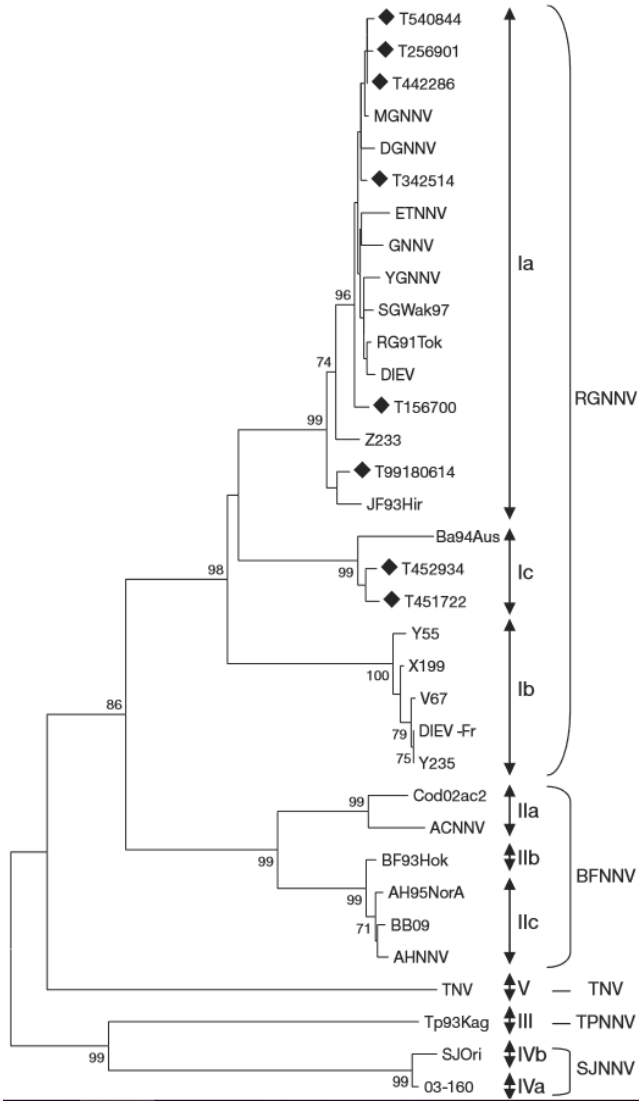
The current species classification of Betanodavirus recognised by the ICTV, named with the original host names of striped jack, red-spotted grouper, barfin flounder and tiger puffer was initially based on analysis of a 606nt conserved region, termed T2 and a 289nt highly variable region, termed T4 of the RNA 2 (Nishizawa et al., 1997). The species divisions align with three distinct serotype divisions determined by antibody reaction to the capsid protein which is encoded by RNA 2 (Nishizawa et al., 1997, Johansen et al., 2004 and Mori et al., 2003) (Table 3.1). Numerous serological studies have reported a lack of *in vitro* and *in vivo* cross-reactivity between two species namely the RGNNV and SJNNV (Mori et al., 2003; Chi et al., 2003 and Pascoli et al., 2019). However, there is some cross reactivity between RGNNV and BFNNV (Mori et al., 2003).

In recognition of an expanded host range beyond those of the original species names, Thiery et al. (2004) proposed a division of the Betanodavirus into subgroup clusters based on analysis of the highly variable region (T4) of RNA 2. The cluster proposal retained TPNNV and SJNNV as distinct single clusters, Clusters III and IV respectively but further differentiated the RGNNV into two, namely Ia and Ib and BFNNV into three: IIa, IIb and IIc. The clusters, based on nucleotide comparison of the T4 region, also tended to align with temperature and geographic origin (Thiery et al., 2004) (Table 3.1).

Table 3-1: Betanodavirus Type Species, Serotypes and Thiery cluster assignments based on conserved capsid protein amino acid

Acronym	Betanodavirus species	Conserved aa motif	Serotype	Thiery cluster
SJNNV	Striped Jack Nervous Necrosis Virus	PAN	A	IV
RGNNV	Red spotted Grouper Nervous Necrosis Virus	PDG	C	Ia,Ib
BFNNV	Barfin Flounder Nervous Necrosis Virus	PEG	C	IIa, IIb,IIIc
TPNNV	Tiger Puffer Nervous Necrosis Virus	PPG	B	III
**TNNV*	*Turbot Nervous Necrosis Virus	PTG	untested	
Compiled from:	(Nishizawa et al. 1997) & (Johansen et al. 2004) & (Mori et al. 2003) & (Thiery et al. 2004)			
	**proposed as an additional species by Johansen et al. 2004*			

Although the ICTV has not adopted the model proposed by Thiery et al. (2004), subsequent authors have applied the cluster proposal in taxonomic comparison of NNV strains (Olveira et al., 2009 & Moody et al., 2009). Phylogenetic comparison between NNV strains from Australian fish species are limited to two publications. (Moody et al., 2009 & Hick et al., 2013). Moody et al. (2009) reported a phylogenetic comparison between the T2 region of four Betanodavirus strains collected from tropical Australian species. Based on T2, all Australian strains clustered within the RGNNV assignment of Nishizawa (1994) or the equivalent 1a of Thiery et al. (2004). Comparative analysis between nine, smaller, T4 region sequences, divided the Australian strains into two clusters, Ia and Ic based on the Theiry et al. (2004) model. Moody et al. (2009) also noted the clusters retained some general geographic alignment which included strains from tropical and cold-water species (Figure 3-1) although this was not consistent within 1a.



Details of Australian sourced strains

Strain Id	Host species	Location and year
T540844	*Epinephelus coioides*	QLD Aus 2005
T256901	*Latris lineata*	TAS Aus 2002
T442286	*Cromileptes altivelis*	QLD Aus 2004
T342514	*Lates calcarifer*	QLD Aus 2003
T156700	*Lates calcarifer*	NT Aus 2001
T99180614	*Oxyeleotris lineolata*	QLD Aus 1999
T452934	*Lates calcarifer*	SA Aus 2004
T451722	*Macquaria novemaculata*	NSW Aus 2004
BA94Aus	*Lates calcarifer*	Aus 1994

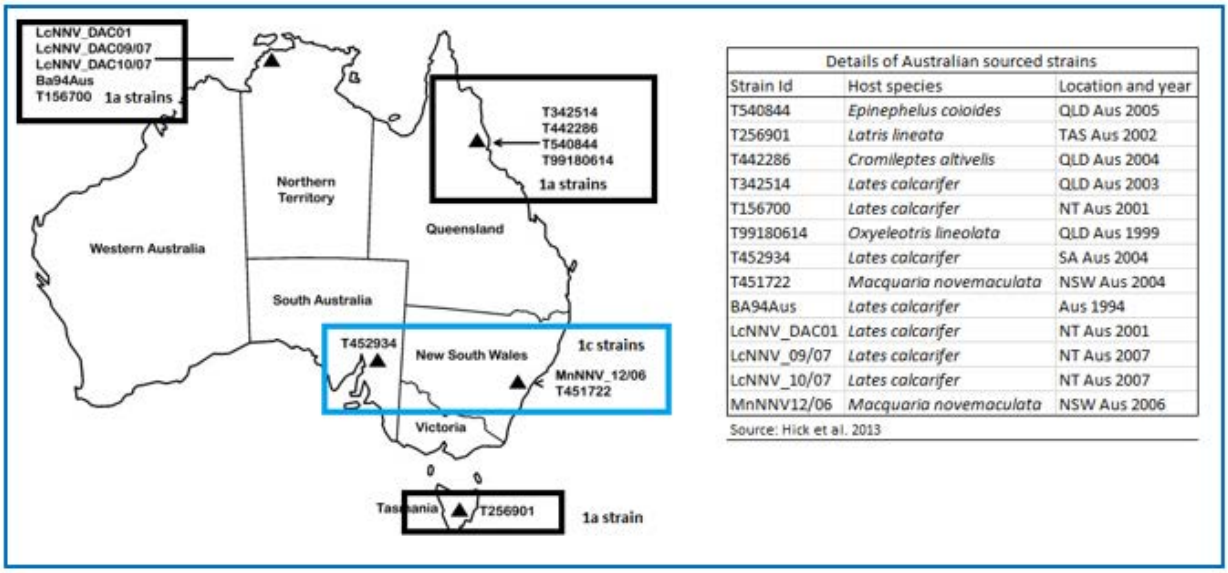
Source: Moody et al. 2009

Ba94AUS and sequences indicated with ◆ are sequences derived from Australian fish species.

Figure 3-1: Unrooted phylogenetic tree produced from partial T4 region (289 nt) of RNA 2 reproduced from Moody et al. (2009) including strain identifiers and source of strain.

Due to the early focus on the T4 and T2 regions of RNA 2 for a taxonomic division of Betanodaviruses, most sequences published within the NCBI database before 2008 are incomplete genome sequences. Subsequent research indicated regions outside of T4 on RNA 2 and also regions on RNA 1 that relate to virulence, infectivity and adaption to temperature increases (Costa et al., 2007; Hata et al., 2010; Souto et al., 2018 and Souto et al., 2019). Further, chimeric recombinants of RNA 1 and RNA 2 between RGNNV and SJNNV species have been detected in diseased fish (Olveira et al., 2009). Noting the inherent weakness in conducting comparative analysis based on the T4 or T2 region, more recent works have compared the entire RNA 1 and RNA 2 sequences of NNV. (Hick et al., 2013 and Olveira et al., 2009). For Australian strains, comparative analysis including complete mRNA sequences obtained from VER outbreaks in *Lates calcarifer* in Northern Territory and *Macquaria*

novemaculata in New South Wales have been reported. (Hick et al., 2013). The authors formed a similar conclusion to Moody et al. (2009) regarding the taxonomic division of Australian NNV strains with an exception of aligning the Ba94Aus strain, collected from barramundi, within the tropical strains 1a rather than 1c temperate strains. (Figure 3.2). Although the divisions of Australian NNV strains tend to align with geographic source, the strain collected from striped trumpeter, *Latris lineata*, from Tasmania aligns more to the tropical species division. It is possible the divisions between 1a and 1c in Australian strains relate to other factors such as marine v freshwater systems. The striped trumpeter is a marine species whereas the Australian bass, *M.novemaculata* is a freshwater species. Although barramundi, *L.calcarifer* are naturally found in tropical waters, as a euryhaline species, it has been translocated into temperate regions in Australia where is cultured within systems that have access to naturally heated spring water or apply heated spring water to warm marine systems. There was no information in the NCBI database entry to indicate if the strain from barramundi collected from South Australia (T452934) was from a marine or freshwater system.



Details of Australian sourced strains

Strain Id	Host species	Location and year
T540844	*Epinephelus coioides*	QLD Aus 2005
T256901	*Latris lineata*	TAS Aus 2002
T442286	*Cromileptes altivelis*	QLD Aus 2004
T342514	*Lates calcarifer*	QLD Aus 2003
T156700	*Lates calcarifer*	NT Aus 2001
T99180614	*Oxyeleotris lineolata*	QLD Aus 1999
T452934	*Lates calcarifer*	SA Aus 2004
T451722	*Macquaria novemaculata*	NSW Aus 2004
BA94Aus	*Lates calcarifer*	Aus 1994
LcNNV_DAC01	*Lates calcarifer*	NT Aus 2001
LcNNV_09/07	*Lates calcarifer*	NT Aus 2007
LcNNV_10/07	*Lates calcarifer*	NT Aus 2007
MnNNV12/06	*Macquaria novemaculata*	NSW Aus 2006

Source: Hick et al. 2013

Figure 3-2: Summary of strain classification based on NNV RNA 2 sequences derived from Australian fish species (including strain reference identified and source). Modified from Hick et al. (2013). The "tropical" strains are aligned within 1a cluster (Black rectangle) and "temperate" strains aligned within 1c cluster (blue rectangle).

Whilst there has been no detection of any Betanodavirus strain other than RGNNV in Australia, the division of strains into clusters indicates a degree of variability between Australian NNV strains. The scarcity of complete sequence data of tropical NNV strains within prior work and the large financial cost of vaccine production, necessitates careful investigation into the complete genome sequences of strains causing VER in tropical grouper species and other

species within their geographic range of grouper culture. This study reports on the complete mRNA sequence of *Betanodavirus* associated with natural VER outbreaks in marine finfish grow-out systems in Northern Queensland and their phylogenetic position within the collection of NNV strains affecting Australian fish species. Knowledge of the complete mRNA sequences of the strains of NNV that cause VER in grouper in North Queensland is required to ensure the therapies to be prepared within this project specifically target those strains that are present in Northern Queensland.

3.2 Materials and Methods

3.2.1 Collection of samples from natural VER outbreaks

Samples of brain and eye were collected from fish from three aquaculture farms in Northern Queensland displaying clinical signs of VER. Viral Encephalopathy and Retinopathy was confirmed by the Queensland Government Biosecurity Sciences Veterinary Diagnostic laboratory (BSL) (farmers *personal comms*.). In this study, RT-PCR and sequencing analysis was performed to confirm VER. A summary of the sample source is provided in Table 3.3.

3.2.2 Sequencing of Betanodavirus from natural disease outbreaks

Total viral nucleic extracts were prepared by dissecting eye and brain tissues from frozen fish collected during natural VER outbreaks. Extraction was completed using the High Pure™ Viral Nucleic Acid kit (Roche 11858874001) according to the manufacturer's instructions. Cloning and sequencing was conducted as previously described in 2.1.3 to 2.1.7 using the primers details in Table 2.1 (Section 2.1.4).

3.2.3 Additional Sequences obtained for phylogenetic analysis

Sequences of RNA 1 and RNA 2, were compared with strains obtained from the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) nucleotide database including reference strains of Betanodavirus species and similar geographic source (Australia). Sequences of NNV from each of the reference strains of Betanodavirus species are indicated in Table 3.2. Sequences of NNV obtained from Australian fish were collected from the NCBI data base and other researchers as indicated in Table 3.3.

Table 3-2: Details of Reference strains and sequences of NNV type species used in this study including species, RNA segment, strain identification, NCBI reference number, length of sequence, host species, year and location of strain and publication relating to the sequence.

Species	Strain Id	NCBI accession	length (nt)	Host species	Location and year	Publication
RNA-1						
RGNNV	SGWak97	AY324869	3105	Red-spotted grouper	Japan 1997	Iwamoto et al., 2001
SJNNV	SJNNV	AB025018	3081	Striped Jack	Japan 1999	Nagai & Nishizawa np.
TPNNV	TPKag93	EU236148	3112	Tiger Puffer	Japan 1993	Okinaka *np.
BFNNV	JFwa98	EU236146	3100	Barfin Flounder	Japan 1998	Okinaka *np.
RNA-2						
RGNNV	SGWak97	AY324870	1434	Seven Band grouper	Japan 1997	Iwamoto et al., 2001
RGNNV	RGNNV	AY690596	1432	Redspotted grouper	China	
SJNNV	SJNNV	NC003449	1421	Striped Jack	Japan 1999	Iwamoto et al., 2001
TPNNV	TPKag93	EU236149	1422	Tiger Puffer	Japan 1993	Okinaka *np.
BFNNV	BF93Hok	EU826138	1433	Barfin Flounder	Japan 1993	Okinaka *np.

*np. Indicates sequence is a direct submission to NCBI and has no published journal article.

Table 3-3: Details of Australian sourced NNV sequences used in this study including species, RNA segment, strain, NCBI reference number, length of sequence, host species, year and location of strain and publication relating to the sequence.

Strain Id	NCBI accession	length (nt)	Host species	Location and year	Publication
RNA-1					
P14-10380	KT390712	2871	*E. lanceolatus*	QLD Aus 2014	Agnithortri et al. (2015)
Lc3NQAus	**MH181161**	**3090**	***Lates calcarifer***	**QLD Aus 2013**	**Condon et al. (2019)**
MnNNV12/06	GQ402012	2998	*M. novemaculata*	NSW Aus 2006	Hick et al. (2013)
LcNNV_09/07	GQ402010	2998	*Lates calcarifer*	NT Aus 2007	Hick et al. (2013)
Ec2NQAus		**3090**	***E. coioides***	**QLD Aus 2015**	**This study**
El1NQAus		**3090**	***E. lanceolatus***	**QLD Aus 2013**	**This study**
RNA-2					
T540844	EF591369	606	*E. coioides*	QLD Aus 2005	Moody et al. (2009)
T256901	EF591370	289	*Latris lineata*	TAS Aus 2002	Moody et al. (2009)
T442286	EF591371	832	*Cromileptes altivelis*	QLD Aus 2004	Moody et al. (2009)
T342514	EF591372	832	*Lates calcarifer*	QLD Aus 2003	Moody et al. (2009)
T156700	EF591367	289	*Lates calcarifer*	NT Aus 2001	Moody et al. (2009)
T99180614	EF591368	832	*Oxyeleotris lineolata*	QLD Aus 1999	Moody et al. (2009)
T452938	EF591366	294	*Lates calcarifer*	SA Aus 2004	Moody et al. (2009)
T451722	EF591365	294	*M. novemaculata*	NSW Aus 2004	Moody et al. (2009)
LcNNV_09/07	GQ402011	1017	*Lates calcarifer*	NT Aus 2007	Hick et al. (2013)
MnNNV12/06	GQ402013	1017	*M. novemaculata*	NSW Aus 2006	Hick et al. (2013)
P14-10380	KT390714	990	*E. lanceolatus*	QLD Aus 2014	Agnithortri et al. (2015)
Lc3NQAus	**MH017207**	**1017**	***Lates calcarifer***	**QLD Aus 2013**	**Condon et al. (2019)**
Ec2NQAus			***E. coioides***	**QLD Aus 2015**	**This study**
El1NQAus			***E. lanceolatus***	**QLD Aus 2013**	**This study**

E. indicates *Epinephelus M.* indicates *Macquaria.* Sequences obtained within this study are indicated in **bold.**

3.2.4 Sequence alignment

Sequencing data was analysed as previously described in 2.1.6 and 2.1.7. The depth of sequence coverage was a minimum of 3 sequences reads per nucleotide over the entire published sequence. Phylogenetic comparison between sequences was conducted using the Geneious v9.1 software, using the default algorithm settings. Neighbourhood joining trees were prepared using the Tamura-Nei genetic distance model (Biomatters http://www.geneious.com).

3.3 Results

Complete mRNA sequences were obtained from the *Lates calcarifer* (ref Lc3NQAus) , *Epinephelus coioides* (ref Ec2NQAus) and *Epinephelus lanceolatus* (ref El3NQAus) VER outbreaks. The sequences from the *Lates calcarifer* outbreak were lodged in the NCBI nucleotide database as accessions MH181161 (RNA 1) and MH017207 (RNA 2) (Condon et al., 2019). Phylogenetic analysis of RNA 1 and RNA 2 indicate all three NNV strains collected in this study display greatest homology to the RGNNV species.

3.3.1 RNA-1

Comparison of the complete mRNA of the RNA 1 segment from the VNN strains collected in this study indicate the strains display strong nucleotide sequence homology (99-97%) to each other (Refer to Table 3.4). Comparison with the Betanodavirus species type strains indicates all 3 NQAus strains display strongest homology to the RGNNV reference strain (95-97%) with lesser homology to BFNNV (82%), SJNNV (82%) and TPNNV (82%) reference strains. (Figure 3.3 and Table 3.4). Comparison with the other Australian strains in the NCBI database indicates strong homology between all of the Australian strains (95-98%) and the original RGNNV isolated from Sevenband grouper in Japan in 1997 (Table 3.5).

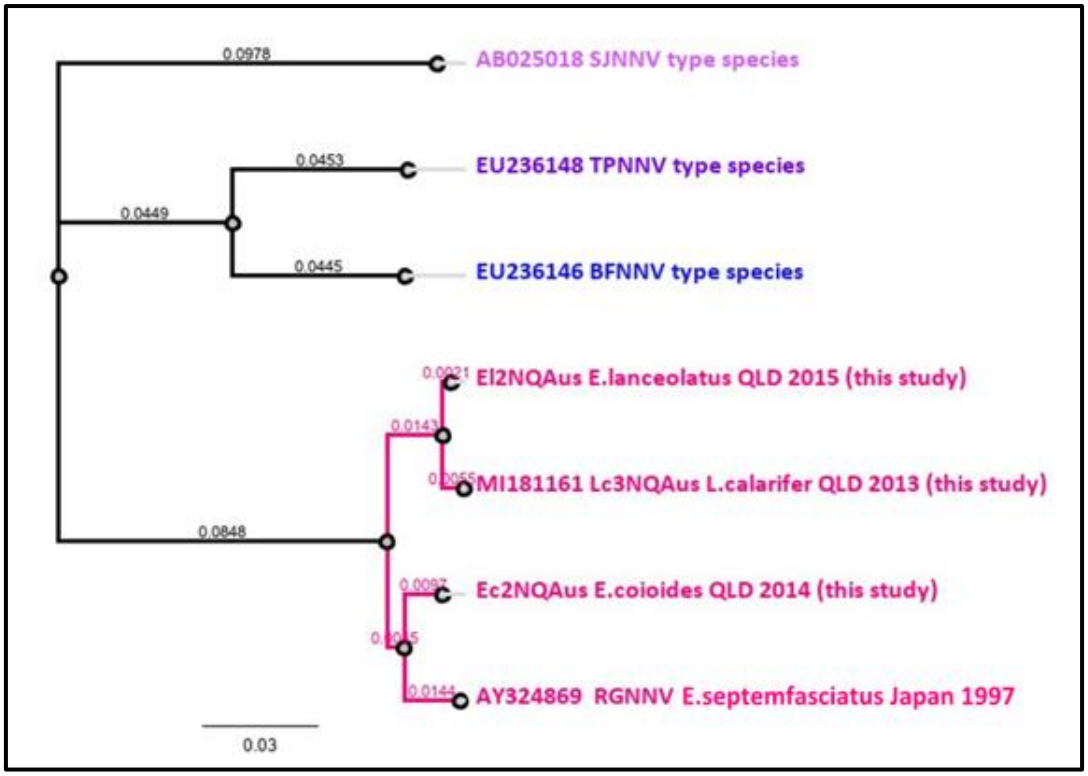


Figure 3-3: Neighbour-joining tree of mRNA RNA 1 segment of the Betanodavirus from this study aligned against the reference species strains of NNV.

Created by Geneious Prime (Biomatters) http://www.geneious.com. Strains coloured pink are RGNNV aligned.

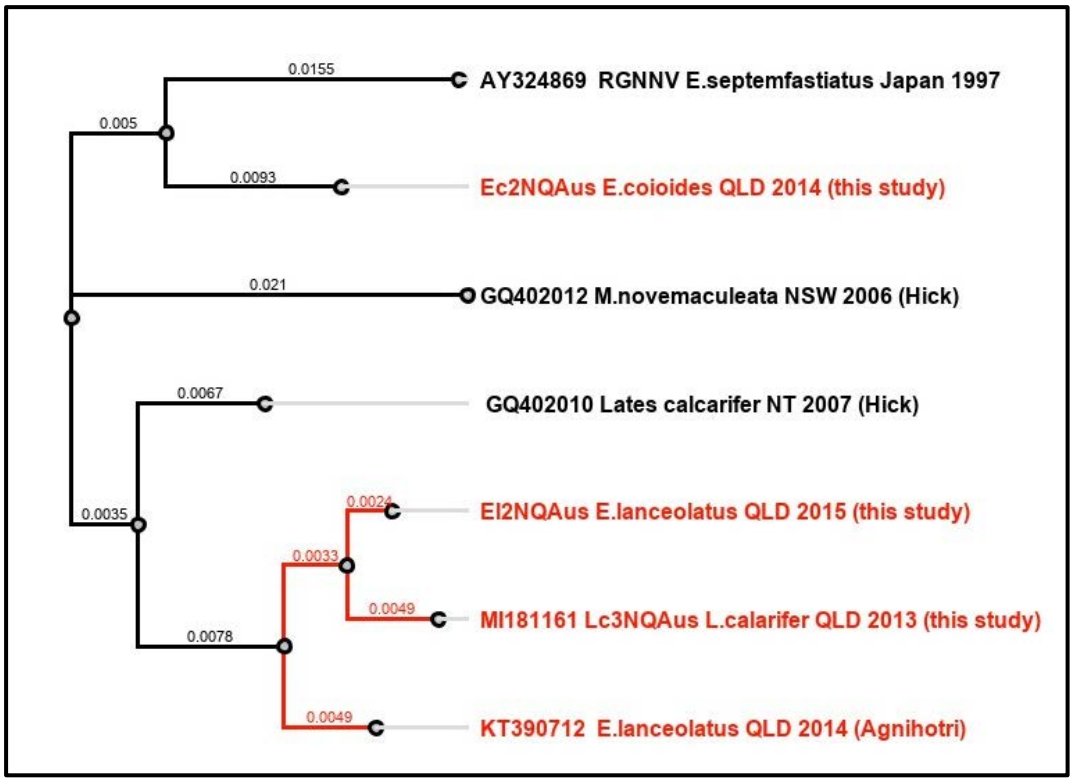


Figure 3-4: Neighbour-joining tree of mRNA RNA 1 segment of Australian strains of Betanodavirus determined by the Tamura-Nei genetic distance model

Created by Geneious Prime Biomatters http://www.geneious.com. Strains indicated in red were sourced within a ~500km coastal zone in North Queensland.

Table 3-4: Displaying Percentage Homology between mRNA sequences of RNA 1 segments from the three NNV strains sequenced in this study (El2NQAus, Lc2NQAus and Ec2NQAus) in comparison to the NNV type species RGNNV, SJNNV, BFNNV and TPNNV including details of NCBI reference, host species and location and year of collection.

Sequence Source details					**This study**			Species allocation and reference identifiers			
Strain	NCBI ref.	Length (nt)	Host species	Location	**El2NQAus**	**Ec2NQAus**	**Lc3NQAus**	AY324869	AB025018	EU236148	EU236146
								RGNNV	SJNNV	TPNNV	BFNNV
El2NQAus		**3030**	***E.lanceolatus***	**QLD Aus 2015**		**97.27**	**99.17**	**96.30**	**82.17**	**82.73**	**82.50**
Ec2NQAus		**3029**	***E.coioides***	**QLD Aus 2014**	**97.27**		**97.06**	**97.55**	**82.09**	**82.61**	**82.81**
Lc3NQAus	**MH181161**	**3090**	***L.calcarifer***	**QLD Aus 2013**	**99.17**	**97.06**		**95.69**	**81.95**	**82.53**	**82.04**
Redspotted Grouper NNV (RGNNV)	AY324869	3105	Sevenband grouper	Japan 1997	96.30	97.55	95.69		82.12	82.62	82.83
Striped Jack NNV (SJNNV)	AB025018	3081	Striped Jack	Japan 1999	82.17	82.09	81.95	82.12		82.62	83.08
Tiger Puffer NNV (TPNNV)	EU236148	3112	Tiger Puffer	Japan 1993	82.73	82.61	82.53	82.62	82.62		91.26
Barfin Flounder NNV (BFNNV)	EU236146	3101	Barfin Flounder	Japan 1993	82.50	82.81	82.04	82.83	83.08	91.26	

Strains obtained in this study are indicated in **bold** and grey shading.

Table 3-5: Displaying Percentage Homology and number of nucleotide differences between mRNA sequences of RNA 1 segments from NNV strains sequenced in this study (El2NQAus, Lc2NQAus and Ec2NQAus) in comparison to the other Australian-sourced strains of NNV including NCBI reference, host species, location and year of reference.

Source of Sequence			Global Zone, Habitat type and % homology in RNA 1 sequences						
			Tropical	**Tropical**	Tropical	Tropical	**Tropical**	Temperate	Temperate
			marine	**marine**	marine	marine	**marine**	freshwater	marine
Host species	**Location/Year**	**Reference No.**	**El2NQAus**	**Lc3NQAus**	KT390712	GQ402010	**Ec2NQAus**	GQ402012	AY324869
Epinephelus lanceolatus	**QLD Aus 2013**	**El2NQAus**		99.20	98.83	97.98	97.27	96.38	96.30
Lates calcarifer	**QLD Aus 2013**	**Lc3NQAus**	99.20		98.78	97.70	97.06	96.03	95.72
Epinephelus lanceolatus	QLD Aus 2014	KT397012	98.83	98.78		98.19	97.49	96.17	96.20
Lates calcarifer	NT Aus 2007	GQ402010	97.98	97.70	98.19		97.13	97.20	97.03
Epinephelus coioides	**QLD Aus 2015**	**Ec2NQAus**	97.27	97.06	97.49	97.13		95.96	97.59
Macquaria novemaculata	NSW Aus 2006	GQ402012	96.38	96.03	96.17	97.20	95.96		96.63
Seven Band grouper	Japan 1997	AY324869	96.30	95.72	96.20	97.03	97.59	96.63	

Heat map indicating closest relatedness by percentage similar (%).

3.3.2 RNA-2

Comparison of the complete mRNA of the RNA 2 segment from the VNN strains collected in this study indicate the strains display strong nucleotide sequence homology (98-99%) to each other (Refer to Table 3.6). Comparison with the Betanodavirus species type strains indicates all 3 NQAus NNV strains display strongest homology to the RGNNV reference strain (97-99%) with lesser homology to BFNNV (81-83%), SJNNV (77-78%) and TPNNV (77-78%) reference strains. (Figure 3.3 and Table 3.6). Comparison with the other Australian strains in the NCBI database reflect a similar pattern to that described by Moody et al. (2009) and Hick et al. (2013) whereby the majority of temperate strains display division from the tropical stains (Figure 3.5 and Table 3.7). Within this analysis, although closely aligned with the other tropical strains (97-98% homology), the freshwater strain collected from sleepy cod (EF591368), displays slightly less homology to the strains collected from the same geographic zone (97.98-99.65%). All other NQ region strains were collected from marine fish culture systems. The majority of Australian strains (Cluster 1a) align more closely to the *E.septemfsciatus* (Cluster 1a) strain collected from Japan in 1997 than those collected from the Australian "temperate"species within Australia (Cluster 1c).

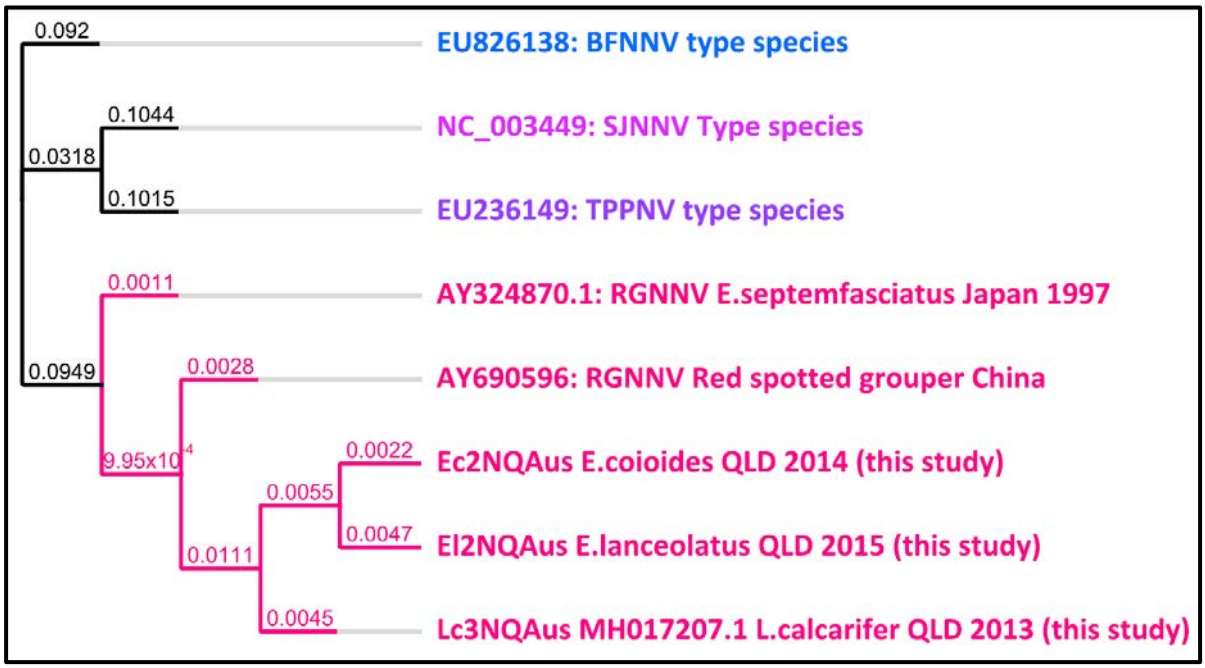


Figure 3-5: Neighbour-joining tree of mRNA RNA 2 segment of the Betanodavirus from this study aligned against the reference species strains of NNV including species and NCBI reference.
Alignment determined by the Tamura-Nei genetic distance model, created by Geneious version 9.1 Biomatters http://www.geneious.com

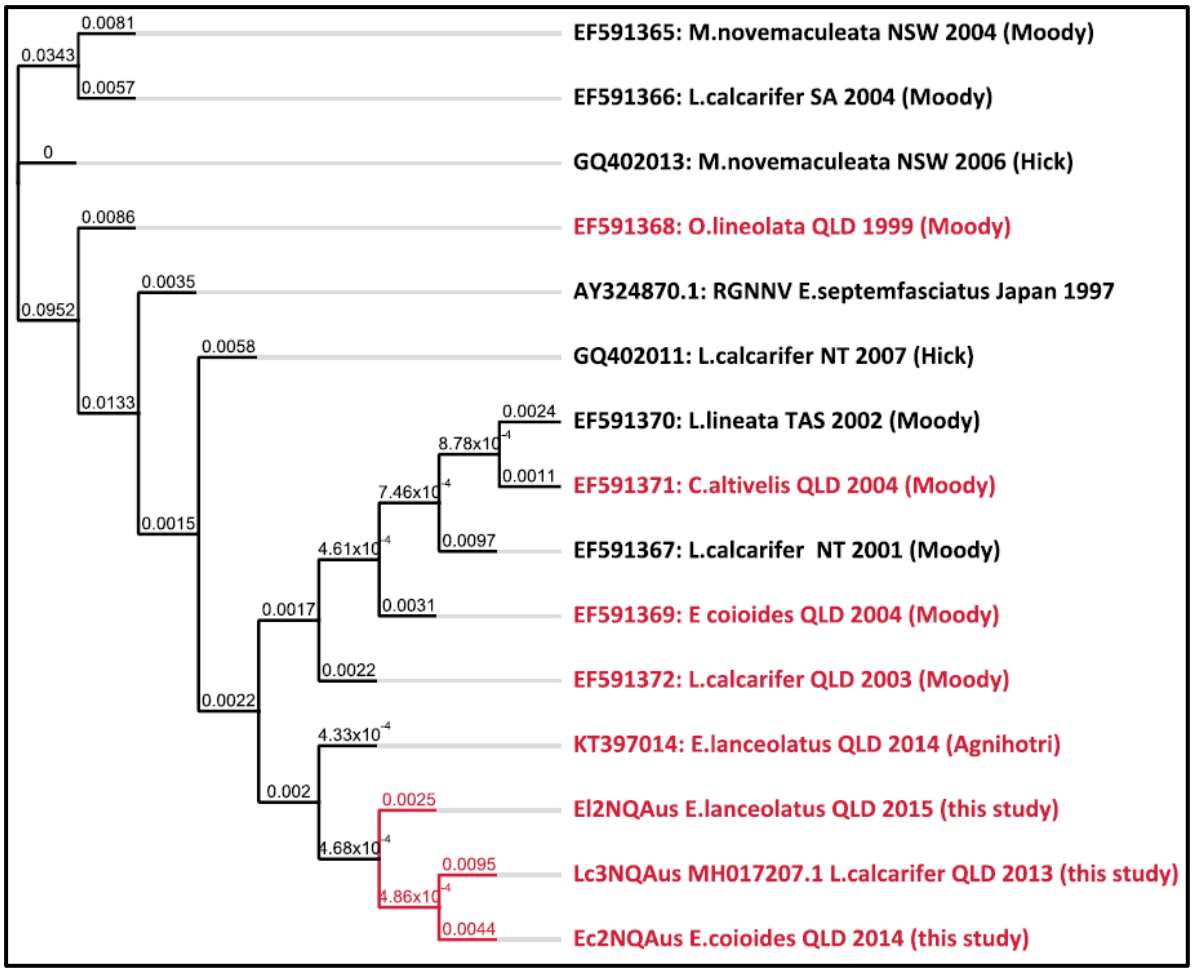


Figure 3-6: Neighbour-joining tree of mRNA RNA 2 segment of Australian strains of Betanodavirus from the NCBI database aligned against the strains collected from this study Determined by the Tamura-Nei genetic distance model created by Geneious version 9.1 using the default parameters. Biomatters http://www.geneious.com. Strains indicated in red were sourced within a ~500km coastal zone in North Queensland.

Table 3-6: Displaying Percentage Homology between mRNA sequences of RNA 2 segments from NNV strains sequenced in this study (El2NQAus, Lc2NQAus and Ec2NQAus) in comparison to the NNV type species including RGNNV, SJNNV, BFNNV and TPNNV, NCBI reference, length of sequence, host species, location and year of collection.
Strains sequenced in this study are highlighted in grey and bold.

Sequence Source details					This study			Species allocation and reference identifiers				
					Ec2NQAus	**El2NQAus**	**Lc3NQAus**	AY690596	AY324870.1	EU826138	NC_003449	EU236149
Strain	NCBI ref.	Length (nt)	Host species	Location				RGNNV	RGNNV	BFNNV	SJNNV	TPNNV
Ec2NQAus (this study)		**1032**	***E.coioides***	**QLD Aus 2015**		**99.32**	**98.62**	**97.86**	**97.96**	**82.49**	**78.26**	**78.24**
El2NQAus (this study)		**1030**	***E.lanceolatus***	**QLD Aus 2013**	**99.32**		**98.72**	**97.58**	**97.87**	**81.88**	**77.86**	**77.65**
Lc3NQAus (this study)	**MH017207**	**1017**	***L.calcarifer***	**QLD Aus 2013**	**98.62**	**98.72**		**98.23**	**98.33**	**82.50**	**78.32**	**78.20**
Redspotted grouper NNV (RGNNV)	AY690596	1432	Red-spotted grouper	China	97.86	97.58	98.23		99.44	82.47	78.46	79.29
Redspotted grouper NNV (RGNNV) SGWak97strain	AY324870	1434	Seven Band grouper	Japan 1997	97.96	97.87	98.33	99.44		82.73	78.59	79.28
Barfin Flounder NNV (BFNNV) BF93Hok strain	EU826138	1433	Barfin Flounder	Japan 1993	82.49	81.88	82.50	82.47	82.73		78.46	79.16
Striped Jack NNV (SJNNV)	NC_003449	1431	Striped Jack	Japan 1999	78.26	77.86	78.32	78.46	78.59	78.46		82.01
Tiger Puffer NNV (TPNNV) TPKag93 strain	EU236149	1422	Tiger Puffer	Japan 1993	78.24	77.65	78.20	79.29	79.28	79.16	82.01	

Table 3-7: Displaying Percentage Homology and number of nucleotide differences between mRNA sequences of RNA 2 segments from NNV strains sequenced in this study (El2NQAus, Lc2NQAus and Ec2NQAus) in comparison to the other Australian sourced strains of NNV from the NCBI database including Host species, NCBI reference, location and year of collection.

Source of Sequence			Global Zone, Habitat type, Reference No. and Matrix of homology (%) and Difference in RNA 2 sequences														
			Temperate	Tropical	Tropical	Tropical	Tropical	Tropical	**Tropical**	**Tropical**	**Tropical**	Tropical	Tropical	Tropical	Temperate	Temperate	Temperate	Zone
			marine	marine	marine	marine	marine	marine	**marine**	**marine**	**marine**	marine	marine	freshwater	freshwater	not recorded	freshwater	Habitat
Host species	**Location/Year**	**Reference No.**	EF591370	EF591371	EF591367	EF591369	EF591372	KT397014	**El2NQAus**	**Lc3NQAus**	**Ec2NQAus**	GQ402011	AY324870	EF591368	EF591365	EF591366	GQ402013	
Latris lineata	TAS Aus 2002	EF591370		99.65	98.62	99.31	99.31	99.31	98.96	98.27	98.96	98.62	98.62	96.19	85.81	85.81	85.81	% homology
Cromileptes altivelis	QLD Aus 2004	EF591371	1		98.96	99.51	99.4	99.16	99.16	98.2	98.68	98.56	99.04	97.24	86.05	86.05	92.19	
Lates calcarifer	NT Aus 2001	EF591367	4	3		98.62	98.62	98.62	98.27	97.58	98.27	97.92	97.92	96.89	86.51	86.51	86.51	
Epinephelus coioides	QLD Aus 2005	EF591369	2	3	4		99.51	99.18	99.18	98.19	98.68	98.52	99.18	96.87	85.81	85.81	90.59	
Lates calcarifer	QLD Aus 2003	EF591372	2	5	4	3		99.28	99.28	98.32	98.8	98.68	99.16	97.36	86.05	86.05	92.43	
Epinephelus lanceolatus	QLD Aus 2014	KT397014	2	7	4	5	6		99.7	98.89	99.39	98.89	99.19	97.12	85.71	86.4	92.22	
Epinephelus lanceolatus	**QLD Aus 2013**	**El2NQAus**	3	7	5	5	6	3		98.72	99.32	98.62	97.87	97.12	85.37	86.05	92.33	
Lates calcarifer	**QLD Aus 2013**	**Lc3NQAus**	5	15	7	11	14	11	13		98.62	98.13	98.33	96.15	84.69	85.37	91.84	
Epinephelus coioides	**QLD Aus 2015**	**Ec2NQAus**	3	11	5	8	10	6	7	14		98.53	97.96	96.88	85.37	86.05	92.33	
Lates calcarifer	NT Aus 2007	GQ402011	4	12	6	9	11	11	14	19	15		98.82	96.76	85.71	86.4	92.33	
Seven Band grouper	Japan 1997	AY324870.1	4	8	6	5	7	8	22	17	21	12		97.48	85.71	85.71	92.33	
Oxyeleotris lineolata	QLD Aus 1999	EF591368	11	23	9	19	22	24	24	32	26	27	21		87.76	87.08	91.83	
Macquaria novemaculata	NSW Aus 2004	EF591365	41	41	39	41	41	42	43	45	43	42	42	36		98.64	97.96	
Lates calcarifer	SA Aus 2004	EF591366	41	41	39	41	41	40	41	43	41	40	42	38	4		97.96	
Macquaria novemaculata	NSW Aus 2006	GQ402013	41	65	39	57	63	77	78	83	78	78	78	68	6	6		
			Number of differences in sequence															

Heat map indicating closest relatedness by percentage similar (%) and number of different nucleotides. Sequences obtained in this study are indicated in **bold** and grey shading. Differences and homology of sequences collected in this study are enclosed by the rectangle.

3.4 Discussion

Prior to this study, knowledge of the VNN strains present in Australia, were not sufficient to allow robust decisions to support vaccine development nor design targeted dsRNA for attempted knockdown of NNV. The acquisition of complete genome sequences of RNA 1 and RNA 2 collected from strains associated with VER outbreaks in marine aquaculture systems in North Queensland indicate the highest level of conservation to the RGNNV. Unfortunately, comprehensive comparison of RNA 1 segments across Australian species are limited. There are only three RNA 1 segment sequences from Australian species in the NCBI database (Hick & Whittington 2010; Agnihotri et al., 2015). Nonetheless, within the comparison containing six sequences, the RNA 1 segments from Australian strains of NNV display a very high level of conservation, exceeding 97%, across the tropical geographic, and 96% across temporal zones. Similarly, the sequences of RNA 2 segments of NNV are highly conserved between strains that have been associated with VER outbreaks in Australia since 2001. High conservation extends not only across Australian strains but also across diverse geographic (Japan, China and the Mediterranean) and temporal sources (earliest strains collected in 1997). Previous researchers have noted the conservation of the capsid protein. The high level of conservation of RNA 2 which contains the mRNA for the capsid protein, the antigen target for vaccine preparation, indicates a vaccine against the Australian strains could have wider global application. Temporally, the capsid protein gene has a low evolutionary rate (Thiery et al., 2004). In particular, the RNA 2 sequences obtained in this study display 97.8 to 98.3% homology to the RNA 2 segment of the type species for RGNNV, namely SGWak97, that was collected from *E.septemfasciatus* in Japan in 1997 (Iwamoto, 2001). The NQAus NNV strains collected in this study have less than 20 nucleotide differences from the original RGNNV strain or less than 1.31 changes per 1000 nt per year. Considering the nucleotide differences translate to very few changes in the capsid protein during the past 17+ years, it is also likely a vaccine will be suitable for a long period of time. Further investigation regarding the conservation of the protein motifs will be discussed in the next chapter.

3.5 Conclusion

The following outcomes and conclusions were drawn from this Chapter:

- NNV was confirmed to be present in high copy number in the viral extract.
- Complete sequence of Betanodavirus RNA 1 and RNA 2 was obtained from three VER outbreaks in North Queensland.
- Comparative phylogenetic analysis indicates the strains are of the RGNNV species.
- Bioinformatic analysis indicates the RNA 1 and RNA 2 sequence and theoretically, the translated proteins are highly conserved in the RGNNV species associated with VER in tropical marine species in North Queensland.
- Comparative analysis with RGNNV from the NCBI database indicates the RNA 1 and 2 sequence and theoretically, translated proteins display remarkable conservation across a broad global species, geographic and temporal range.
- Sequence of mRNA 2 was obtained for application to produce standard RT-qPCR control plasmids and recombinant protein expression.
- Sequence of mRNA 1 was obtained which indicated the strains causing VER are pure RGNNV genomes and the lack of chimeric recombination with other NNV strains.
- Gene sequence of RNA 1 was obtained for application to produce standard RT-qPCR control plasmids.

Data presented in this chapter was published:

Condon K., Bochow S., Ariel E., and Miller T., (2019) Complete sequence of Betanodavirus from Australian barramundi, *Lates calcarifer*. Microbiology Resource Announcements 8. https://doi.org./10.1128/MRA.00081-19

CHAPTER 4. REVIEW AND IDENTIFICATION OF THE FUNCTIONAL MOTIFS OF THE BETANODAVIRUS GENOME

Background

- Amino acid motifs that are critical to the production of neutralising antibodies against RGNNV in grouper have been identified in the capsid protein.
- Amino acid motifs that are linked with increased virulence, or critical to viral replication of Betanodavirus have been identified in the capsid Protein, Protein 1 and B1 and B2 proteins.
- Amino acid motifs that are linked with host species specificity have been identified in the RGNNV capsid protein structures.
- Confirmation the strain of RGNNV to be used in this study contains virulence-associated motifs is required for vaccine preparation and to have confidence in the translation of the project results to industry application.
- Knowledge of the functional motifs is required to direct targeted knockdown of the RGNNV using dsRNA.

Aims of this Chapter

- Confirm that motifs which are critical targets of neutralising for antibodies are present in the viral extract.
- Confirm that the motifs which are critical targets for neutralising antibodies will be encoded by the gene sequence used for recombinant protein expression and vaccine preparation.
- Confirm the viral extract being used in this project contains the factors that have been identified to be associated with virulence.
- Determine a region/s in the RGNNV genome to target for knockdown with dsRNA.

4.1 Introduction

Although the previous chapter identified a remarkable level of conservation in the genome of RGNN strains, there are also studies that identify significant loss in virulence associated with single amino acid mutations (Souto et al., 2015). Furthermore, there are a number of short amino acid motifs that have been determined to be critical to the production of neutralising antibodies in grouper species (Chen et al., 2015). In other fish species, including Senegalese sole, single amino acid mutations in the capsid protein have been demonstrated to significantly affect viral replication kinetics in SJNNV (Hata et al., 2010 and Souto et al., 2015).

To ensure an effective translation of this project's outcomes to the grouper aquaculture industry, it is important to ensure the therapies and challenges being conducted within this project are performed using the most virulent and competent viral strains. The production of a vaccine against a viral strain of reduced virulence would not be effective as a long-term management tool to prevent VER in grouper farms, in fact, it may contribute to the selection of more virulent strains within culture systems (Kennedy et al., 2016).

In addition to the preparation of a vaccine, this project is attempting to apply dsRNA to prevent VER outbreaks. The targeted knockdown of portions of the viral genome that are critical to replication or disease is a fundamental requirement for the successful application of dsRNA as a therapy. A range of functional motifs have been identified in the Alphanodavirus and Betanodavirus genome.

This work summarises the findings of previous researchers that have identified functional activity of specific regions of Nodavirus genomes. Considering the paucity of data available on Betanodaviruses, some Alphanodaviruses are included as a model for explaining the general replication characteristics of the Betanodavirus.

The aim of this chapter is to identify motifs of the Betanodavirus genome that are critical for viral replication and virulence in grouper species and confirm the presence of such in the NQAus NNV strains that are being investigated in this project. The collective findings are illustrated in Figure 4.1 Schematic representation of the functional motifs for the Betanodavirus Protein A (p.85) and Figure 4.2 Schematic representation of the functional motifs of the Betanodavirus capsid protein (p.88) (Condon, this work).

4.1.1 Viral Replication Characteristics

Although the small size of the nodavirus genome would suggest a simple replication process the mechanism of viral and gene replication and expression involves a sophisticated regulatory system that is not completely understood (Petrillo et al., 2013). The restricted protein

encoding capacity of the small genome means the nodaviruses must utilise many host-regulatory processes to complete the replication cycle (Castorena et al., 2010). Successful viral replication, in the case of nodaviruses, requires completion of several critical actions. The processes are presented as:

- Attachment to susceptible host cells
- Entry of viral particle to host cells
- Formation of the replication complex
- Expression of viral proteins
- Replication of viral RNA segments
- Formation of viral particles
- Exit of viral particles from host cell.

The processes do not occur as separate steps but rather a regulated system and presumably the inhibition of any of these critical actions could form the basis for the development of new treatments or therapies. For clarity, the processes will be discussed separately.

4.1.1.1 Attachment to susceptible host cells

All non-enveloped viruses use receptor mediated endocytosis to enter host cells (Chang & Chi 2015; Smith & Helenius 2004). Three reports indicate the Betanodavirus mode of attachment to host cells (Liu et al., 2005) (Ito et al., 2008) (Chang & Chi 2015). A Sialic acid moiety of unknown location on the capsid of RGNNV is required for cell infectivity in vitro (Liu et al., 2005).

4.1.1.2 Entry of viral particles to host cells

Although the mode of viral attachment is unknown, the mode of entry of the nodaviruses into cells has been determined. The Betanodavirus, Dragon Grouper NNV (DGNNV), penetrates Striped Snakehead (SSN-1) cells via a spherical pit and membrane-ruffling pathway suggestive of the use of macro- and micropinocytosis pathways (Liu et al., 2005). The inhibition of DGNNV replication was blocked in vitro by chlorpromazine, which suggests clathrin-mediated endocytosis as an entry pathway (Liu et al., 2005). This is the common pathway of cell entry by many unenveloped viruses and requires an acidic environment in the endosome (Liu et al., 2005). 5-(N-ethyl-N-isopropyl) amiloride (EIPA) is a selective inhibitor of sodium/hydrogen ion exchange, disrupts glucose-induced acidification and is a micropinocytosis-specific inhibitor (Huang et al., 2014). EIPA reduced cell death *in vitro* and significantly reduced the expression of both RNA 1 and RNA 2 of RGNNV. The detection of RNA 2 was affected more significantly

than RNA 1 by EIPA (Huang et al., 2014). Following entry into cells, nodaviruses form viral replication complexes (VRCs) (Nagy & Pogany 2012).

4.1.1.3 Formation of the Replication Complex

All the characterised +ssRNA viruses assemble a viral replication complex (VRC) (Nagy & Pogany 2012). The expression of viral proteins, replication of the viral genome and packaging of viral particles occurs within the cell at a viral replication complex (VRC). The nodavirus VRC is located at the mitochondria and results in conformational changes in the organelle to form spherules, which are "viral replicating factories" (Kopek et al., 2007). The spherules appear between the outer and inner mitochondrial membranes and are invaginations of the outer mitochondrial membrane with interiors connected to the cytoplasm through small open necks (Kopek et al., 2007). Motifs on Protein A are responsible for the location of the VRC at the mitochondria. In the Alphavirus, Flock House Virus (FHV), spherule formation does not occur unless the viral RNA dependant RNA polymerase (RdRp) is active and a replication competent FHV RNA strand is present (Kopek et al., 2010).

Docking of the Betanodavirus VRC in the mitochondrion relies on interaction between Protein A, secondary structures in the viral RNA strands and the modulation of host cellular co-factors (Weeks et al., 2010; Young et al., 2003). The interactions are not fully understood however, host cellular co-factors including heat shock proteins (Hsp) and phospholipids have been implicated. Heat shock protein 90 (Hsp90) and heat shock protein 70 (Hsp70) were recognised as important cytosolic chaperones for docking onto a special domain on the import receptor of the outer mitochondrial membrane (TOM) specifically Tom70 (Young et al., 2003). Hsp40, Hsp70 and Hsp90 have been implicated as important co-factors for nodavirus replication (Weeks et al., 2010). Deletion of a SSZ1 gene in yeast that encodes an atypical Hsp70, involved in translational fidelity as part of the ribosome-associating complex (RAC), resulted in a 30-fold increase in FHV3 RNA 3 accumulation (Weeks et al., 2010). Deletion of similar genes encoding RAC Hsp40 reduced FHV accumulation (Weeks et al., 2010).

Hsp70 and Hsp90 have been implicated as important co-chaperones facilitating replication in several other RNA virus infections including human immunodeficiency virus (HIV) , Influenza A, severe acute respiratory syndrome (SARS) and hepatitis C (Mine et al., 2012). A plant infecting, +ssRNA virus, tomato bushy stunt virus (TBSV) like the Alphanodaviruses, replicates in yeast (Nagy 2015). In yeast, the formation of the VRC by TBSV requires the recruitment of a number of cellular co-factors including Heat shock protein 70 (Hsp70), proteosomal Rpn1 1p metalloprotease, eukaryotic elongation factor 1A (eEF1A), endosomal sorting complexes required for transport protein (Vps23p ESCRT), 60 Bro1p ESCRT-associated protein, Vps4p AA

A+ ATPase and Cdc34p E2 ubiquitin conjugating 61 enzyme (Nagy 2015). In addition, TBSV channels sterols and phospholipids to the sites of replication (Nagy 2015). The activation of TBSV RNA polymerase within the VRC was demonstrated to require Hsp70 and was enhanced by the phospholipids phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (Pogany & Nagy 2015). The role of lipids in nodavirus VRCs has been reported as essential but rarely investigated (Castorena et al., 2010) (Qui et al., 2014a). FHV replication in Drosophila S2 cells *in vitro* was associated with the upregulation of several lipid metabolism genes (Castorena et al., 2010). The down regulation of chaperone containing T-complex proteins (Cct), Cct1 and Cct2, that encode essential enzymes for phosphatidylcholine biosynthesis, supressed FHV RNA replication (Castorena et al., 2010). In contrast, formation of the VRC by WhNV was not improved with PE exposure (Qui et al., 2014a). WhNV Protein A was enhanced by 1, 1', 2, 2'-tetraoleoyl cardiolipin (CL), 1, 2-dioleoyl-sn-glycero-3-phosphate (PA) and 1, 2-dioleoyl-sn-glycero-3 – (phosphoro-rac-(1-glycerol)) (PG) which are present in mitochondrial membranes (Qui et al., 2014a). Further research into the role of cellular chaperones in Betanodavirus and other viral infections is required.

4.1.2 Expression of Viral Proteins

In eukaryotic cells, many of the processes of RNA replication and capping which allow efficient protein translation to occur are contained within the nucleus (Ahola & Karlin, 2015). Considering their cytoplasmic location, nodaviruses must encode their own enzymes to ensure viral RNA replication and protein translation success (Ahola & Karlin, 2015). Nodaviruses express 4 proteins during replication namely Protein A, B1 and B2 and the capsid protein-α. In the positive sense ssRNA viruses, the RNA dependant RNA polymerase (RdRp) is always translated first (Miller & Koev, 2000). Protein A, B1 and B2 are expressed early in the infection stage and capsid protein-α later. Functional motifs of the RNA 1 strand are illustrated by Figure 4.1

4.1.2.1 Protein A:

Protein A is ~1000 aa and contains multiple functional domains including nucleolar locating domains, mitochondrial targeting domains (MTD/MLS), a transmembrane domain (TMD), a RdRp, a self-interacting domain and an RNA capping domain (Bai et al., 2011). Additionally, upstream of the RdRp domain is a hypothetical methyltransferase-guanylyl transferase (MTase-GTase) region, which includes the MTD/MLs and TMD (Ahola & Karlin 2015). Wang et al. (2013) demonstrated Protein A also displays N-terminal transferase activity. Unlike many of

the +ssRNA viruses Protein A is produced as a single replication protein that is not cleaved into several functional proteins.

4.1.2.2 Nucleolar Localising domains of Protein A

The C-terminus of Protein A (aa 972 to 978) is similar in aa sequence (RPRRQRR) to the nuclear location signal (NLS) of HIV-1 Trans-activator of Transcription (Tat) and Rev proteins (Mezeth et al., 2007). No reason for a nuclear localisation signal in Protein A has been proposed. In addition, no Nuclear Export Signal (NES) has been proposed in the nodavirus genome (Guo et al., 2003a).

4.1.2.3 Mitochondrial-interacting proteins of Protein A

Early expression of Protein A coincides with the formation of the nodavirus viral replicating complex (VRC) (Miller & Ahlquist 2002). The VRC complex locates to the mitochondria via a mitochondrial locating signal (MLS) and embeds within the mitochondria via the mitochondrial transmembrane domain (TMD) (Miller & Ahlquist 2002). The RdRp is maintained in close association with both the spherule and the RNA strand by RNA binding motifs and RdRp self-interacting motifs. Many of these tasks occur *in vitro* through amino acids within the MLS and MTD motif. In the nodavirus group, the mode of membrane association by Protein A is taxon specific (Ahola & Karlin 2015). In FHV the mitochondrial location signal (MLS), contained within the N-terminal end of Protein A (aa 1 to 46), is responsible for locating the VRC at the mitochondrion (Miller & Ahlquist 2002). Genetic replacement of the mitochondrial localisation sequence (MLS) region of FHV Protein A with an endoplasmic reticulum (ER) targeting sequence results in the formation of the VRC at the ER with increased viral protein production *in vitro* (Miller et al., 2003). Once localised to the mitochondria, a region within the N-terminal end of Protein A, termed the transmembrane domain (TMD) becomes inserted in the intermembrane space of the mitochondria and the C-terminus is exposed to the cytoplasm (Miller & Ahlquist 2002). In FHV, the TMD is predicted to be aa 15 to 36 LLVGIATVSGCGAVVYCIS (Miller & Ahlquist 2002). One or more other regions, which were C-terminal to aa 230, also possessed some MLS/TMD activity (Miller & Ahlquist 2002). Recently, both N-and C-terminal TMDs were studied (Qui et al., 2014b). In Wuhan nodavirus (WhNV), a self-interaction of Protein A occurs between aa 1 to 254 and 255 to 480. The interaction occurred between 3 amino acid regions. Homotypic interactions between the same regions were noted for aa 1 to 254 (75 %) and 255 to 240 (55 %). The region aa 481-659 also formed heterotrophic interactions between aa 1 to 254 (30 %) and aa 255 to 480 (22 %). The self-interaction of Protein A was lost when mutations were created at K91A, W92A, R93A,

S163, R165A and Y169A. The mitochondrial membrane binding was not affected by the mutations (Qui et al., 2014b). Amino acid motifs between aa 660 to 839, which is within the RdRP domain, and aa 840 to 1014 of Protein A also display RNA binding activity that has not been fully investigated (Qui et al., 2014a).

Protein A of GGNNV localised to intracellular membrane compartments in the cytoplasm (Guo et al., 2004). Bioinformatic analysis identified two hydrophobic aa residues that could hypothetically act as TMDs at aa 153 to 173 and 229 to 249 (Guo et al., 2004). The region consisting of aa 215 to 255 was demonstrated to contain an MLS and a TMD (229 to 249) (Guo et al., 2004). The MLS was not determined within the aa 215 to 255 region. The TMD mode of association was proposed be through embedding into the phospholipid bilayer of the organelle. In Atlantic Halibut NNV, four hypothetical transmembrane domains were identified (Mezeth et al., 2007). The regions consisted of aa residues 6 to 26, 148 to 169, 225 to 247 and 647 to 665. Regions aa 6 to 26 and aa 225 to 246 were demonstrated to be MLS (Mezeth et al., 2007). Proteins comprising aa 225 to 246, if expressed alone, localised to the golgi apparatus in some cells (Mezeth et al., 2007).

4.1.2.4 MTase-GTase domain of Protein A

In close association with the MLS and MTD is a proposed MTase-GTase domain. A MTase-GTase domain is essential for RNA capping and is related to the formation of spherules (Ahola & Karlin 2015). Alphaviruses, another family of +ssRNA viruses that replicate in the cytoplasm, contain a unique type of RNA capping enzyme that has combined methyltransferase-guanylyl transferase (MTase-GTase) activity. The MTase-GTase structure, rather than the sequence, is proposed to convey the functional properties of the enzyme. The N-terminal moiety of the Alphanodavirus- Nodamura Virus Protein A (aa 1 to 460) is homologous in functional secondary structure to the Alphavirus MTase-GTase and contains a hypothetical membrane-associating region (Ahola & Karlin 2015). MTase-GTase is comprised of the N-terminal core and the downstream “iceberg region” region (Ahola & Karlin 2015). The “iceberg region” is synonymous with the MLS/MTD domain already discussed. Ahola and Karlin (2015) did not refer to the region as a domain because it did not form a separate unit. Specific mutations within either the core or iceberg region can abolish the MTase-GTase activity.

In the Betanodaviruses, a genus-specific insertion contributes to membrane association and mitochondrial targeting or “Iceberg region” (Ahola & Karlin 2015). This occurs in an area between aa 108 to 255 and concurs with the results of Guo et al. (2004). In FHV the C-terminal aa 1 to 200 were demonstrated to possess the ability to interact with components of the Protein A (Dye et al., 2005). Specifically, mutations to aa N203, W220, W222 and S231 induced

significant reduction in Protein A self-binding and RNA replication (Dye et al., 2005). Mutations introduced at aa Y207, W215 and E227 did not affect Protein A self-binding but did reduce RNA replication to 8, 5 and 4 % of wild type (WT) respectively (Dye et al., 2005). The Y207, W222 and S231 are conserved at the same location in the Betanodaviruses however; the E227 is replaced with M227. The W222, S231 and M227 could relate in function as an MLS for the downstream MTD. Guo et al. (2004) or Ahola & Karlin (2015) did not solely differentiate the MLS function.

Between the MTD/MLS and self-binding motifs and RdRP motif of Protein A is a region that relates to temperature sensitivity in the Betanodaviruses (Hata et al., 2010). Temperature sensitivity of the Betanodaviruses involves a combination of RNA 1 and RNA 2. The region on the RNA 2 has not been investigated. The region on the SJNNV RNA 1 genome was located from nt 84 to 1419 or aa 1 to 445 Protein A (Hata et al., 2010). More specifically, the region nt 1088 to 1419, aa 335 to 445 was particularly important in the temperature sensitivity of RGNNV (Hata et al., 2010). Souto et al. (2019) demonstrated point mutations of nucleotide sequence of SJNNV RNA 1 that lead to single specific amino acid changes to resemble that of RGNNV RNA 1 at aa 41, 48, 218, 223, 238 and 239 lead to reduced infectivity of the recombinant strain compared to the wild type SJNNV at 25 °C in experimental challenge in sole. The authors were not able to test if the aa changes improved infectivity at the RGNNV optimal temperature of 30°C due to the inability of sole to tolerate the higher water temperature.

4.1.2.5 RNA dependant RNA polymerase of Protein A

In the positive sense ssRNA viruses, the RNA dependant RNA polymerase (RdRp) is always translated first (Miller & Koev 2000). Betanodavirus RdRP was detected in the barramundi cell line from 12 hours post infection (hpi) with peak expression at 24 hpi and declining expression 48 to 72 hours hpi (Wu et al., 2010). In *Dicentrarchus labrax*, Protein A of GGNNV was detected from 5 hpi, increased to 14 hpi and stabilised until 23 hpi whereby it slightly declined (Guo et al., 2004). The RdRp has an essential requirement for Mn2+ (1mM optimal and above 4mM inhibitory and an optimal performance at pH 8 to 9. (Guo et al., 2004). The nodavirus RdRP contain 6 motifs that are preserved in many ssRNA virus RdRps (Johnson et al., 2001). The conserved RdRp motifs have been identified in the Betanodavirus RdRP (Johnson et al., 2001). The RdRp can replicate the nodavirus genome in the absence of cellular co-factors (Wang et al., 2013). However, protein translation and virion formation rely on the formation of the VRC. The RdRp recognises the nodavirus genome via cis-acting elements and secondary structures rather than any sequence specific recognition factor (Wang et al., 2013).

4.1.2.6 Terminal Transferase in Protein A

The RdRp is unable to replicate the viral genome if more than 3 nt are removed from the 3' end of the negative strand of RNA 1 sequence (Wang et al., 2013). Protein A has terminal nucleotransferase (TNT) activity that adds nt to the 3' sequence if 2 nt are missing (Wang et al., 2013). The TNT recognises the last 191nt of +/-RNA 1. Adding additional nucleotides to the 3' of the 191 nt sequence does not affect RdRp activity provided the addition be not too long such as that of an 18nt poly a tail. Like the RdRp, the TNT relies on the presence of the 3' end of the positive or negative strand of RNA 1 and the presence of the GDD motif (Wang et al., 2013). The location of the TNT motif on Protein A has not been determined.

4.1.2.7 B1 Protein

B1 is a 111 aa protein. B1 is dispensable for RNA replication in mammalian and yeast cells *in vitro* (Chen et al., 2009). In Betanodaviruses, B1 cDNA and B1 expression was detected at 12 hours, peaked at 24 hours and declined over 72 hours post-transfection in 3 fish cell lines (grouper liver cells (GL-av), grouper fin cells (GF-1) and zebrafish liver cells (ZLE) (Chen et al., 2009). B1 was demonstrated to be a novel anti-necrotic protein that may serve to maintain mitochondrial function to allow viral replication (Chen et al., 2009). How B1 prevents cell death has not been determined. However, it has been demonstrated to not be a member of the Bcl-2 family, which is a host-encoded antagonist of apoptosis (Chen et al., 2009).
The aa 1 to 70 are highly homologous across the RGNNV, AHNNV and SJNNV strains studied by Chen et al. (2009). A N-glycosylation site is present at aa 16 to 19 (NKTS) as is a nuclear locating sequence (NLS) between aa 33 to 38 (PRRARAA). The PRRARAA aa sequence is retained by the grouper isolated RGNNV genotypes but present as a PRRART in the AHNNV and SJNNV genotypes. An additional NLS is located at aa 66 to 70 (KRPRR) (Chen et al., 2009). Although not demonstrated to be a result of the proposed NLS, B1 linked to a reporter protein were observed to be transported to the nucleus (Chen et al., 2009). The purpose of the nuclear transport has not been discussed in literature. Investigation into functional motifs in B1 identified possible protein modification sites including a N'myristoylation site (aa 30 to 55 GGVTAI), a protein kinase C phosphorylation site (aa 58 to 60 SRR), a protein kinase II phosphorylation site (66 to 69 TVIE) and a mitochondrial specific sequence (45 to 52 TFVISHAA A) (Su et al., 2009). Amino acid sequence between the Nodaviruses was not conserved within the hypothetical protein modification sites proposed by Su et al. (2009). When the nucleotide sequence identity of the B1 region RGNNV TN1 strain (EU118118) was compared with other species, there was a 92 to 96 % homology with other grouper isolated strains, 81 % with

AHNNV (AJ401165), and 70 % with SJNNV (AB025018) (Chen et al., 2009). No functional explanation for the homology between strains was proposed.

4.1.2.8 B2 Protein

B2 is a 75 aa multifunctional protein whose role in nodavirus replication has not been fully exposed (Petrillo et al., 2013). The protein is not required for viral RNA replication however it is required for accumulation of the viral RNA and the production of infectious virions (Settles & Friesen, 2008). B2 of RGNNV was expressed in grouper liver cells (GL-av) 12 hpi with increased expression between 24 and 72 hpi (Su et al., 2009). The B2 protein has been demonstrated to possess dsRNA-binding activity, pro-apoptosis properties, be involved in the formation of the VRC and promote translation of the capsid protein (Venter and Schneemann, 2008). There is little aa homology between B2 of the Alpha-and Betanodaviruses however due to their functional similarity some homologous regions have been identified (Ou et al., 2007). B2 plays a critical role in the silencing of the RNA interference pathway, which is involved in innate immune responses (Su et al., 2009). B2 binds dsRNA to both prevent the cleavage of long dsRNA by Dicer-2 and inhibit the loading of short interfering RNAs (siRNA) into the RNA silencing complex (RISC).

B2 recognition of dsRNA occurs in a sequence independent manner. The mode of recognition relies on an A-type duplex RNA, specifically 2 successive minor grooves with an intervening major groove on the other side of the RNA duplex (Venter & Schneemann, 2008). The dsRNA binding activity of B2 is based on electrostatic forces (Petrillo et al., 2013). Mutation of 3 positively charged aa including R36, L47and L62 to A or D reduced or inhibited the ability of FHV B2 to bind dsRNA. In the presence of the mutant viruses, the FHV RNA accumulated in cytoplasmic granules, preventing the translation of the RNA into protein (Petrillo et al., 2013). A substitution mutation in NoV B2 of aa R59 was defective in binding both 44bp dsRNA and 21 nt siRNA (Aliyari et al., 2008).

B2 also acts as a structural component of the VRC to limit the effect of the iRNA pathway to combat viral production (Aliyari et al., 2008). In RGNNV a mitochondrial targeting sequence (MTS) of B2 (aa 41 to 50) is reported as RTFVISAHAA (Su & Hong 2010). Residues critical to maintaining the dsRNA binding ability of Betanodaviruses have been identified (Fenner et al., 2007). Arginine (R) residues, R26, R53 and R60 are essential for the binding of short 40-bp dsRNA (Fenner et al., 2007). R53 and R60 in GGNNV were essential for the accumulation of long (600bp) dsRNA in vitro (Fenner et al., 2007). An R55, which is only present in AHNNV, and GGNNV not SJNNV was also identified to have dsRNA binding ability (Fenner et al., 2007). The R53 and R60 were confirmed to be critical for B2 dsRNA binding and RNAi-inhibition in a

grouper isolated VER strain in Taiwan (Ou et al., 2007). Four aa residues were identified as necessary for the mitochondrial targeting of an RGNNV strain namely V44, I45, R52 and R53 (Su & Hong 2010). Although no nuclear localisation signal has been identified Fenner et al. (2006) noted the accumulation of GGNNV B2 within the nucleus during the late stages of GGNNV infection.

Comparative analysis of the 27 B2 Betanodavirus sequences published on NCBI reveal homology of 51/70 aa (73 %) across the entire protein (this work). Within B2 the first 50 aa are highly conserved displaying homology of 42/51aa (82 %). A motif of unknown significance at aa 5-19 is completely conserved in the N-terminal arm of the Betanodavirus B2 aa 5-QQAIDQHLVELEQLF-19 (this work). The E14 residue along with D24 facilitates long dsRNA binding (Fenner et al., 2007). In addition, the aa 16-20 LEQL is conserved between the Betanodaviruses and NoV (Ou et al., 2007 and this work). The MTS, aa 41 to 50 (Su & Hong 2010), is almost completely conserved in the Betanodaviruses. All SJNNV species have a S48 motif rather than H48. Three strains have a single aa change within the motif that has not been linked with any clinical change in VER. Only 8/24 (33 %) aa of the C-terminal end of B2 are conserved. Conserved motifs are R53, L54, L57, R60, P52, E70, P71 and M72 (This work). The R59 motif identified by Aliyari et al. (2008) as essential for NoV B2 is conserved in all the 25 Betanodavirus B2s except the BFNNV strains. An A69 motif is conserved between FHV, NoV and the Betanodaviruses, except AHNV. The effect of variation in these sequences on virulence or species specificity was not proposed.

In vitro monitoring of capsid protein demonstrated B2 is necessary for efficient translation of the capsid protein (Petrillo et al., 2013). The translational efficiency was linked to B2 playing a role in ribosomal occupancy of RNA 2 (Petrillo et al., 2013). B2 has also been demonstrated to possess pro-apoptotic activity (Su et al., 2009). B2 upregulated the expression of Bax which is an antagonist of apoptosis leading to mitochondria-mediated necrotic cell death at the late to mid stage (24 to 48 hpi) of viral replication of RGNNV (Su et al., 2009).

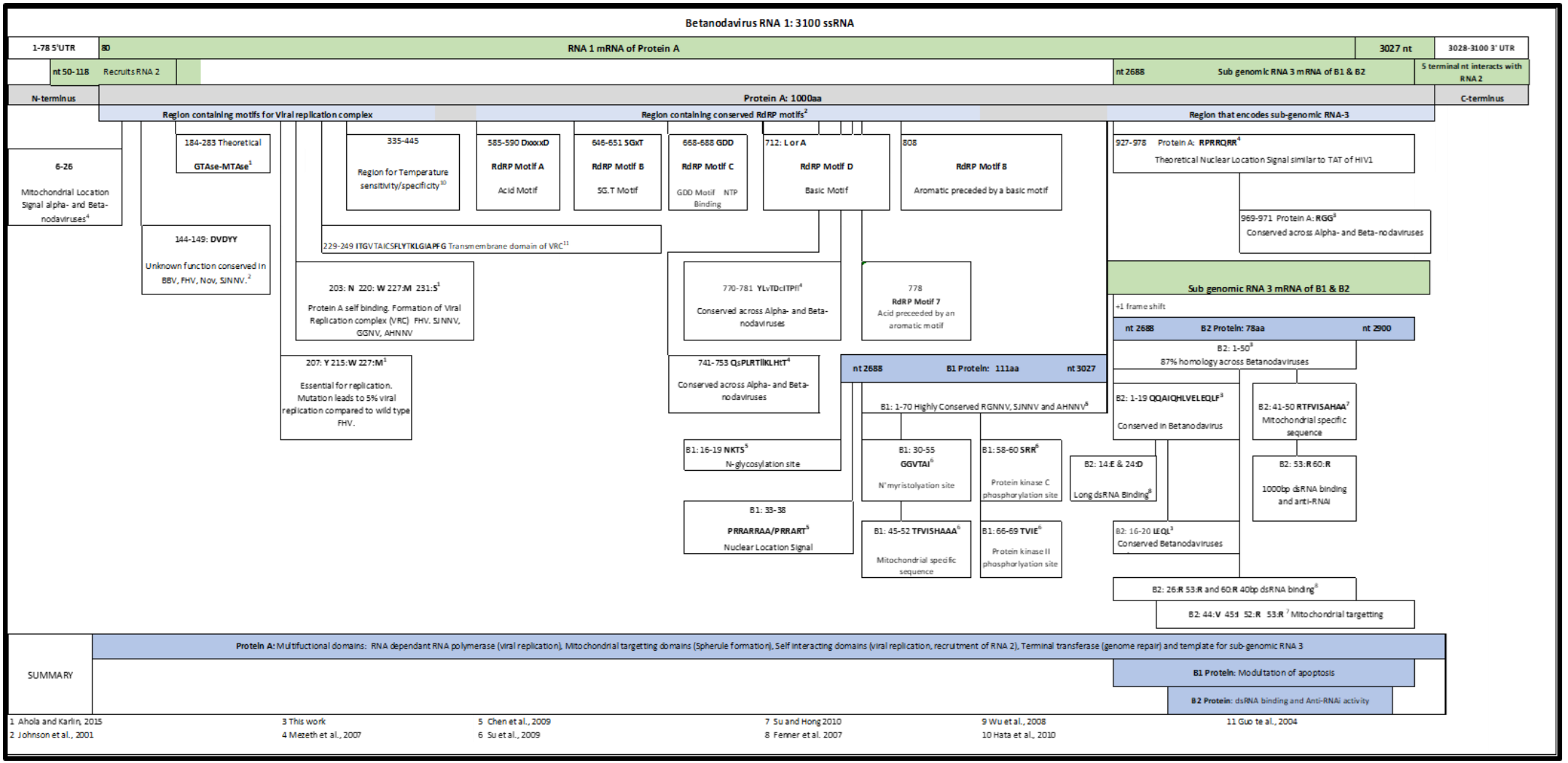


Figure 4-1: Schematic drawing of RNA 1, mRNA of Protein A indicating location (number) , function and amino acid sequence of functional motifs described in the literature and source of reference.

4.1.2.9 Capsid Protein

The RNA 2/capsid protein of the Betanodavirus has been proven to determine host range (Iwamota et al., 2004). The Betanodavirus capsid protein has also been reported to possess Nuclear Location signal activity, pro-apoptosis activity and contain a motif that relates to species-specific virulence and the recognition of virus by neutralising antibodies (Guo et al., 2003a; Ito et al., 2008 and Chen et al., 2015). A schematic diagram of the Betanodavirus capsid protein is presented in Figure 4.2.

The Betanodavirus capsid protein consists of 340 aa. The translation of the capsid protein requires both UTRs of RNA 2 and the 5'UTR contains a Kozak sequence of ACAA TGG (3ANNATGG+4) (Huang et al., 2007). The capsid protein has a mw of ~37 to kDa which is slightly higher than that predicted by their amino acid sequences (Lin et al., 2001). The higher molecular weight is consistently observed and could possibly be caused by host cell factors causing polyadenylation *in vitro* (Huang et al., 2007). Expression of the capsid protein was reported to increase from 12 to 72 hpi and was significantly greater than the peak RdRP expression (Wu et al., 2010). Studies with recombinantly expressed mouse grouper NNV (MGNNV) indicated the capsid protein packages multiple RNA strands ranging in size from 100 to 4500 nt (Guo et al., 2003b). Expression of the capsid protein of GGNNV *in vitro* induced activation of caspase-3-like and caspase-8-like proteases which ultimately leads to apoptosis via the "extrinsic" pathway (Guo et al., 2003b). The induction of apoptosis serves as a mechanism to release progeny virus from infected cells (Guo et al., 2003b).

The Betanodavirus capsid protein displays an N-terminal arm aa 1 to 52, a highly conserved region T2 aa 83 to 216, a variable region T4 aa 235 to 351 and an area referred to as the C-terminal arm aa 288 to 338. The N-terminal aa 23 to 31 RRRANNRRR of the capsid protein from GGNNV were demonstrated to perform nucleolar localisation (NLS) functions in both mammalian (Cos-7) and Asian seabass *D.labrax* (SB) cell lines (Guo et al., 2003a). The N-terminal aa 2 to 10 RKGEKKLAK of GGNNV capsid protein also displayed NLS activity (Guo et al., 2003b). No role of the NLS in the replication of the Betanodavirus genome has been proposed. The N-terminal arm of the capsid protein contains a high percentage of basic residues and is involved in binding the negatively charged phosphate backbone of the viral genomic RNA within the capsid (Lu & Lin 2003). There are two stretches of RRR residues and D75 residue. The D75 residue represents a catalytic residue (Grotmol et al., 2000). The RRR residues are involved in the binding of the viral genomic RNA to the internal capsid wall of the host cell. The aa R23 to 25 and R 29-31 were critical in the formation of viral like particles (Lu &

Lin 2003). Also, within the N-terminal arm aa 127 to 140 is the highly conserved DxxDxD motif, which in the Betanodaviruses is FLP**DPTDND**HTFDA, is responsible for calcium binding (Wu et al., 2008).

Within the highly variable T4 region a 3 aa motif (254 to 256) is completely conserved within each NNV genotype (Nishizawa et al., 1997). The motif is responsible for neutralising epitopes that are associated with viral infectivity (Ransangan & Manin 2012). The species present as 3 serotypes by analysis with monoclonal antibodies raised against the capsid protein (Mori et al., 2003). The amino acid motifs and serotypes were discussed in Chapter 3 (refer to Table 3.1).

The RNA 2 and/or the encoded coat protein controls host specificity in SJNNV and RGNNV (Iwamoto et al., 2004). Recombinant studies combining RNA 1 and different RNA 2 regions of SJNNV and RGNNV identified regions on RNA 2 which related to virulence (Ito et al., 2008). Virulence in striped jack and seven band groupers was retained when the RNA 2 segment nt 693 to 1054 (RGNNV) and 694 to 1061 (SJNNV) was retained respectively. *In vitro* all the viral hybrids replicated indicating the loss in infectivity was related to a host factor rather than critical mutation in the virus. Significantly, *in vitro* activity was demonstrated via transfection with lipofectamine which facilitates entry of DNA or RNA to cells (Cardarelli et al., 2016) and by-passes the requirement of attachment and entry to cells. The loss of virulence *in vivo* could indicate the regions identified by Ito et al. (2008) relate to attachment and entry of host cells. However, other mechanisms could also account for the variation in infectivity between *in vivo* and *in vitro* studies.

In RGNNV the region between nt 694 to 758 (aa 223 to 245) appears to affect species specificity. In SJNNV, the region between nt 695 to 765 affects species specificity (Ito et al., 2008). The nt and aa homology of the two viruses within the critical regions were 60 and 55 % respectively. How the region influences species specificity is unknown. Near this region a combination of either both or two single amino acid mutations of S247A or S270N in a SJNNV capsid protein lead to mortality of 60 % compared to 100 % compared to wild type virus in experimental infection of Senegalese sole (Souto et al., 2015). *In vitro*, the viral mutations reached similar viral titres albeit with slower replication kinetics (Souto et al., 2015a).

The capsid protein may also have yet to be discovered functions. The formation of the VRC and the expression of B2 provide a mechanism that protects the viral RNA during synthesis following the expression of Protein A. However, no mechanism for the protection of the RNA 1 strand prior to translation to Protein A has been proposed. Considering the capsid protein is the only viral-derived protein present in the host cell prior to Protein A translation it is likely the capsid protein performs other roles yet to be described.

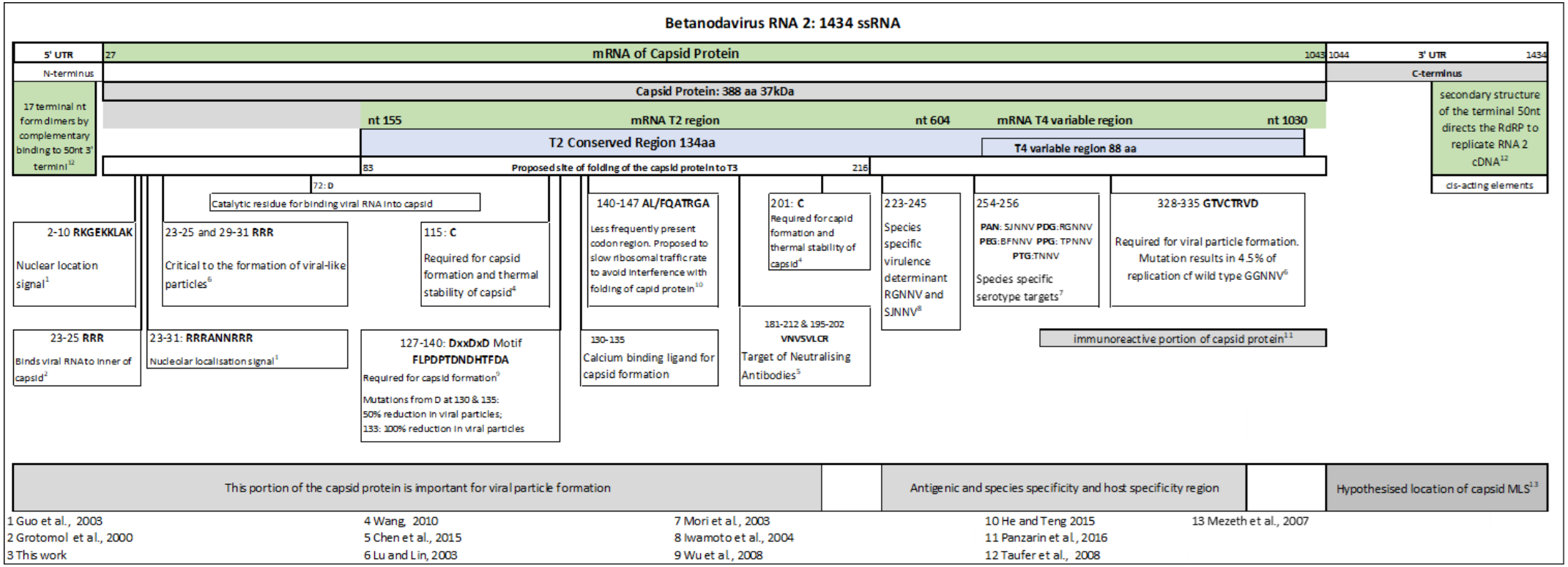


Figure 4-2: Schematic drawing of RNA 2 mRNA of Capsid Protein indicating location (number) , function and amino acid sequence of functional motifs described in the literature and reference source.

4.1.3 Replication of Viral RNA segments

Replication of the Nodavirus RNA involves the production of multiple RNA species in addition to RNA 1, 2 and 3 (Venter & Schneemann 2008). Although packaged as separate strands, during replication, the RNA 1 and 2 are covalently linked as head to tail monomers (Rosskopf et al., 2010). Additional RNA species have been described as defective interfering RNAs (DI-RNAs) and RNA dimers (Venter & Schneemann 2008). RNA dimers consist of head to tail junctions of RNAs 1, 2, 3 or 2 and 3 in both positive and negative strand RNA. DI-RNAs consist of genomic RNAs with internal deletions or sequence rearrangements. A 634 nt DI-RNA of FHV (DI 634) accumulates to higher levels than viral genomic RNAs (Dasgupta et al., 2003). No function has been demonstrated for the DI-RNA. An additional negative sense intermediary formed in the early replication cycle accounts for approximately 1 % of the RNAs replicated (Venter & Schneemann 2008). The production of the negative sense intermediary is independent of an external RNA primer and in the case of WhNV involves the recognition of the last 191nt on the 3' of RNA 1 positive sense or last 201nt on the negative sense RNA 1 (Wang et al., 2013). A D.labrax NNV (termed DIEV in the publication) intermediate, minus strand RNA, was detected in cell culture at 96 hours post-inoculation (hpi). Other researchers report the detection of minus strand RNA 1 of GGNNV in cell culture 12 hpi (Adachi et al., 2007).

How the RNA 1 strand locates and becomes stabilised in the cell prior to packaging in the capsid protein is not established beyond occurring within the VRC. The B2 protein has been shown to be in close association with the RNA 1 and plays a role in the protection of RNA 1 from the RNA interference (RNAi) machinery. B2 is produced well in excess of RNA 1 and could perform additional roles in the replication of the Nodaviruses (Petrillo et al., 2013).

Within the virion, RNA 1 and 2 of FHV form a single RNA that has been observed in gel electrophoresis. A covalent link is proposed to occur through a small section with nucleotide homology between the 3' end of RNA 1 and 5' end of RNA 2. Deletion of 5 nt from the 3' end of RNA 1 prevents RNA synthesis in the case of FHV (Ball 1995). A single nt substitution of G at 2960 to T in FHV leading to an amino acid change from R to L in the RNA 3 promoter region lead to an absence of B2 and significant downregulation in the translation of the FHV coat protein α (Petrillo et al., 2013). The recruitment of RNA 1 by Protein A at the VRC in WhNV was demonstrated to rely on the presence of a stem loop structure with nt 50 to 118 of RNA 1 being critical (Qui et al., 2014a). The binding ability was increased with increased Protein A concentration with the minimal ratio of Protein A: RNA 1 being 10: 1 (Qui et al., 2014a). The

recruitment of RNA 2 by Protein A in the VRC was also dependant on the presence of a stem loop structure that is located in nt 123 to 164 of RNA 2 (Qui et al., 2014a).

4.1.3.1 RNA 1

Betanodavirus RNAs are of similar function and structure to that of the Alphanodaviruses. RNA 1 is the mRNA for the RdRP (Protein A). The sub-genomic RNA 3 is also present on the 3' end of the RNA 1 and is the mRNA for the B1 and B2 proteins. Both the RNA 1 and 2 molecules are required for infectivity and the viral strands are self-replicating in vitro in the absence of complete virions. The presence of RNA 1 and RNA 2 was detected in vitro at 24 hours post-infection (hpi) and RNA 3 at 96 hours pi (Delsert et al., 1997). Delsert et al. (1997) further noted that the RdRp was associated with the mature capsid. The RNA 1 nt sequence of Betanodaviruses displays ~80 % homology across the genus. Within each species/genotype approximately 90 % nt homology exists although it should be noted that a large proportion of the strains in the NCBI database have not been assigned to genotype level. Within the first ~40nt of RNA 1 of the Betanodaviruses a TPNNV specific insert exists at nt 27 to 34 (UAACUGAA). The insert is positioned in the UTR of RNA 1. No reference to the insert has been located in the published literature. As TPNNV has not been reported since 2007, it could be inferred that TPNNV displays low virulence and adaptability compared to the RGNNV, SJNNV and BFNNV.

4.1.3.2 RNA 2

The RNA 2 strand consists of ~ 1435 nt and contains one open reading frame (nt 27 to 1043) flanked by a 5' 26 nt non–coding/untranslated region (NCR/UTR) and a 3' 392nt UTR (Huang et al., 2007). Secondary structures within the RNA 2 C-terminal region (UTR), also referred to as "cis-acting elements", are required for RNA 2 replication. The secondary structures in UTRs are required by the RdRP of many RNA viruses to allow the recognition and processing of RNA in viral replication (Taufer et al., 2008). Conserved secondary structures were predicted by computer modelling to be present in the 3' terminus of RNA 2 in the nodaviruses including FHV, NoV, SJNNV and GGNNV (Taufer et al., 2008). The 50 nt of the 3' terminal RNA 2 of FHV were sufficient to direct the replication of complementary RNA (Albarino et al., 2003). Similarly, in NoV, 50 nt (1287 to 1336) at the 3' end of the RNA 2 is sufficient to direct the RdRP to replicate a complementary RNA (Rosskopf et al., 2010). RNA 2 also forms head-to-tail dimers during RNA replication (Qia et al., 2011). The dimers are approximately the same size as the RNA 1 and can be difficult to distinguish on gel electrophoresis (Qia et al., 2011). The

RNA dimers of NoV form between the 5' terminal 17 nt and the 3' terminal 54 nt of RNA 2 (Rosskopf et al., 2010).

4.1.3.3 RNA 3

The replication of RNA 3 has been described for the Alphanodaviruses. The replication of RNA 3 does not involve an intermediary negative sense sequence. In the Alphanodaviruses Flock House Virus (FHV), Pariacoto Virus (PaV), Nodamura Virus (NoV) and Wuhan Nodavirus (WhNV), RNA 1 and sub genomic RNA3 (sgRNA3) form homodimers during replication (Qia et al., 2011). It is proposed that RNA 3 binds to viral RNA to prevent the formation of incompatible dsRNA that may affect the formation of the capsid (Schneemann 2006). The homodimers consist of negative strand RNA 1 and positive strand sgRNA3 which results in the initiation of sgRNA3 transcription. The promoter for transcription of sgRNA3 is located on the negative strand of RNA 1. In the case of WhNV, the promoter is located within nt 2758 and 2769 on RNA 1. The production of sgRNA3 also requires the presence of secondary structure that includes the transcription start site of B1 (Qia et al., 2011).

Transcription of RNA 2 suppresses the transcription of sgRNA3 (Qia et al., 2011). The suppression of sgRNA3 by RNA 2 is proposed to occur through RNA 2 having 3 regions of nucleotide complementarity to the negative strand of RNA 1, which both restrict the formation of the secondary structure of RNA 1 and also bind directly to the sgRNA3 promoter region (Qia et al., 2011, Wang 2010). By annealing to the sgRNA3 promoter site on the negative to strand RNA 1, RNA 2 impairs the recognition of the sgRNA3 promoter by the viral RdRp (Wang et al., 2013). The mechanisms of replication of RNA 3 in the Betanodaviruses have not been described.

4.1.4 Formation of viral particles

Nodaviruses do not form empty capsids but package RNA into the capsid being either viral or seemingly random in origin with the final product having approximately the same sedimentation rate as that of the nodavirus virion (Gopal et al., 2014). The capsid protein packages one molecule of RNA 1 and RNA 2 into progeny particles. The RNA 3 is not packaged into virions (Gopal et al., 2014). RNA packaging into virions occurs approximately 30 minutes after synthesis of the capsid protein. Optimal capsid formation in the Betanodaviruses occurs at pH 8.0 that correlates well with the normal pH of ocean water (pH 8.4) (Lin et al., 2001). The capsid consists of 180 copies of the capsid protein α (Gopal et al., 2014).

The Betanodavirus capsid protein is produced as a single protein. Functional motifs have been identified on the capsid at aa 23 to 25, 29 to 31, 83 to 216, 127 to 140, 187 to 201 and C-

terminal aa 4 to 11 (Lu & Lin 2003). At the N-terminal the RRR motifs at aa 29 to 31 and less so aa 23 to 25 and were critical for viral particle formation in vitro (Lu & Lin 2003). Wang (2010) investigated the role of 35 residues at the N-terminus of the DGNNV. R30 and R31 are important for particle formation and particle stability (Wang 2010). Effects of R29 were negligible (Wang 2010).

The middle region of the capsid protein aa 83 to 216 is proposed as the site of folding to form the T=3 capsid. Within this region a conserved aa 140 to 147, AL/FQATRGA, is encoded by less frequently present codons and may serve to slow the ribosomal traffic rate to avoid interference with the folding of the capsid protein (He & Teng 2015). Folding of the single protein to form a functional capsid involves disulphide bonding at highly conserved residues C187 and C201 (Krondiris & Sideris 2002). Amino acid analysis of the coat protein of the Betanodaviruses indicates the DxxDxD motif, which is important for capsid formation, is positioned at aa 127 to 140. Within aa 127 to 140 of DGNNV aa 130 to 135 of the capsid protein was identified as the calcium binding ligand. Point mutations of aa D130, D133 and D135 resulted in a 50, 100 and 50 % reduction in viral particles respectively (Wu et al., 2008). Motifs C115 and C201 are essential for capsid formation of DGNNV and for thermal stability of viral particles (Wang 2010).

In the DGNNV capsid protein, N-and C-terminal motifs are involved in the viral particle formation (Lu and Lin 2003). Deletion of the C-terminal aa 4 to 11 (328GTVCTRVD335) reduced viral particle formation to just 4.5 % of wild type (Lu and Lin 2003). Although viral replication and packaging occurs in membranes associated with the mitochondria, the capsid protein lacks MLS (Mezeth et al., 2007). Signals for mitochondrial targeting maybe present in the UTR of RNA 2 (Mezeth et al., 2007). The covalent linkage proposed by Rosskpf et al. (2010) between the RNA 1 and RNA 2 would facilitate localisation of the capsid protein to both the mitochondria and replicated RNA 1 and 2. How the ssRNA viruses in general regulate viral production with protein synthesis and capsid formation and packaging has not been determined. A form of viral sensing of cellular co-factors in the sub-cellular environment is proposed to regulate the actions within the VRC (Nagy 2015).

4.1.5 Exit of viral particles

The exit of viral particles from the host cell by Betanodaviruses has not been extensively studied. The fore-mentioned induction of apoptosis by either the capsid protein or B2 would facilitate the release of progeny virus from infected cells. The action of B2 to upregulate expression of the pro-apoptotic gene Bax which induced loss of mitochondrial membrane potential and mediated necrotic cell death of 44 % of cells at 72 hpi, is a mechanism that

would facilitate exit of viral particles (Chen et al., 2009). Although cell-to-cell transport is reported for many neurotropic viruses, no such movement of viral particles between cells has been demonstrated in the Betanodaviruses.

4.1.6 Undescribed mechanisms

Acknowledging the limitation of a small genome, the Betanodaviruses complete infection and replication in a very efficient manner. Although the untranslated region of RNA-2 represents a relatively large proportion of the genome (~10% of whole genome and ~30% of RNA 2 segment), limited attention has been directed to the role of the UTR in viral replication. Recently Souto et al. (2018) demonstrated the importance of the 50 terminal nucleotides of the 3' end of the genome in forming the stem loop structure that is essential for replication. The nt region 1398-1421 was broadly identified as more critical in forming the stem loop structure and mutations at 1408 and 1408-1412 but not 1412 alone. Through *in vitro* studies, the functional role of 1408-14212 was demonstrated to affect the interaction with the 3' NCR/UTR of RNA 2 (Souto et al., 2018). Moreover, the significant attenuation of virulence in Senegalese sole through mutation of 1408-1412 indicated the region also plays a role in interaction for RNA 2 with host cellular proteins (Souto et al., 2018).

The importance of viral encoded microRNA (V-miRNA) is recognised as a potent mechanism used by viruses to achieve viral replication, persistence, immune evasion and cellular transformation (Cullen 2009; Grundhoff & Sullivan 2011; Tycowski et al., 2015). V-miRNAs represent a genomically efficient way for viruses to regulate host immune responses. A V-miRNA could target multiple genes in the same host or a highly conserved gene in multiple hosts. Additionally, V-miRNAs are known to target the expression of viral replication process and are involved in the Singapore grouper Iridovirus which encodes at least 16 V-miRNAs, the functions of which are being studied (Guo et al., 2013). In addition, nine v-miRNAs have been detected during replication of the fish-infecting Megalocytivirus (Zhang et al., 2014). Nodavirus V-miRNAs have not been reported. However, the replication of short incomplete copies of Differential interfering-RNA (DI-RNA) by the RdRp has been detected in the early phases of nodavirus replication *in vitro*. During RNA replication, FHV produces, ~ 400bp ds RNA (DI-RNA) from the 5' terminus of RNA 1 which serves as a Dicer-2 substrate (Aliyari et al., 2008). Pyrosequencing of the dsRNA formed 4 days post-FHV infection detected 4371 small RNAs the majority of which have strong homology and presumably target a region comprising the first 400nt of RNA 1 (Aliyari 2008). No functional roles for the DI-RNAs have been demonstrated. The mass replication of the DI-RNAs could be an intermediate step in the production of V-miRNA which could serve to downregulate specific host genes such as those

which regulate immune function or to regulate nodavirus replication. The lower expression of Protein A from RNA 1 compared to the capsid protein of RNA 2 despite both strands being transcribed in equal amounts supports a proposal that portions of RNA 1 may have regulatory roles conferred by the RNA rather than translated protein.

4.1.7 The importance of the functional motifs in the context of this study

There are few studies investigating the functional motifs of the Betanodavirus genome that discuss multiple motifs across multiple Betanodavirus strains. Rather many studies investigate a small portion of the genome in a single strain of NNV. Although this review has identified a large number of motifs that are important for replication, it must be noted that the images prepared are a collective representation from many articles. The aim of this chapter is to confirm the presence or absence of the many functional motifs identified in the literature in the three NQAus NNV strains of RGNNV obtained within this study. Confirmation of the functional motifs serves a three-fold purpose namely:

1. It confirms the presence of the motifs noted from literature are present in strains beyond those used in the published reports.
2. It ensures the viral extract being used in this project to test therapies is theoretically competent and virulent.
3. It ensures the therapies developed in this project are targeted and tested against a competent and virulent strain of RGNNV.

4.2 Materials and Methods

4.2.1 Genome annotation and illustration

The aforementioned critical motifs identified by previous researchers were annotated into the complete RNA 1 and RNA 2 segments obtained from sequencing using the Geneious bioinformatic program. Although the Geneious program is a very useful tool for bioinformatics, the illustration of the overall genome annotation is not easily reproduced in printable, readable single page format from Geneious. Hence, additional schematic maps of RNA 1 and RNA 2 were prepared (Figures 4-1 and 4-2).

4.2.2 Identification of critical motifs in the E.coioides RGNNV viral extract strain.

The critical motifs identified by previous workers were annotated into the RGNNV species type strain using the Geneious bioinformatic program (Biomatters, available from http://www.geneious.com). The sequences of the viral segments obtained from the three NQAus NNV strains described in Chapter 6 were aligned against the RGNNV reference genome described in Chapter 3 using a Geneious global alignment with default parameters. The region

of the RNA 2 that translates into the species specificity region identified by Ito et al.(2008) were compared for all of the Australian strains described in Chapter 3. The conservation or lack thereof motifs in the strains collected in this study were recorded.

4.3 Results

4.3.1 Genome annotation and illustration

A summary of the functional motifs from the translated amino acid sequence from the Betanodavirus are illustrated in Figures 4.1 and 4.2.

4.3.1.1 RNA 1, mRNA of Protein A

All of the motifs that are translated from the mRNA of RNA 1 and identified in the literature as critical for viral replication in the RGNNV species were conserved in each of the three strains investigated in this study. (Table 4.1). The motifs that have been identified as essential for viral replication complex formation were 100% conserved in the species sequenced in this study (aa 6 through to 249 in Table 4.1). A common aa motif of SGxxxxV was retained between the RGNNV, and all of the Betanodaviruses and some Alphanodaviruses in the Mitochondrial location signal motif (aa 6-26 in Table 4.1). The RdRP motifs, where identifiable, were conserved (aa 594-740 in Table 4.1). Two regions in the Protein A sequence (aa750-761 and 780-791) were also 100% conserved across the three strains sequenced in this study. No reference to the potential function of these motifs has been identified (Refer to Table 4.1). All motifs proposed as critical in the B1 and B2 protein were also conserved (Table 4.1). The TVIE motif proposed as a protein kinase II phosphorylation site (aa 66 to 69) identified by Su et al. (2009) was not identified in any of the strains or the RGNNV reference sequence.

4.3.1.2 RNA 2, mRNA of Capsid Protein

All of the motifs that are translated from the mRNA of RNA 2 and identified in the literature as critical for viral replication in the RGNNV species were conserved in each of the three strains investigated in this study (Table 4.1). Most critically to this project, the minimal essential epitopes required for antibody production in grouper namely, aa 181-212 VNVSVLCR were identified (Chen et al., 2015). The region of the RNA 2 identified by Ito (2008) which relates to species specificity of the RGNNV strains was conserved between the RGNNV reference strain and all 3 NQAus NNV strains collected in this study (Figure 4.3). Within the Ito et al. (2008) region, a difference of 3aa across all of the Australian collected sequences was identified (Figure 4.3). Immediately adjacent to the region of Ito et al. (2008), an additional motif GAVF

was also conserved within the strains of this study and the RGNNV reference genome (Figure 4.3). A variant motif of GAIF was present in some of the strains (Figure 4.3).

Table 4-1: Conserved motifs critical for replication of the RGNNV genome identified from translated mRNA of RNA 1 obtained from this study. Including strain, function, amino acid sequence and location on the protein.

	Protein A						
Sequence Id.	Function	Stem loop structure	Mitochondrial Location Signal	Important for Viral Replication complex formation. Essential for replication, point mutations lead to reduced virulence.			
	aa motif		6 – 26	184-229			
	Sequence in literature	**DVDYY**	**MRRFEFALARMSGAAFCVYTGYRLLTSKWLADRVEDYRQRVI**	DDSVH**Y**RVAGGKDVRHRI**W**NYNQNT**M**YVC**S**RPRGFWANLMQILRD			
Ec2NQAus	*E.coioides* this study	160-164 DVDYY	MRRFEFAL**A**RM**SG**AAFC**V**YTGYRLLTSKWLADRVEDYRQRVI	DDSVH**Y**RVAGGKDVRHRI**W**NYNQNT**M**YVC**S**RPRGFWANLMQILRD			
EI3NQAus	*E.lanceolatus* this study	160-164 DVDYY	MRRFEFAL**A**RM**SG**AAFC**V**YTGYRLLTSKWLADRVEDYRQRVI	DDSVH**Y**RVAGGKDVRHRI**W**NYNQNT**M**YVC**S**RPRGFWANLMQILRD			
MI181161	*L.calcarifer* this study	160-164 DVDYY	MRRFEFAL**A**RM**SG**AAFC**V**YTGYRLLTSKWLADRVEDYRQRVI	DDSVH**Y**RVAGGKDVRHRI**W**NYNQNT**M**YVC**S**RPRGFWANLMQILRD			

	Protein A						
		RdRP conserved motifs					
Sequence Id.	Function	Transmembrane Domain of Viral Replication Complex	A: Acid motif	B: SG.T motif	C: GDD motif: NTP binding	D: Basic motif	Motif 7 & 8
	aa motif	229 – 249	594-599	655-660	695-697	740	
	Sequence in literature	**ITGVTAICSFLYTKLGIAPFG**	**DxxxxD**	**SGxxxT**	**GDD**	**Lysine or Arginine**	Description not sufficient for alignment
Ec2NQAus	*E.coioides* this study	ITGVTAICSFLYTKLGIAPFG	DYSKFD	SGSALT	GDD	L	
EI3NQAus	*E.lanceolatus* this study	ITGVTAICSFLYTKLGIAPFG	DYSKFD	SGSALT	GDD	L	
MI181161	*L.calcarifer* this study	ITGVTAICSFLYTKLGIAPFG	DYSKFD	SGSALT	GDD	L	

	Protein A		
Sequence Id.	Function	Unknown function	Unknown function
	aa motif	750-761	780-791
	Sequence in literature	**QsPLRTIlKLHtT**	**YLvTDclTPfI**
Ec2NQAus	*E.coioides* this study	QSPLRTLLKLHTT	YLVTDSKTPFIG
EI3NQAus	*E.lanceolatus* this study	QSPLRTLLKLHTT	YLVTDSKTPFIG
MI181161	*L.calcarifer* this study	QSPLRTLLKLHTT	YLVTDSKTPFIG

		B1 Protein (111aa) same reading frame as Protein A					
Sequence Id.	Function	N-glycosylation site	Nuclear Location signal	Hypothetical Nuclear Location signal	Protein kinase C phosphorylation site	Protein kinase II phosphorylation site	Nuclear Location signal
	aa motif	869-899	912-918	984-988	929-931	66 - 69	946-950
	Sequence in literature	**NKTS**	**PRRARAA**	**RPRRQRR**	**SRR**	**TVIE**	**KRPRR**
Ec2NQAus	*E.coioides* this study	NKTS	PRRARAA	RQRRR	KRSRR	not found	KRPRR
EI3NQAus	*E.lanceolatus* this study	NKTS	PRRARAA	RQRRR	**R**RSRR	not found	KRPRR
MI181161	*L.calcarifer* this study	NKTS	PRRARAA	RQRRR	**R**RSRR	not found	KRPRR

		B2 Protein (72aa) 1 frame shift from Protein A mRNA				
Sequence Id.	Function	Conserved in all the Betanodaviruses	Conserved with NoV	Long dsRNA binding	40bp (short) dsRNA binding motifs	Mitochondrial specific sequence
	aa motif	5-20	16 - 20	14,24	26	41 - 50
	Sequence in literature	**QQAIDQHLVELEQLF**	**LEQL**	**E14, D24**	**R26**	**RTFVISAHAA**
Ec2NQAus	*E.coioides* this study	QQAIDQHLVELEQLF	LEQL	E14, D24	R27	RTFVISAHAA
EI3NQAus	*E.lanceolatus* this study	QQAIDQHLVELEQLF	LEQL	E14, D24	R27	RTFVISAHAA
MI181161	*L.calcarifer* this study	QQAIDQHLVELEQLF	LEQL	E14, D24	R27	RTFVISAHAA

		B2 Protein (72aa) 1 frame shift from Protein A mRNA continued					
Sequence Id.	Function	N'myristolyation site	Mitochondrial specific sequence	Mitochondrial targetting			
	aa motif	30 -55	38-47	R60	V44	I45	R52
	Sequence in literature	**GGVTAI**	**TFVISHAAA**	**R60**	**V44**	**I45**	**R52**
Ec2NQAus	*E.coioides* this study	26-31 GGVTAI	TFVISHAAA	R60	V44	I45	R52
EI3NQAus	*E.lanceolatus* this study	26-31 GGVTAI	TFVISHAAA	R60	V44	I45	R52
MI181161	*L.calcarifer* this study	26-31 GGVTAI	TFVISHAAA	R60	V44	I45	R52

		B2 Protein (72aa) 1 frame shift from Protein A mRNA continued			
Sequence Id.	Function	Mitochondrial targetting	1000bp (long) ds RNA binding motifs and anti- RNAi	Conserved with NoV but not BFNNV	Conserved with NoV, FHV but not AHNV
	aa motif	53	**53,60**	**59**	**69**
	Sequence in literature	**R53**	**R53 R60**	R59	A69
Ec2NQAus	*E.coioides* this study	R53	R53 R60	R59	A69
EI3NQAus	*E.lanceolatus* this study	R53	R53 R60	R59	A69
MI181161	*L.calcarifer* this study	R53	R53 R60	R59	A69

Table 4-2: Conserved motifs critical for replication of the RGNNV genome identified from translated mRNA of RNA 2 obtained in this study including strain, function and amino acid motif and location.

Sequence Id.	**Function**	Nuclear location signal	Binds with viral RNA	Critical in the formation of viral like particles	
	aa motif	2 to 10	23-25	23-25	29-31
	Sequence in literature	**RKGEKKLAK**	**RRR**	**RRR**	**RRR**
Ec2NQAus	*E.coioides* this study	RKGEKKLAK	RRR	RRR	RRR
El3NQAus	*E.lanceolatus* this study	RKGEKKLAK	RRR	RRR	RRR
MI017207	*L.calcarifer* this study	RKGEKKLAK	RRR	RRR	RRR

Sequence Id.	**Function**	Nucleolar localization signal	Required for capsid formation and thermal stability of capsid	Required for capsid formation	50% reduction in viral particle
	aa motif	23-31	115	127-140	130 & 135
	Sequence in literature	**RRRANNRRR**	**C**	**DxxDxD**	**D**
Ec2NQAus	*E.coioides* this study	RRRANNRRR	C	DPTDND	D
El3NQAus	*E.lanceolatus* this study	RRRANNRRR	C	DPTDND	D
MI017207	*L.calcarifer* this study	RRRANNRRR	C	DPT**G**ND	D

Sequence Id.	**Function**	100% reduction in viral particles	Proposed to slow ribosomal processing	Required for capsid formation and thermal stability of capsid	Minimum essential epitopes for antibody target	Region within Ito et al. 2008; SGIV homology; GTPAse homology
	aa motif	133	140-147	201	181-212	223-245
	Sequence in literature	**D**	A**L/F**QATRGA	**C**	**VNVSVLCR**	**LSTND**
Ec2NQAus	*E.coioides* this study	D	ALQATRGA	C	VNVSVLCR	LSTND
El3NQAus	*E.lanceolatus* this study	D	ALQATRGA	C	VNVSVLCR	LSTND
MI017207	*L.calcarifer* this study	**G**	ALQATRGA	C	VNVSVLCR	LSTND

Sequence Id.	**Function**	Mutation lead to reduction in mortality by 40%	Serotype/Species determinant	Variance leads to lack of Pathogenecity in *D.labrax*	C terminal region. Viral particle formation
	aa motif	S247>A	252-254	254-257	328-335
	Sequence in literature	**S**	**PDG**	**GAVF**	**GTVCTRVD**
Ec2NQAus	*E.coioides* this study	S	PDG	GAVF	GTVCTRVD
El3NQAus	*E.lanceolatus* this study	S	PDG	GAVF	GTVCTRVD
MI017207	*L.calcarifer* this study	S	PDG	GAVF	GTVCTRVD

Figure 4-3: Nucleotide sequence and translated amino acid sequences of Australian strains of NNV aligned with Ito species specific region including strain identification, nucleotide and translated protein sequence and arrows indicating points of variance. Prepared using Geneious Prime. Biomatters http://www.geneious.com.

4.4 Discussion

Acknowledging previous works and targeting therapies that are directed at critical motifs may improve the efficacy in preventing VER outbreaks. This study reviewed the collection of literature on the functional motifs of the Betanodavirus genome and confirmed the majority of the motifs that have been identified as critical have been conserved in the NQAus RGNNV, strains collected in this project. No similar review and confirmation of the motifs across an entire RGNNV Betanodavirus genome has been published. The conservation of motifs identified from the translated mRNA-1 indicate all of the motifs considered essential for viral replication have been retained by the strains in this study. Specifically, the mitochondrial location signals and motifs that are essential for the formation of the viral replication complex were identified. The RdRP motifs were also conserved. Individual motifs that locate the B1 and B2 proteins to the VRC and nucleus and also ensure binding of dsRNA to prevent the degradation of the viral genome by host factors were also identified. Interestingly, four additional motifs were identified to be conserved between the species in this study and the RGNNV reference genome. Two motifs namely 750-761aa QSPLRTLLKLHTT and 780-791aa YLVTDSKTPFIG were identified by Johnson et al. (2001) to be conserved in both Alphanodaviruses and Betanodaviruses (specifically: Flock house virus, Nodamura virus, SJNNV, Black beetle virus and Pariacoto virus) but had not previously been identified in the RGNNV. In the same study the authors also identified aa 969-971 RGG motif conserved across the same species but the RGG motif was not identified in this study. No function for any of the three motifs were proposed. The other two motifs noted as highly conserved in this study are aa 5-20 QQAIDQHLVELEQLF of the B2 protein and within this motif a LEQL motif. The LEQL is conserved within the Betanodaviruses and the Alphavirus, Nodamura Virus, which significantly has a wide host range including insects, pigs, suckling mice and hamsters (Gant et al., 2014). No function of this motif has been reported in the literature relating to Nodavirus replication. However, an LEQL motif is an important epitope recognised by the endoplasmic reticulum aminopeptidase (ERAP 1) to target Major Histocompatibility complex class 1 presented epitopes for degradation. ERAP 1 is stimulated by interferon (Hearn et al., 2009). The LEQL motif is also an important epitope of TRIM 21 which is a member of the Tripartite motif superfamily (Al-Majdoub et al., 2013). TRIM 21, in particular, is expressed upon interferon stimulation and is also an important component of innate immunity and neurological disorders (Al-Majdoub et al., 2013). Kim et al. (2017) reported the upregulation of TRIM 21, 29, 39, 25, 14, 16 and 47 following infection of Sevenband grouper to NNV. *In vitro* upregulation of TRIM

21 in human microglial cells attenuated the replication of Japanese encephalitis (JE), via regulation of the type 1 interferon response (Manocha et al., 2014). JE is similar to NNV in that it affects the brain of host species. Damage due to inflammation is associated with mortality in many neurotropic viruses. Additionally, TRIM 21 is activated by antibody-coated viral particles, binding to the Fc receptor and targets virions for degradation. It is possible the LEQL motif of the B2 performs roles not currently identified to prevent both the innate and adaptive immune response to RGNNV infection. The targeting of TRIMs by Betanodavirus B2 would be a very efficient mechanism to successfully mitigate both a humoral (antibody) and innate immune response. However, no work has been published relating to TRIM and B2 protein. Petrillo et al. (2013) noted, B2 is produced well in excess of RNA 1 and could perform additional roles in the replication of the Nodaviruses.

The majority of the motifs proposed to be critical for viral replication that are associated with the translated RNA 2 were also identified in all three strains of RGNNV studied. Importantly for the vaccine development, the motifs of the capsid protein that were identified to be critical for neutralising antibody production in grouper were present in all three strains obtained in this study (Chen et al., 2015, discussed in Chapter 1.8). The region identified by Ito et al. (2008) to confer virulence in host species was also conserved between all strains collected within this study. Notably comparison with all of the Australian collected strains indicated a variance of 3 amino acids within the Ito et al. (2008) region. Specifically, at aa 233-237 an L**S**T**N**D motif is present in all of the strains identified as "tropical (1a) strains" in Chapter 3. Whereas L**A**T**S**D was conserved in the sequences assigned to the 1c cluster (Section 3.1). No mechanism for how the sequences may impact on host or environmental selection have been identified. Also, immediately adjacent to the PDG motif which confers the Betanodavirus serotype assignment an additional motif variation was noted between the Australian strains. Within Australian RGNNV strains, the four strains that slightly differentiate from the other Australian strains based on nucleotide comparison of RNA 2 (Chapter 3) display a GA**I**F motif. Interestingly, the tropically freshwater cultured sleepy cod, *O.lineolata* sourced strain displayed a hybrid pattern of motifs. The *O.lineolata* strain retained the LSTND motifs of the tropical 1a strains yet shared the GAIF motif of the "temperate Ic strains", proposed by this worker to possibly reflect a freshwater habitat rather than temperate v tropical division (Chapter 3). Investigating how these motifs affect virulence to different species, or the same species within different habitats is beyond this study. However, confirmation that the strains in this study have the retained of the factors that have been identified to affect viral replication or host specificity provides confidence to advance further work using the strains collected. Confirmation of the protein

motifs is rarely considered in experimental challenge experiments. Although analysis completed in Chapter 3 indicated the RGNNV genome segments were highly conserved, between 13-21 nucleotide differences were noted between the Australian strains collected in this study and also the RGNNV reference genome (Table 3.2). Considering a single nucleotide variation could also translate to change in a critical protein motif, it was important to confirm the conservation of the motifs in the strains being used in this project. Variation on a single motif could lead to reduced virulence, which if were transferred into the project would reduce the effectiveness in translation of the outcomes of this study to industry. Lin et al. (2007) reported a relative percent survival of 80-90% in 35 dph *E.septemfasciatus* larvae when challenged by bath exposure to VVN at following 17 days of feeding with Artemia that been orally loaded with *E.coli* expressing a recombinant NNV capsid protein (Lin et al., 2007). However, the control fish displayed between 44-69% survival which is unusually high compared to similar reports of experimental challenge with this species. High survivals in control groups following RGNNV exposure have not been reported in any other research publication involving larval grouper. Despite the impressive survival and obvious economic benefit of this approach as a vaccination strategy, these results have not been translated to a commercial product. Although it cannot be confirmed, it is possible the strain of RGNNV used in the study was less virulent, possibly through lacking a motif that confers increased virulence. From the analysis completed in this chapter, considering all of the strains hosted the critical motifs, and the volume of infectious material available, the RGNNV extract prepared from *E.coioides* (Ec2NQAus) is considered an acceptable source of NNV for the remaining study in this project (and is henceforth referred to as the RGNNV viral extract).

4.5 Conclusion

The aims of this chapter were met in the following manner:

- The motifs demonstrated to critically impact on viral replication or host specificity in Betanodaviruses have been identified.
- The motifs have been confirmed to be present in the strains used within this project.
- The production of a recombinant vaccine based on the RNA 2 sequence of the *E.coioides* RGNNV strain will contain the antigenic epitopes that were identified as critical to the production of neutralising antibodies by grouper recognised by Chen et.al., (2015)
- dsRNA can be specifically designed to target functional motifs that are confirmed to be present in the strain used in this project.

Data from this Chapter is planned for publication entitled “Review of the functional motifs of the Betanodavirus genome”.

CHAPTER 5. DEVELOPMENT OF QPCR STANDARD CONTROL MATERIAL TO ALLOW INITIATION OF VALIDATION OF RT-QPCR ASSAYS TO DETECT AUSTRALIAN STRAINS OF REDSPOTTED GROUPER NERVOUS NECROSIS VIRUS (RGNNV).

Background

- Hick & Whittington, (2010) reported the development of RT-qPCR, qR2T, that detects RNA 2 of RGNNV in two Australian sourced NNV strains.
- The qR2T assay is the assay recommended in the Australian and New Zealand Standard Diagnostic Protocols for the detection of NNV.
- The assay has not been validated as fit for purpose on grouper tissues.
- There are various novel biotechnology applications that aim to produce NNV vaccine via delivery of the viral capsid protein.
- Some biotech applications aim to produce an antigen that can be delivered via oral dispersal of the antigen within larval fish feeds.
- The widespread dispersal of RNA 2 constructs into the fingerling production systems will confound the current method of verification of freedom from RGNNV with the RT-qPCR targeting the RNA 2 segment.
- Due to discovery of chimeric reassortment between RNA 1 and RNA 2 of SJNNV and RGNNV some researchers advocate the application of qPCRs that target both viral segments to study VER outbreaks.
- Hick & Whittington (2010) reported an additional RT-qPCR that detects RNA 1 of RGNNV but did not extensively validate the assay.

Aims of this Chapter

- Develop RT-qPCR control material to assist in the validation of the currently recommended Australian and New Zealand Standard Diagnostic Protocol RT-qPCR that targets RNA 2 for the detection of the RGNNV from grouper brain and eye tissue.
- Develop RT-qPCR control material to assist in the validation of an additional assay that targets the RNA 1 for the detection of the RGNNV genome from grouper brain and eye tissue.

5.1 Introduction

The absence of a sound understanding of the factors that trigger VER disease outbreaks in grouper grow-out aquaculture, currently limits management options to the stocking of ponds with fingerlings that are free of NNV. The development of reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) assays has significantly improved the opportunity for industry to obtain NNV free fish (RT-qPCR negative) and rapidly diagnose a VER disease outbreak (RT-qPCR high load positive). Detection by RT-qPCR is the only method currently recommended for all applications including targeted surveillance of larvae, juveniles and adult fish and presumptive and confirmatory diagnosis of VER disease (Anon. OIE 2018). The OIE Aquatic Animal Manual describes two RT-qPCR assays to detect NNV that have undergone significant validation namely the qR2T (Hick & Whittington 2010) and RNA 2 assay (Anon. OIE 2018). The area targeted by each assay overlap on the redspotted grouper nervous necrosis virus (RGNNV) RNA 2 segment. The qR2T assay, is incorporated into the Australian New Zealand Standard Diagnostic Procedures (ANZSDP) for the detection of NNV (Hick & Whittington, 2010; Moody & Crane 2012; Anon. OIE 2018). The RNA 2 assay was developed by the OIE Reference Laboratory for VER and has been adopted for proficiency testing across five European laboratories (Panzarin et al., 2010; Anon. OIE 2018;). Both assays are reported to detect all NNV species although qR2T is noted to optimally target RGNNV (Hick & Whittington, 2010 and Anon OIE 2018).

With their emergence as high value aquaculture species, the economic impact of VER in sea bass aquaculture has prompted the development of two commercially available vaccines to protect against VER (ALPHA JECT micro® 1 Noda PHARMAQ and ICTHIOVAC® VNN HIPRA Laboratories). The ALPHA JECT micro® 1 Noda vaccine is a cell culture- derived- formalin-inactivated antigen reported to induce effective immunity against the RGNNV genotype. The ICTHIOVAC® VNN is inactivated Betanodavirus strain 1103 (HIPRA Laboratories https://www.hipra.com/portal/en/hipra/animalhealth/species/fish). To avoid stress associated with injectable vaccines, several biotech applications have been attempted with oral delivery of recombinant NNV capsid protein (Cho et al., 2017 and Gonzalez-Silvera et al., 2019). Oral vaccination, through consumption of recombinant yeast, *Saccharomyces cerevisiae* expressing RGNNV capsid protein induced the production of neutralizing antibodies in *Epinephelus septemifasciatus* (Thunberg, 1793) (Cho et al., 2017). The inclusion of TOPO® transformed *E. coli* with RNA 2 segment of RGNNV into diet was recently reported to provide protection to sea bass against viral challenge with RGNNV (Gonzales-Silvera et al., 2019).

Although oral delivery offers advantages of practicality of delivery and reduced handling of fish, it may lead to inaccurate assessment of fish health status if the recombinant RNA 2 genome segments are detected by RT-qPCR. The potential development of orally delivered vaccines based on expressed proteins from RNA 2 constructs necessitates an RT-qPCR targeting the RNA 1 segment to differentiate between NNV infected fish and those exposed to orally delivered recombinant capsid proteins. Further, the discovery of strains of NNV that consist of chimeric recombination of RGNNV and SJNNV segments highlights a need to apply assays that detect both RNA segments in epidemiology studies, particularly in zones where more than one genus of NNV is endemic (Panzarin et al., 2016).

RT-qPCRs that detect the RNA 1 have been described but not widely adopted in Australia (Hick & Whittington, 2010 and Baud et al., 2015). Neither the qR1T or qR2T assay was validated for application on grouper tissues. The OIE Aquatic Animal Health Manual notes that it is important to revalidate an assay when it is being applied beyond the scope of the original intended purpose of the assay such as application to an additional host species (OIE Anon. 2014). The recommendations of the OIE are also adopted by authorities in Australia that oversee quality control in testing for aquatic animal pathogens namely, the sub-committee for aquatic animal health (SCAAH) and the National Association of Testing Authorities (NATA). The validation of an assay is a step-wise process. Initial steps determine if the assay platform is the best fit for purpose format. For reasons of quantitation, high throughput, rapid turnaround, sensitivity and specificity, RT-qPCR is the assay of choice for many applications in aquatic disease management. An initial step in the implementation of an assay is to produce stable qPCR controls, of sufficient volume and stability that can be applied to monitor the performance of the assay over time (Fig 1. Anon OIE 2017).

The aim of this chapter is to develop the standard positive controls for the qR1T and qR2T assays of Hick & Whittington, (2010). These controls are required to support further application of the assays within the activities of this project to assess the effectiveness of prophylactic measures against RGNNV; to gain a better understanding of the pathogenesis of VER in grouper and to validate the assays as suitable for application beyond the scope which was demonstrated by Hick & Whittington (2010).

5.2 Materials and Methods

5.2.1 Preparation of plasmid control for quantitative real-time polymerase chain reaction

Synthetic control for quantitative real-time polymerase chain reaction was prepared from PCR amplicons obtained from NNV infected fish. Positive PCR amplicons were produced using the primers R1F1/R1R5 and R2F1/R2R1 described by Hick & Whittington (2010) (Table 5.1). The amplicons were visualised and cut from agarose gels as described in Chapter 2.1.4-2.1.7. The PCR product was cleaned using a High Pure PCR Product Purification Kit (11732668001 Roche, NSW) as per manufacturer's instructions. Purified PCR product was cloned into One Shot TOPO chemically competent *E. coli* using the pCR4-TOPO TA vector (Cat. K4575-01 Life Technologies, VIC) as per manufacturer's instructions. Transformed *E. coli* were grown overnight at 37 °C on lysogeny broth (LB) (Bertani, 1951) agar supplemented with 50 μg mL^{-1} ampicillin (Cat. A9393-5G Sigma-Aldrich, NSW). Three white colonies were picked from the agar and grown overnight at 37 °C in LB supplemented with 50 μg mL^{-1} ampicillin (Cat. A9393-5G Sigma-Aldrich, NSW) and shaken (Bioline incubator shaker 8500 Edwards Instruments, NSW) at 150 rpm. Plasmid DNA was extracted using a High Pure Plasmid Isolation kit (Cat. 11754777001 Roche, NSW) as per manufacturer's instruction. Plasmid extracts were submitted to Macrogen Inc. (Seoul, Korea) for sequencing. Sequence analysis confirmed the respective plasmids were the RNA 1 and RNA 2 derived products. The clones and extracts became the positive plasmid controls for the quantitative real-time PCRs (qPCR).

Table 5-1: Details of primer sequences used to produce RT-qPCR control sequences including assay type, primer name, primer sequence, primer target, primer position and expected size of positive amplicon.

Assay name	Primer Name	Sequence (5'-3')	RNA segment Target	Position~	amplicon size (bp)
qR1T	R1F1*	CACTTACGCAAGGTTACCG	1	1	1515
	R1R5*	TCTGCTGCTCCTCGACATAC	1	1525	
qR2T	R2F1*	CATATGGTACGCAARGGTGA	2	3	1020
	R2R1*	CTCGAGTTAGTTTTCCGAGTCA	2	1023	
Source of All primer sequences Hick & Whittington (2010)					
~Position with reference to NCBI MI181161/RNA-1 or MH017207/RNA-2					

5.2.2 Standard curve preparation from plasmid controls

A standard curve was prepared for the qPCR using the positive plasmid controls generated from section 5.2.1. The clones were cultured for 18 hours in 30 mL of LB supplemented with 50 μg mL^{-1} ampicillin (Sigma-Aldrich) at 150 rpm (Bioline incubator shaker 8500). A 100 μL aliquot of culture was added to 12 x 10-fold serial dilutions while the 30 mL culture was frozen at -80 °C to prevent further bacterial growth. Copy number was calculated by plating triplicate 20 μL

aliquots of each dilution on LB agar plates supplemented with 50 µg mL^{-1} ampicillin (Sigma-Aldrich) and incubating for 18 hours. The number of white colony forming units (CFU) were counted for each dilution and used to calculate the plasmid copy number in the 30 mL culture, assuming one CFU represents one plasmid copy.

5.2.3 RT-qPCR analysis of standard control serial dilutions

The 30 mL culture that was frozen during the CFU counting procedure was thawed and plasmid DNA extracted and eluted in 100 µL elution buffer as previously described (3.3.4). A 50 µL aliquot of DNA extract was used to prepare 9 x 10-fold serial dilutions in DEPC-treated water (BIO-38030, Bioline) and stored at -20 °C for qPCR. A 2.5 µL aliquot of each serial dilution was used as template in the qR1T and qR2T RT-qPCRs to construct the quantified standard curve. The qPCR mix consisted of 2.5 µL DNA extract in Bioline SensiFAST probe No ROX mix (BIO-86050) prepared according to the manufacturer's specifications, containing Forward and Reverse Primer (20 pmol) and Probe (5 pmol) per reaction (Table 5.2). The qPCR was completed on a Qiagen Rotor-Gene™ with thermal cycling consisting of 95 °C for 3 mins followed by 40 cycles of 95 °C for 15 s and 60°C for 25 sec. Fluorescence was acquired at the 60°C for 25 sec step of each cycle using the green and yellow filters of the machine.

Table 5-2: Primer and Probe sequences used in RT-PCR assays including assay type, primer name, primer sequence, RNA target, position on target and primer melt temperature.

PCR format	Primer Name	Sequence (5'-3')	RNA segment Target	Position~	Tm Primer
RT-qPCR	qR1T-F	GCTACCGCCTGTTGACCTC	1	140	61
	qR1T-R	TTGTTTCTTCTCAGCGATGATGC	1	219	64
	qR1T-Probe	TGGCGAATCCTCAACACGTCC	1	171	
RT-qPCR	qR2T-F	CTTCCTGCCTGATCCAACTG	2	401	62
	qR2T-R	GTTCTGCTTTCCCACCATTTG	2	476	61
	qR2T-Probe	CAACGACTGCACCACGAGTGG	2	454	
Source of All primer and probe sequences Hick & Whittington (2010)					
~ Position with reference to NCBI GQ904198/RNA-1 or GQ904199/RNA-2					

5.3 Results

5.3.1 Confirmation of sequence of the RNA 1 and RNA 2 plasmid

Plasmid sequences were confirmed to be 100% homologous to the *E.coioides* RGNNV strains discussed in Chapter 3. Although shorter sequences would have been sufficient to act as qPCR

controls, the longer sequences were selected to align more closely with the characteristic of viral sequence that would be present in host tissues during viral replication.

5.3.2 Plate count of CFU of each dilution of plasmid controls

A number of the serial dilutions of plasmid contained too many colonies to allow accurate counting of colonies. The plates that contained less than 100 colonies were used to calculate the plasmid CFUs. Plate counts indicated the stock cultures of each plasmid contained ~ 12.6 x 10^9 (RNA-1) and 13.8 x 10^9 (RNA-2) CFU mL^{-1}.

5.3.3 Quantitative real-time polymerase chain reaction

The target sequences were detected in the standard curve dilutions from 10^0 to 10^9 range for qR1T and 10^0 to 10^8 range for qR2T. The standard curves prepared displayed a strong linear correlation between estimated plasmid copy number and Ct value (R^2 value=0.99 in each assay). (Figure 5.1). The qR1T assay had a reaction efficiency of 98% which was slightly less than that of qR2T (99%) (Figure 5.1). The Cycle threshold value, plasmid copy number and typical standard series amplification curve for the RNA 1 and RNA 2 segment plasmid control are provided in Figure 5.1.

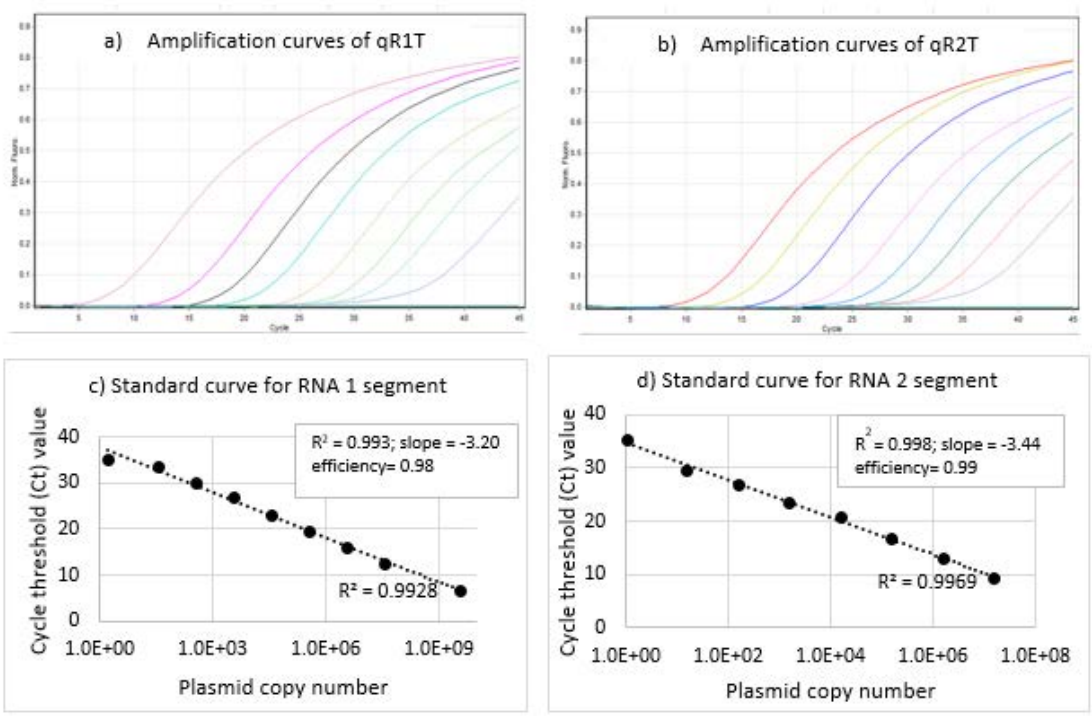


Figure 5-1: RT-qPCR amplification curves of qR1T (a) and qR2T (b) and linear correlation between Ct value v plasmid copy number determined from bacterial plate counts of qR1T (c) and qR2T plasmid (d) controls.

5.4 Discussion

The data collected in this study indicates the RT-qPCR assays that detect RNA 1 (qR1T) and RNA 2 (qR2T) perform consistently to accurately detect the specific segments of the Ec2NQAus genome on the standard constructed DNA plasmid controls. The operational range of each assay detected a minimum of ~10 copies of the plasmid target. In this study, consistent Ct values were obtained for plasmid dilutions targeted by each assay across a gene copy number ranging from ~10 to 10^8 copies mL^{-1}. Although a calculated copy number greater than 10^8 is detected by both assays, there is considerable variation in the calculated copy number at higher concentration of plasmid (data not shown) and higher concentrations were not included in the standard curve regressions. As the calculated copy number that applies to such high concentration of plasmid genome would only be detected in severe VER outbreaks where management decisions are not reliant on accurate discrimination between a 10^8 or $10^{9 \text{ or } 10}$ copies of viral genome, the omission of the high copy number control within the standard curve is not expected to be a major impediment to the application of either RT-qPCR assay. The OIE devotes a chapter in the Manual of Diagnostic Tests for Aquatic Animals to discuss the principles and methods of validation of diagnostic assays for infectious diseases (OIE 2019).

Whilst Hick & Whittington, (2010) validated the performance of the qR2T assay against the criteria discussed in the OIE Aquatic Manual, their analysis was based on cell cultures and barramundi derived tissue samples. In addition, very limited assessment of the qR1T assay that targeted the RNA 1 was performed. Neither assay has been reportedly applied to grouper species (Hick & Whittington 2010). The OIE recommends validation and verification of assay performance is conducted when analysis is applied to tissue matrix that is beyond that which the assay was validated (OIE 2017).

One of the initial steps in assay validation is the implementation of standard positive controls (OIE 2017). This current work has developed a plasmid containing the respective target sequences to act as a standard control for each assay. The use of a constructed plasmid as a standard curve control reduces laboratory biosecurity risk by avoiding a need for staff to handle viable viral particles and also negates a need for cell culture capability within a laboratory. However, further monitoring of assay performance on a similar analyte matrix to that which it will most typically be applied, namely eye and brain tissue of known NNV status, is required for assay validation (OIE 2017). The stepwise approach to assay validation which has been adopted in this project is typical within the framework recommended by a number of authorities responsible for animal health laboratory standards including the OIE, the sub-committee for animal health laboratory standards (SCAAHL) and the National Association of Testing Authorities (NATA) in Australia (OIE 2017). The development of the standard controls discussed in this chapter are a crucial requirement for managing the quality of the quantitative data that will be collected in this project. Further application of the assays on grouper brain and eye tissues to monitor the pathogenesis of VER and to assess the efficacy of prophylactic measures against RGNNV will be discussed within subsequent chapters of this thesis.

5.5 Conclusion

The following outcomes were achieved in this chapter:

- Standard plasmid controls for RT-qPCRs to detect RNA 1 (qR1T) and RNA 2 (qR2T) of RGNNV described by Hick & Whittington, (2010) were developed.
- Both assays, qR1T and qR2T were demonstrated to detect the respective standard plasmid controls.
- The linear regression of standard curve preparations was determined for each assay, with high correlation between Ct value and calculated copy number.

Data collected in this chapter contributed to the validation documentation to support application by the JCU AquaPATH laboratory for NATA accreditation in the field of animal health. Scope of application detection of viruses by nucleic acid detection.

CHAPTER 6. DEVELOPMENT OF PROPHYLACTIC MEASURES TO PREVENT VIRAL ENCEPHALOPATHY AND RETINOPATHY (VER)

Background

- Grouper in grow out pond culture are susceptible to NNV for the entire duration of pond stocking.
- VER outbreaks in pond grow out impose severe mortality and economic losses that threaten the viability of the grouper aquaculture industry in Australia.
- A number of experimental vaccines to prevent VER have been reported.
- There is no VER vaccine available for use in Australia.
- The capsid protein has been demonstrated as the target of neutralising antibodies.
- Treatment with dsRNA has been proposed as a treatment for difficult to prevent viral diseases.

Aims of this Chapter

- To develop a recombinant clone and express RGNNV capsid protein.
- To prepare a vaccine containing the recombinantly expressed capsid protein.
- To develop a dsRNA construct to knock-down replication of RGNNV.

6.1 Introduction

The development of a vaccine that prevents VER outbreaks in *E.lanceolatus* is a primary goal of this study. Vaccination strategies that prevent VER have been reported with varying success in experimental settings (Tanaka et al., 2001;Nishiwawa et al., 2012; Kai and Chi 2008). Various antigen presenting configurations have been developed to produce vaccines against VER. Antigen configurations include the presentation of live virus (Nishizawa et al., 2012), inactivated virus (Kai and Chi 2008), recombinantly expressed DNA or capsid protein (Tanaka et al., 2001), provision of virus-like particles in live *Artemia* (Lin et al., 2007) recombinantly expressed virus-like particles (VLPs)(Lai et al., 2014) and most recently, viral capsid protein expressed in a cell-free system (Kim et al., 2015). All strategies report improved survival measured over a period of weeks. Some studies don't include viral challenge but rather detect

the expression of immune components following vaccination (Lai et al.2014). Despite the reported progress, there is no vaccine approved for use or commercially available for use in Australia.

Previous research into immunization and the potential of dsRNA as an additional option to prevent VER is discussed within this chapter. In addition, the methods used to prepare experimental therapies that will be evaluated as a means to prevent VER within this project, namely a vaccine and dsRNA constructs, are described.

6.1.1 Live Virus Vaccine

"Vaccinating" fish with live virus at a lower rearing temperature has been reported as an effective way to reduce mortality to subsequent Betanodavirus exposure (Nishizawa et al., 2012). Seven-band grouper "vaccinated" with live RGNNV at $10^{4.3}$ $TCID_{50}$ displayed no mortalities at 17 °C compared with ~47 % mortality in fish held at 20 °C and 93-100 % in fish reared at 23 °C and 26 °C respectively (Nishizawa et al., 2012). NNV titres varied over the 21-day trial period at the different rearing temperatures. Nishizawa et al. (2012) hypothesised the slower replication of NNV in fish at lower water temperature enabled sufficient time for the fish to mount an effective immune response before NNV reached critical a threshold level of 10^{10} $TCID_{50}$ and death occurred. The long-term outcome of this vaccination strategy was not investigated.

6.1.2 Inactivated-Virus Vaccine

Protection from VER has been reported through the use of vaccination based on the IM or IP-injection of formalin-inactivated (Yamashita et al., 2009) and binary ethylenimine (BEI)-inactivated (Kai & Chi 2008) RGNNV. Survival, antibody presence and antibody titre in the formalin-inactivated vaccinated fish exposed to challenge dose of 10^5 $TCID_{50}$ was dependant on the dose of RGNNV in the vaccine. Yamashita et al. (2009) did not measure the neutralising antibody level in vaccinated fish over a period beyond 28 days. As NNV is believed to be endemic in the waterways around Japan, fish would likely been exposed to NNV which would serve as a natural booster in immunity (Yamashita et al., 2009).

6.1.3 DNA vaccine: recombinantly expressed viral protein

Protection from NNV infection has been reported through the use of vaccines based on recombinantly expressed Betanodavirus capsid protein since 2000/2001 (Nakai 2002 and Tanaka et al., 2001). Tanaka et al. (2001) reported the successful vaccination of seven band grouper (28 g) against RGNNV using IM. injected recombinantly expressed coat protein (60 µg

fish^{-1}) and challenged by intramuscular injection 20 days post vaccination (dpv). Vaccinated fish that were challenged with a dose of $10^{4.4\text{-}5.4}$ $TCID_{50}$ displayed 65 % mortality compared to the control fish which suffered 100 % mortality within 4 days post challenge (dpc) (Tanaka et al., 2001). Vaccinated fish exposed to a lower challenge dose $10^{3.4}$ $TCID_{50}$ had 10 % mortality compared to 85 % mortality in the unvaccinated group. Results of fluorescent antibody testing (FAT) on the lower challenge dose survivors revealed only 1/18 of the vaccinated fish was positive for the detection of VER capsid protein compared to 1/3 control fish. Neutralising antibody titres were detected in the vaccinated fish peaking at 1:400-1:500 at days 20 to 30 and declining to 1:200 to 1:260 at days 70 to 110 dpv (Tanaka et al., 2001). The minimum neutralising antibody giving protection is approximately 1:200 to 300 (Yamashita et al., 2009). The application of recombinantly expressed capsid protein to protect against VER continues to be reported with no advance towards the production of a commercial vaccine (Vimal et al., 2014a). Most recently a vaccine based on an IM injection (50 µg fish^{-1}) of recombinantly expressed RGNNV capsid was reported to provide 76 % relative percent survival (RPS) to juvenile *L.calcarifer* (10 to 15 g) (Vimal et al., 2014a). No mortalities occurred in the vaccinated group until 16 days post-VER challenge. Mortality in the non-vaccinated group was 80 % at the same time point. Notably mortalities in the vaccinated group were on an upward trend from day 22 until when the trial was terminated at day 30 post-VER challenge. Serum collected from vaccinated fish displayed anti-viral activity capable of neutralising 50µl of 10^2 $TCID_{50}$ in *D. labrax* kidney cells (Vimal et al., 2014a). Using the same plasmid attached to chitosan-tripolyphosphate (CS/TPP) in an oral vaccination (feed) survival of ~50 % was achieved in *L. calcarifer* following injection with RGNNV (Vimal et al., 2014b). Immunofluorescent detection of the plasmid demonstrated the nanoparticles were delivered to the gills, heart, intestine, muscle and liver. Antibodies against RGNNV were detected in serum diluted 1:1000 (Vimal et al., 2014b). Unlike the previous report, all of the fish injected with RGNNV displayed relatively good survival until day 15 (~70 %) suggesting the exposure to plasmid offered some degree of protection against VER. However, from day 15 mortality in all of the control groups displayed a marked increase which reached approximately 80 to 90 % at day 30 pi. The mortality in the CS/TPP "vaccinated" group displayed an upward trend at day 30 pi. Considering the upward trend observed in both experiments at 30 dpi the long-term protection offered by the vaccines is questionable, but could perhaps be improved by boosting.

6.1.4 Viral Like particle (VLP) Vaccine

Viral like particles (VLP) formed by the expression of RGNNV RNA 2 in *E. coli* have been used as experimental vaccines to prevent VER. VLPs, IM-injected (1 to 10 µg^{-1} fish) into malabar

grouper and dragon grouper (average body weight {abw}~20g) lead to the production of specific antibodies within 4 weeks post-injection (Liu et al., 2006). The innate immune response of grouper following exposure to RGNNV VLPs was measured in orange-spotted grouper *E. coioides* (0.36 g) (Lai et al., 2014). VLP injection (1.5 $\mu g g^{-1}$ FBW) was demonstrated to be effective in the production of neutralising antibodies. Titres detected within one-week post-exposure could neutralise more than 10^8 $TCID_{50}$ of virus *in vitro* (Lai et al., 2014). Changes in the expression of humoral (CD_4, MHC11a) and cellular (TCR-β, MHC1a, CD8, CCC3) immunity factors, members of the antiviral pathway (Mx, TNFR14, ISP16), and the cellular chaperone HSP90, were recorded in various organs. The immune factors measured from the brain and eyes displayed minor increases in expression levels. Measurements in the kidney, spleen and liver displayed varying responses, none of which were prolonged beyond 48 hours (Lai et al., 2014). As the fish were not exposed to viral challenge, the significance of the differential immune response data cannot be determined. The lack of detectable changes in the brain and eye could indicate the VLPs were never presented to those organs. Nonetheless, the data provides some indication the capsid protein of RGNNV stimulates both cellular and humoral immune pathways.

6.1.5 Cell-free vaccine production

The commercial production of vaccines based on recombinant technologies is reportedly hindered by the tedious and labour-intensive processes involved in production (Kim et al., 2015). Kim et al. (2015) reported such issues can be overcome through the production of a RGNNV capsid based vaccine using cell-free protein synthesis (rNNV-CP). Seven band groupers (20 g) were injected with rNNV-Cp (20µg $fish^{-1}$) followed by injection with NNV $10^{2.8}$ $TCID_{50}$, two weeks later. Seventeen days post-VER challenge 10% of vaccinated fish displayed mortality comparing favourably to 50 % of unvaccinated fish (Kim et al., 2015).

6.1.6 Consideration of path for approval of use of a vaccine against VER

The Australian Pesticides and Veterinary Medicines Authority (APVMA) regulates the registration of veterinary vaccines in Australia (https://apvma.gov.au/node/1108). The registration process is lengthy, and in some cases, requires demonstration of compliance across multiple authorities. Vaccines containing cell-culture derived antigen must be prepared in compliance with Australian Government Department of Agriculture and APVMA (pers. comms from APVMA). There are no fish cell lines in Australia with approval for vaccine production. Consequently, in Australia, the development of vaccines containing antigens

produced from recombinant expressed viral proteins is underway and considered an attractive longer-term approach to viral vaccine production of fish species (Norwood, 2018).

6.1.7 dsRNA as an alternative or complement to vaccines

Many vaccines have been reported with varying success in reducing mortality due to VER. However, all report the reduction, rather than elimination, of NNV titres over the short term (Kim et al., 2015). Based on previous reports, successful vaccination of grouper although preventing disease expression, results in the production of sub-clinical carriers following exposure to NNV (Kim et al., 2015). Considering the factors which lead to VER in grow out grouper systems are unknown; vaccination may not be the remedy to the challenge NNV poses to aquaculture. If disease in grow out grouper is caused when naïve individuals are exposed to Betanodavirus, vaccination will be a useful management tool. However, if disease outbreaks are due to other modulating factor/s inducing sub-clinical fish to express disease, vaccination may not be the solution to prevent losses due to VER in grouper grow out aquaculture. The limited availability of any commercially produced vaccine against VER, despite their reported successful application in experimental conditions since 2001, suggests the pathogenesis of VER disease in grouper grow-out culture is more complex than anticipated.

Attempts to combat human RNA viral infections have led to the development of vaccines by a variety of protocols. Although often safe and effective in the short term, many fail to be effective long-term and require multiple doses which are not cost effective. The production of live-attenuated vaccines, although overcoming the need for boosting, presents some risk in that RNA viral genomes are particularly unstable and vaccine strains could become virulent. Additionally, in human applications, there is a lack of success in producing vaccines which are protective against a variety of viruses that, like VNN, display phases of latency such as Epstein Barr virus, Human Immunodeficiency Virus and Herpes Simplex Virus (HSV) or a range of viruses that sequester in neural tissues such as Dengue Fever, Zika virus and West Nile virus. The use of specific microRNAs, termed dsRNAs, is emerging as effective means to overcome the limitations of vaccines (Heiss et al., 2010). Successful downregulation of a multitude of viruses has been reported *in vitro* using synthetic RNA (sRNA) to prime the RNAi pathway vaccines (Heiss et al., 2010). There are no reported attempts to down-regulate Betanodavirus replication using sRNA. However, when Grouper Heat Shock Protein 70 (GHsp70), which was discussed in Chapter 3 as an important site for viral attachment to cells, was silenced with iRNA, NNV expression of RNA 2, as measured by SYBR green RT-qPCR, was reduced (Chang & Chi 2015). Although Chang & Chi (2015) report the target of the iRNA was GHsp70 the

designed iRNA (iRNA sequence: CGGUGUUCCUCAGAUUGA) conserved regions of homology with many RGNNV RNA 1 sequences (eg. KP455643.1) (Table 6.1). Successful knockdown of NNV RNA may have been achieved by direct action of the iRNA on NNV replication rather than knockdown of the critical Hsp70.

Table 6-1: Shared homology between anti GHSP-70 iRNA and Spotted grouper RNA 1 segment (NCBI reference KP455643.1), including homologous sequence, nucleotide position and orientation and functional motif of NNV genome (if known).

Source of sequence and alignment		Details of homology to RGNNV genome		
GHsp70 iRNA	SGNNV (KP455643.1) (RNA1)	nt/nt	%	Function on VNN Protein A if known
2 GGTGTTCC 9	550 GGTGTTCC 557	8/8 +/+	100	Region associated with temperature sensitivity
9 CTCAGAT 15	248 CTCAGAT 254	7/7 +/+	100	Region associated with temperature sensitivity
10 TCAGATT 16	745 TCAGATT 739	7/7 +/-	100	Mitochondrial location signal of Mezeth
4 TGTTCCT 10	1663 TGTTCCT 1657	7/7 +/-	100	
12 AGATTGA 18	2354 AGATTGA 2360	7/7 +/+	100	
5 GTTCCTC 11	2963 GTTCCTC 2957	7/7 +/+	100	B1/B2

Effective prevention or treatment of viruses requires the dsRNA to have exact complementary alignment to the viral target gene sequence to ensure the target sequence is loaded into the RNAi silencing complex (RISC) and template degraded rather than translated into protein (Chakraborty et al., 2017). It is equally important to ensure that the gene in target is essential for viral replication or virulence and the dsRNA does not have off-target effects such as down-regulating host genes that are required to retain health (Chakraborty et al., 2017).

In Chapter 4 the processes known to occur during replication of NNV and the genome regions that translate to the functional motifs of the genomes of the NNV strain being used in this study were identified. As discussed in Chapter 4, the mRNA 1 is the first strand translated during viral replication. The initial stages of viral replication occur within viral replicating complexes that are contained within the mitochondrion. Also, during the early stage of viral replication, the B2 protein is expressed. B2 binds short and long dsRNA and inhibits iRNA activity presumably by preventing any viral genomic material that is not within the VRC from being targeted by Dicer-1 and also abrogate the genome recognition by Argonaute and prevent loading of viral genome into the RNA Silencing Complex for degradation. B2 is downregulated when the capsid protein mRNA is upregulated. Additionally, the capsid protein isn't reported to locate within the protection of VRC.

Because of the forementioned processes this study considered the capsid protein mRNA to be theoretically more accessible during viral replication and therefore it was considered a target for dsRNA. Specifically, the region that encoded the LSTND motif, contained within the species

specificity region identified by Ito et al. (2008). The motif was retained by the RGNNV reference genome and the NQAus NNV strains that display virulence to the tropical grouper species in Northern Queensland (Chapter 4.3 and 4.4). Examination of the nucleotide region of this motif indicates, changes to host specificity associated with this region may not be related to the translated amino acids but alternately affect species specificity through action a viral micro-RNA that targets host immune factors (Weber et al., 2004; Workenhe et al.2010; Shen et al.2015; Tycowski et al.2015). An align two sequences function within the NCBI tools using the nucleotide sequence that encodes this region namely, CTTTCCACAAATGACTTCAAGTC indicated the first 12 nt sequence encoding for LSTND is conserved with Singapore Grouper Iridovirus https://blast.ncbi.nlm.nih.gov/Blast.cgi. A BLASTx search with the nucleotide sequence that encodes this region reveals a 22nt region (CTTTCCACAAATGACTTCAAGTC) 22/23nt homology in the plus/minus orientation to *Haplochromis* and teleost species interferon-induced very large GTPase 1-like mRNA. Additionally, smaller 8 to 12 nt regions of this sequence display 100% homology to the mRNA of various immune factors of *Epinephelus* sp. including RAB7 GTPase (JQ08543.1), TLR3 (HQ857748.1), Tumour necrosis factor 2 (HQ011926.1), interleukin enhancer binding protein factor 2 (HM185492.1) beta-2 macroglobulin (HQ441036.1). The homology may be coincidental, however Mx, which is a GTPase is one of the immune factors known to be significantly upregulated and associated with survival to NNV in sea bream *S.aurata*) (Poisa-Beiro et al. (2008) mentioned p.44 Chapter. 1.8). Carballo et al. (2016) demonstrated prior infection of *D.labrax* with SJNNV (which has variance to RGNNV at this nucleotide- aa region) increased survival to RGNNV exposure from 24% to 96%. Mx was significantly upregulated in the SJNNV exposed fish compared to the RGNNV exposed fish. No mechanism for the ensued protection or differential immune response was provided. There is a reported lack of cross-protection by neutralising antibodies between SJNNV and RGNNV (Pascoli et al., 2019). One mechanism of improved survival against SJNNV may be a loss in ability by the SJNNV to downregulate Mx through mismatch to the target gene if this region acts as a v-miRNA.

Extending on the novel use microRNA to combat viral infections, Heiss et al. (2010) recently reported that engineering of a neurotropic flavivirus to include microRNAs (mir-9 or mir-124a) that are exclusively expressed in neural tissue, was sufficient to restrict their replication in the CNS of immunodeficient mice. The application of microRNA to combat Betanodavirus warrants further investigation.

The aims of this investigation are twofold namely:

1. Prepare the expressed capsid protein from the *Ec2NQAus* NNV strain described in Chapters 2 through to 4.
2. Prepare dsRNA constructs designed to down regulate targeted portions of the virus will be employed in viral challenge studies (*Ec2NQAus* NNV).

6.2 Materials and Methods

6.2.1 Nucleic acid extraction and reverse transcriptase polymerase chain reaction

Nucleic acid extraction and RT-PCR was performed as described in 2.1.2 to 2.1.4 using primers R2F1/R2R1 (Hick & Whittington 2010) (indicated by * in Table 2.1).

6.2.2 Cloning into replication plasmids

PCR products of complete mRNA of RNA 2 were cloned into TOPO vectors as previously described in Chapter 2.1.7.

6.2.3 Confirmation of clone sequence

Ten plasmid extracts containing the DNA sequence of the complete mRNA strand of RNA 2 segment were screened by PCR using the primers R2F1/R2R1 (Hick & Whittington 2010) (indicated by * in Table 2.1). Amplicons of the expected size were cut from agarose gels and purified using the Bioline gel PCR purification kit. Amplicons were subjected to PCR amplification using the primers that contained an additional EcoR1 target (on the R2F1 primer) and a Hind111 target (on the R2R1 primer). The amplified segment was ligated into the PRSET B expression backbone that contained T7 promoter, Lac operon, ribosome binding site state codon, capsid protein sequence, stop codon and T7 terminator (GeneART ThermoFisher) according to the manufacturer's instructions. An aliquot of the purified PCR product was submitted to AGRF for sanger sequencing analysis. Sequence was confirmed to have 100% homology to the EcNQAus NNV strain and also contain the T7 promoter, Lac operon, ribosome binding site state codon, capsid protein sequence, stop codon and T7 terminator in appropriate location to ensure correct reading frame for protein expression (codon optimisation).

6.2.4 Cloning into expression vector and protein expression

The DNA amplicon containing the N-terminal histone tag, T7 promoter, Lac operon, ribosome binding site state codon, capsid protein sequence, stop codon and T7 terminator were cloned into BL21 (D3) Chemically competent *E.coli* following the manufacturer's instructions for heat shock method (ThermoFisher C60003). Five cultures were prepared from single cloned colonies and incubated in 50ml SOC media under protein induction by 0.4 mM IPTG for 6

hours. After incubation, 1ml of culture was subjected to plasmid extraction and subjected to PCR analysis using the T7/T3 primer set. Three cultures that were contained high copy number of the target amplicon (determined by correct size) were harvested by low speed centrifugation at 5000g x 10 mins, resuspended in 5ml RNAse free PBS and frozen at -20°C.

6.2.5 Purification of Capsid Protein

Protein was extracted from the frozen cultures prepared as described in 6.2.6. Protein was extracted using the B-PER®Bacterial Protein extraction reagent as per the manufacturer's instructions (90079 ThermoFisher Scientific). Briefly, 10mL of B-PER II Reagent, containing 20ul of lysozyme and 20ul of DNase was added to the bacterial pellet and incubated at RT for 10-mins. Solutions were centrifuged at 15, 000 g x 15mins. Total protein extracts were viewed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Fifty microlitres of each protein extract were mixed with 2 x loading dye and loading buffer and boiled for 5 mins. Samples were loaded to a polyacrylamide gel and the electrophoresis chamber (BioRad), containing Tris-glycerine-SDS buffer was subjected provided with electrical current at 150 V for ~ 1 hour. Gels were fixed in silver fixative and washed 5 times for 1 minute. Gels were then stained by 10-minute incubation in silver nitrate solution (0.1% $AgNO_3$ in distilled water), washed three times in distilled water and developed for sufficient time to allow resolution of bands (by eye). Gels were rinsed three times in 6.25% of acetic acid solution (in distilled water). Gels were patted dry and photographed with I-phone. I-phone images were downloaded and edited to display the protein ladder and the rows that allowed visualisation of protein bands. The soluble capsid protein fraction was purified from the total protein fraction by affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) spin purification kits (3mL) according to the manufacturer's instructions (88229 ThermoFisher Scientific). The concentration of purified protein was determined using the Pierce BCA protein assay kit following the 96 well plate protocol (23225 ThermoFisher Scientific) following the manufacturer's instructions.

6.2.6 Preparation of Vaccine

Vaccine and Placebo vaccine were prepared using Freund's incomplete adjuvant. Viral vaccine was prepared by combining 15ml of purified capsid protein diluted to 500 mg mL^{-1} with sterile phosphate buffered saline (PBS) and 15ml Freund's incomplete adjuvant (Sigma F5506). The mixture was homogenised using a sterile homogenized using an Ultra-Turrax T 25 (IKA works) at 20,000 rpm in 3 x 10-minute intervals minutes until adjuvant emulsified. Triplicate tubes of vaccine were prepared. The mixture was contained within 3 x 50ml sterile tubes and held within a beaker containing ice during the homogenisation process. Placebo vaccine was

prepared by combing 15ml of sterile PBS and Freund's incomplete adjuvant (Sigma F5506) and homogenising until emulsification as described for preparation of vaccine. Vaccine was stored at 3°C overnight prior to use.

6.2.7 Preparation of dsRNA to target RGNNV genome.

Constructs of synthetic dsRNA were purchased from Integrated DNA technologies. A dsRNA construct with homology to the prawn virus *Penaeus merguiensis* hepandensovirus PmeDV which had no homology to NNV was used as the non-specific dsRNA construct, and herein refered to as the non-NNV construct (Owens et al., 2015). The NNV spec designed and purchased from the Integrated DNA technologies custom dicer-substrate siRNA design tool from the region of RNA 2 nt 721 to 780 of Ec2NQAus. (https://sg.idtdna.com/site/order/designtool/index/DSIRNA_PREDESIGN). The design tool selected the sequence **cacaaaugacuucaaguccauccuc** as the positive sense strand as a construct design. Sense and anti-sense contructs were purchased and prepared according to the manufacturers' instructions. Briefly, individual constructs were provided as tubes containing lyophilised ssRNA constructs 20 x 10 nmol (minimum yeild) (NNV specific) or 3 x 10 nmol (minimum yeild non-NNV specific) individual positive and negative strand constructs. Constructs were resuspended in RNAse free water at a concentration of 100pmol $\mu l^{-1.}$. On the day of injection into experimental fish, the constructs were combined in equal volume and incubated for 2 hours to allow annealing to dsRNA. Fish were injected IM with 100µl of 50pmol μl^{-1} dsRNA.

6.3 Results

6.3.1 dsRNA Construct design

The alignment of the positive strand of the dsRNA corresponds to the RNA 2 segment nt 722 to 738 which is the mRNA encoding the LSTND motif of the viral capsid protein (Figure 6.1).

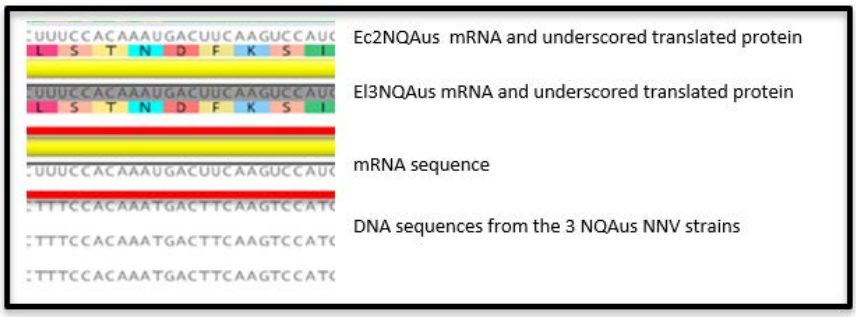


Figure 6-1 Alignment of the positive strand of the dsRNA construct designed to target the mRNA that encodes the LSTND motif. Alignment illustrated using Geneious prime (Biomatters.)

6.3.2 PCR Amplification of the mRNA encoding the Capsid Protein

Nine of the ten plasmid extracts that were screened for the DNA sequence equivalent to the capsid protein mRNA were positive for the sequence (Figure 6.2). Plasmids 5, 6 and 7 were selected for progression to capsid protein expression protocols.

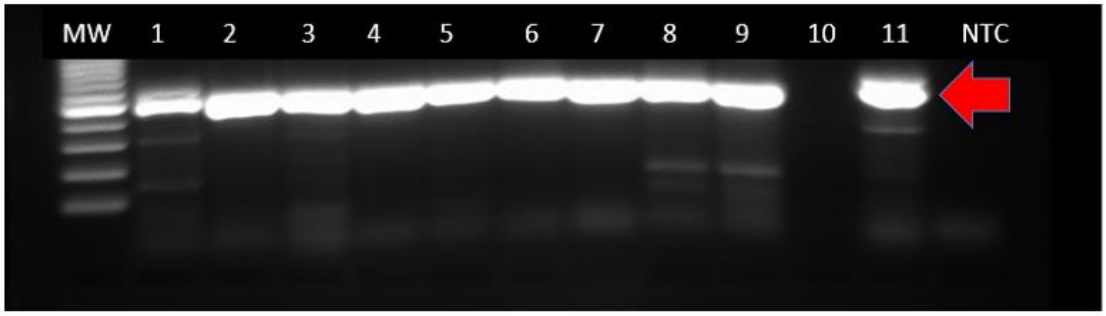


Figure 6-2 Image from gel electrophoresis screening of plasmid extracts for DNA sequence equivalent to the capsid protein mRNA. MW indicates molecular weight marker (200bp increments). Lanes 1 to 10 are amplicons (or lack thereof) from plasmid extracts analysed by PCR using R2F1 and R2R4 primers of Hick & Whittington (2010). Lane 11 is amplicon from positive control (Ec2NQAus NNV viral extract). NTC indicates no template control. Red arrow indicates positive amplicon of expected size in positive control (1034nt).

6.3.3 Purification of Capsid Protein

The total protein fraction from the 3 cultures selected for expression were examined using SDS PAGE (Figure 6.3). The 42kDA protein of the capsid protein was evident as the darkened band with IPTG expression. The capsid protein purified from the Ni-NTA spin columns were adjusted with sterile PBS to a final concentration of 1000µg ml^{-1}. Purified protein was frozen at -60°C until use.

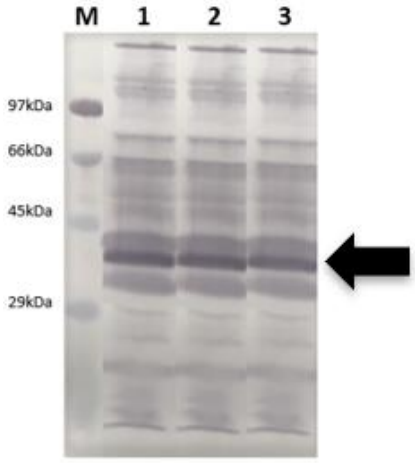


Figure 6-3 Silver stained SDS PAGE gel from protein purification of recombinant expression of capsid protein. M indicates Protein marker ladder with labelled protein bands. Lane 1, 2 and 3 are total protein extracts from the IPTG induced cultures. Arrow indicates band corresponding to capsid protein (~42kDa).

6.4 Discussion

Vaccine and dsRNA constructs targeting the NNV capsid protein/mRNA were produced in this study. In Australia, the application of vaccines produced from recombinant expressed viral proteins is underway and considered an attractive longer-term approach to viral vaccine production for fish (Norwood, 2018). Interfering RNAs syn dsRNA are considered some of the most important biopharmaceuticals as future medicines (Chakraborty et al., 2017). In addition to the aligning with the target sequence and not having off-target effects on host genes, the success of dsRNA treatment is determined by effective delivery of the dsRNA construct (Chakraborty et al., 2017). However, the design and delivery of dsRNA has not been applied to many studies involving fish (Gotesman et al., 2015). Considering the lack of understanding relating to dsRNA in fish, the design and delivery of dsRNA into a fish host in this project is considered highly experimental. This study has adapted a dsRNA approach that was used to reduce viral load in crustaceans (Owens et al., 2014).

The design and preparation of dsRNA and a vaccine based on expressed recombinant NNV capsid protein has been discussed in this chapter. The effectiveness of either/both of these agents as prophylactic measures to prevent VER in *E.lanceolatus* following experimental challenge are described in chapters 8 and 10.

6.5 Conclusion

The aims of this chapter were met in the following manner:

- Complete sequence of the RNA 2 segment of RGNNV obtained from diseased *E.coioides* was cloned into the expression vector.
- Capsid protein was expressed, purified and incorporated into a vaccine.
- dsRNA constructs targeting RGNNV RNA 2 were designed and prepared.

CHAPTER 7. EXPERIMENTAL CHALLENGE VIA CO-HABITATION WITH MARINE LEECH, *ZENYLANICOBDELLA ARUGAMENSIS*

Background

- Challenge of fish via injection does not represent the natural pathway of transmission of NNV in natural disease outbreaks.
- Experimental reproduction of VER outbreaks simulating a more natural route of exposure, namely co-habitation with infected individuals or water borne exposure does not yield consistent results.
- Although naturally occurring disease outbreaks are reported following periods of "stress", a trigger that consistently induces VER in aquaculture systems has not been identified.
- In temperate finfish aquaculture, leeches have been proposed as vectors of viral disease.
- Infestation by the marine leech, *Zeylanicobdella arugamensis,* has been reported to affect the health of grouper within earthen ponds in North Queensland.
- Some authors report the detection of NNV from blood of infected fish.
- Whilst *Z.arugamensis* have been reported to be vectors of marine fish trypanosomes, there has been no reported investigation of their role in transmission of viruses.

Aims of this Chapter

- Investigate if RGNNV can be transmitted to fish held in a co-circulation system with fish exposed to by IM challenge.
- Investigate if exposure to the marine leech, *Zeylanicobdella arugamensis*, in combination with co-habitation with RGNNV infected fish leads to RGNNV infection or VER outbreaks in juvenile *E.lanceolatus*.
- Investigate if the marine leech, *Zeylanicobdella arugamensis*, has potential to act as a vector of RGNNV.

7.1 Introduction

Although injection methods induce a consistent expression of VER, they are artificial and do not reflect a natural infection path leading to outbreaks of VER (Kim et al., 2018). Ideally,

challenges to assess the efficacy of vaccines should mimic the natural path of infection. Whilst natural disease outbreaks, which presumably do not involve an intramuscular injection of NNV, often lead to mass mortality, the reproduction of VER via co-habitation with infected animals and water borne exposure is inconsistent and leads to reduced levels of mortality and disease in fish that are aged beyond the larval stages (Anderson & Moody 2004, Kim et al., 2018). Anderson and Oakey (2008) stated: "Our feeling is that the project test results do not support the suggestion that nodavirus infections are readily transmitted from one adult barramundi to another sharing the same tank." Attempted bath exposure of freshwater fish species (silver perch, golden perch and sleepy cod and Barcoo grunter (6 and 12-week-old) to VNN did not display any signs of infection despite morbidity of 46%, 15%, 98% and 7% when fish were exposed via injection (Anderson and Moody 2004). RGNNV has been isolated in cell culture and detected by RT-qPCR in water containing fish with clinical VER (Jaramillo et al., 2017). Hodneland et al. (2011) added brain homogenate from severely infected sea bass to an aquarium containing 15 sea bass (40g) and co-habitated fish with IM injected sea bass (1:3 ratio infected: cohabitated). None of the cohabitated fish displayed clinical signs or were positive for the detection of RGNNV RNA 2 by RT-qPCR during the 5-week incubation period. Whereas 85% of the IM infected fish died within 10 days. Fish exposed to brain homogenate in the water displayed 33% mortality from day 17-35 post exposure (Hodneland et al., 2011).

The inconsistency in disease expression via water exposure to viruses, including NNV, in experimental systems compared to the process that leads to natural disease outbreaks is proposed to be due to the presence of "a stress event" as a precursor to trigger natural disease outbreaks. The "stress events" remain largely un-investigated beyond simple manipulations of water temperature and salinity or retrospective studies that identify risk factors (Juniar et al., 2018). Artificial attempts to mimic stress and induce immunosuppression of Atlantic cod, *Gadus morhua* by injection with prednisolone-acetate failed to induce disease following IM challenge with NNV isolated from Atlantic halibut, *Hippoglossus hippoglossus* (Korsnes et al., 2009). Conversely, exposure to tributyltin, a component of boat hull anti-foul, lead to increased mortality, with lower exposure dose to RGNNV in Japanese medaka *Oryzias latipes* (Kitamura et al., 2017).

Parasite infestations are frequently encountered in marine fish culture systems and, in high numbers, may place a stress burden on host fish. Infestation of grouper and barramundi by the marine leech *Zeylanicobdella arugamensis* have occurred within marine farm grow-out systems in Northern Queensland (Vaughan, 2018). This leech has a wide geographic range and infection of marine groupers have been reported from Philippines, Sri Lanka, Malaysia,

Singapore, India, Japan, Iran and China (Murwantoko et al., 2018). In temperate fish farming systems leeches have been implicated as vectors of some viruses (Salimi and Abdi 2016 and Steinbauer et al., 2019). Although no viral transmission studies have been conducted with the species, *Z.arugamensis* has been demonstrated to be an efficient vector of the marine trypanosome, *Trypanosoma nudigobbi* (Hayes et al., 2014.) The case of VER in cobia described by Chu et al. (2013) noted the presence of *Z.arugamensis* along with monogeneans (*Benedenia* sp.) and copepods (*Caligu*s sp.). Yet, there was no suggestion of any role the parasites may have played in relation to the mortalities and there are no published reports of attempts to detect viruses from leeches from tropical finfish aquaculture systems (PubMed, Science Direct, Google scholar, Scopus databases accessed 2.08.19). In addition to directly transferring viruses, the feeding action of leech and fish scraping and rubbing behavioural in response to leech infestation can cause disruption of the protective skin-scale-mucus barrier. Disruption of the physical barrier of skin, scales and mucus by parasites render fish susceptible to secondary infection by disease-causing organisms such as bacteria, fungi and viruses (Francis-Floyd, 2018). Dermal abrasion prior to water borne exposure to *Vibrio harveyi* was required to induce disease in an experimental challenge of juvenile hybrid groupers *E.fuscoguttatus x E.lanceolatus* (abw 4.15g) (Shen et al., 2017). Infestation with *Z.arugamensis*, and secondary infection with pathogenic bacteria such as *Vibrio alginolyticus* has been associated with mortalities in grouper (Ravi & Yahaya, 2017). The aim of this study is to investigate potential experimental challenge models for future studies to test the efficacy of therapies developed in Chapter 6 to prevent VER. Models include exposure to RGNNV through a shared circulation system (co-circulation). The potential of the marine leech, *Z. arugamensis* to compromise the skin-mucus barrier, act as "a stress factor" or vector to induce VER under co-circulation will also be investigated.

7.2 Materials and Methods

7.2.1 Culture of marine leech, Zeylanicobdella arugamensis

Culture of marine leech *Z. arugamensis* was kindly conducted by JCU PhD Candidate Dr David Vaughan. Approximately 100 leeches were provided for the experiment. Prior to exposure to the experimental fish, leeches were removed from the *E. coioides* host fish and starved for 24 hours.

7.2.2 Fish culture, viral challenge and co-circulation system

Thirty juvenile groupers*, E.lanceolatus* were dispersed evenly into a system consisting of 3 x 60L tanks that were immediately adjacent to each other and sharing the same biological

filtration and recirculation system. There was no shield to prevent aerosol spread between tanks. (Figure 7.1). Water, temperature, light and fish feeding conditions were as described previously in section 2.2. For the viral challenge, ten fish from one tank (Tank C Figure 7.1) were sedated and challenged by IM injection of 100 uL of RGNNV viral extract (6.36 x 10^4 mL^{-1}) as described in 2.2.2. Fish from the RGNNV challenged tanks began displaying clinical signs of VER from day 8 post IM injection. One fish was sampled from the IM challenge group (Tank C) at day 4 prior to the onset of clinical signs. When fish began displaying signs of VER at day 8, fifty leeches *Zeylanicobdella arugamensis* were added to two of the tanks, namely the RGNNV challenged tank (Tank C Figure 7.1) and another tank on the same recirculation system (Tank A Figure 7.1). The leeches had been starved for 24 hours prior to their addition to the tanks (provided by Dr David Vaughan). Fish were observed twice daily for signs of VER. When fish displayed multiple signs of disease (two clinical signs e.g. not feeding and inflated swim bladder), they were removed from the system and euthanased as described in 2.2.1. Any leeches that were attached to euthanased fish were removed and stored in separate 1.5ml RNase free microcentrifuge tubes and frozen at -20°C until the end of the experiment. The experiment was terminated at 40 days post IM challenge. All remaining fish were euthanased at day 40 post IM challenge. All leeches present on fish or in the tank system were stored in pools of 5 animals and frozen at -20°C.

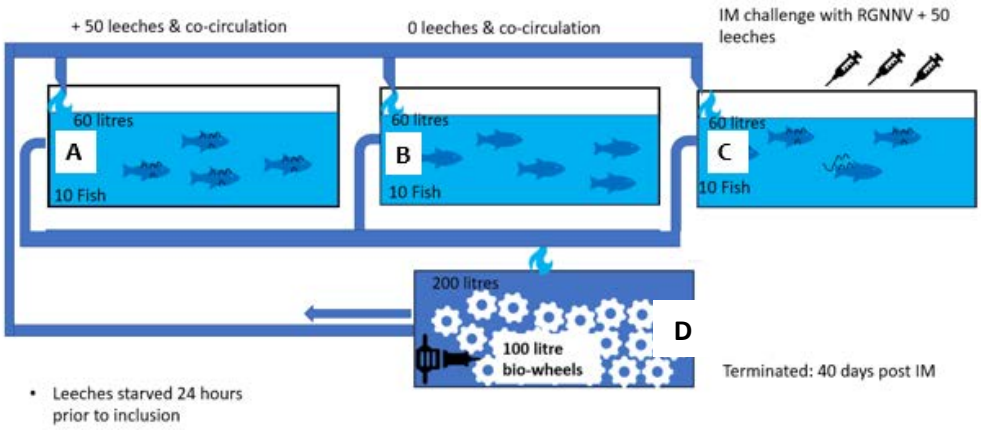


Figure 7-1: Diagrammatic illustration of the experimental aquarium system for co-circulation

Each tank (A, B, C) contained 10 fish within a 60-litre tank. Water circulated between the tanks from a shared bio-filter (D). Tank A fish were exposed to 50 *Z.arugamensis* from the onset of clinical signs of VER in Tank C fish. Tank B fish had no exposure to *Z.arugamensis* nor RGNNV other than that which occurred within the recirculation system. Tank C fish were exposed to an IM challenge of RGNNV and at the onset of clinical signs were exposed to 50 *Z.arugamensis.*

7.2.3 Nucleic acid extraction, CDNA synthesis, RT-qPCR.

Total nucleic acid (TNAs) was extracted from whole eye and whole marine leeches collected from fish following the protocol previously described in 2.1.2. cDNA synthesis and qR1T and

qR2T, were conducted on TNAs as previously described in 2.1.3 and 5.2.3. Leeches were extracted in pools of 5 collected from fish when they were euthanased.

7.3 Results

7.3.1 Expression of VER in juvenile E.lanceolatus.

The fish that were challenged by IM injection of viral extract (Tank C) displayed clinical signs of VER (Figure 7.2). Clinical signs of VER, including hypertrophy of the swim bladder, abnormal swimming behaviour and lack of feeding were evident in the RGNNV injected + leech exposed group from 8 days post IM challenge (Figure 7.2). No clinical signs of VER were evident in fish from either of the co-circulating tanks for the 40 days of the experiment (Figure 7.2). Leeches were observed attached to the fish in Tank A and C (leeches added to the tanks) but no leeches were observed in the Tank B (no leeches added to the tank) during the course of the experiment.

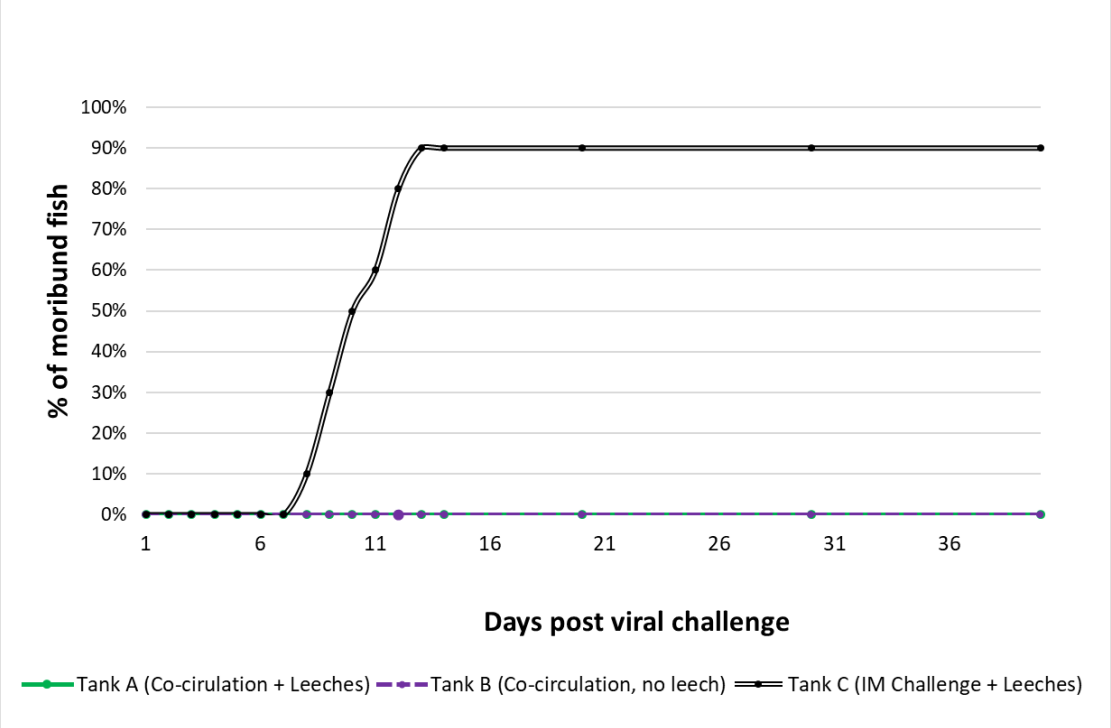


Figure 7-2 Cumulative morbidity (%) of fish from each tank following IM challenge of Tank C fish v days post viral challenge. Tank A contained fish exposed to leeches and on co-circulation with IM challenged fish in Tank C. Tank B contained fish on circulation with IM challenged fish in Tank C. Tank C contained fish challenged with IM injection of viral extract and exposed to leeches from the onset of clinical signs of VER.

7.3.2 Detection of RGNNV by RT-qPCR

RGNNV was detected using qR1T and qR2T from the eye tissue of all fish in the IM challenged + leech experimental group (Table 7.1). RGNNV was detected in the eye tissue of the grouper that was killed at day 4 post challenge (4 days prior to clinical signs). RGNNV was not detected

from the eye of fish that were not subjected to IM challenge (Table 7.1) nor any of the leeches collected during the experimental period (Table 7.1).

Table 7-1 Cycle threshold values from each RT-qPCR from eye and leech samples collected from the three experimental groups at various days post challenge by IM injection.

Tank	Experimental Group and (sample)	Assay Target	Assay Name	Days post challenge								
				0	4	8*	9	10	11	12	13	40
C	IM challenge (grouper)	RNA 1	qR1T	nd	30.2	20.6	16.9	15.3	16.3	15.3	14.9	nt
		RNA 2	qR2T	nd	27.7	16.6	13.4	12.0	12.8	12.0	11.7	nt
B	Co-circulation + only (grouper)	RNA 1	qR1T	nd	nt	nt	nt	nt	nt	nt	nt	nd
		RNA 2	qR2T	nd	nt	nt	nt	nt	nt	nt	nt	nd
A	Co-circulation + leeche (grouper)	RNA 1	qR1T	nd	nt	nt	nt	nt	nt	nt	nt	nd
		RNA 2	qR2T	nd	nt	nt	nt	nt	nt	nt	nt	nd
C	IM challenge (leech)	RNA 1	qR1T	nd	nd	nd	nd	nd	nd	nd	nd	nd
		RNA 2	qR2T	nd	nd	nd	nd	nd	nd	nd	nd	nd
A	Co-circulation + leeche (leech)	RNA 1	qR1T	nd	nd	nd	nd	nd	nd	nd	nd	nd
		RNA 2	qR2T	nd	nd	nd	nd	nd	nd	nd	nd	nd

* indicates day clinical signs of VER became evident

nt = none tested

(grouper) and (leech) indicates type of sample analysed by RT-qPCR

7.4 Discussion

Waterborne exposure, which reflects the route of infection in natural disease outbreaks, would be the preferred model for disease experimental challenges to induce VER and test the efficacy of prophylactic measures. However, variable morbidity and mortality have been reported from waterborne and co-habitation exposure to NNV (Anderson & Moody 2004). In this study, VER disease was not observed when juvenile groupers were held on a shared circulation system for 40 days with a cohort of fish that displayed 90% morbidity following IM challenge with RGNNV extract. Furthermore, RGNNV was not detected from the eye or brain of-fish held in co-circulation using qR1T or qR2T. In contrast, fish challenged by IM injection of viral extract had signs of disease from day 8 post challenge and cycle threshold values of NNV in the range of 14.72 to 20.58 and 11.48 to 16.56 were detected from eye tissue using qR1T and qR2T respectively. Further attempts to promote NNV infection and induce VER disease by introducing an infestation of the leeches, *Z.arugamensis* were also unsuccessful. Leeches have been proposed as mechanical vectors of viruses of fish for many years (Ahne, 1985). Spring Viremia of Carp virus was isolated from the leech *Piscicola geometra* (Ahne, 1985). Viral Haemorrhagic Septicaemia Virus was isolated and detected by RT-PCR from the leech *Myzobdella lugubris* (Faisal & Schulz, 2009). Infectious Pancreatic Necrosis Virus was detected by PCR from two leech species, *Hemiclepsis marginata* and *Hirudo medicinalis* (Salimi & Abdi 2016). Salmonid alphavirus was detected by PCR from *Piscicola geometra* (Steinbauer et al., 2019). In this study, RGNNV was not detected by RT-qPCR from any leech collected from fish displaying clinical signs of VER or from fish within the same co-circulation system as diseased

fishes. Presumably, one of the requirements for leeches to act as mechanical vectors of viral infections relies on the presence of the virus in the blood cells of infected fish. Reports on the detection of NNV from the blood of infected fish are inconsistent. Korsnes et al. (2009) reported the detection of NNV from the blood of Atlantic cod, *Gadus morhua*. Yet, no detection from blood cells have been reported by others (Valero et al., 2018). The inability to induce RGNNV infection or VER disease via co-circulation with diseased fish, either with or without an additional burden of leech infestation indicates waterborne transmission of NNV is not a robust model for future studies to test the efficacy of prophylactic measures. Although injection challenge by-passes one of the primary immune barriers of the fish, investigation into efficacy of prophylactic measures developed in Chapter 6 are proposed to be conducted using IM challenge with RGNNV

7.5 Conclusion

The following outcomes and conclusions were discussed in this chapter:

- RGNNV was not transmitted to naïve fish on a co-circulation system with VER diseased fish during a 40-day experiment.
- Exposure of fish to the marine leech *Zeylanicobdella arugamensis* in addition to co-circulation with VER affected fish did not lead to infection with RGNNV in juvenile *E.lanceolatus*.
- The inability to detect RGNNV from leeches which fed on VER affected animals indicates *Z.arugamensis* is not likely to be an effective vector of RGNNV.
- As VER was not induced via co-circulation or co-exposure to a leech “stress event”, no effective method of water borne challenge has been identified for future trials.
- Although not mimicking the natural route of infection, challenge by IM injection is the only consistent challenge model available for continued testing of the efficacy of vaccine to prevent VER within the time frames of this project.

CHAPTER 8. TESTING EFFICACY OF PROPHYLATIC MEASURES AGAINST VIRAL ENCEPHALOPATHY AND RETINOPATHY (VER) IN *EPINEPHELUS LANCEOLATUS* VIA EXPERIMENTAL CHALLENGE

Background

- A vaccine containing the recombinantly expressed capsid protein of RGNNV has been prepared for assessment as a prophylactic measure to prevent VER (Chapter 6).
- dsRNA designed to target RGNNV RNA 2 has been prepared as an experimental compound to prevent VER (Chapter 6).
- Due to reported inconsistency to induce VER by waterborne or co-habitation, challenge by IM injection with RGNNV is the preferred method of challenge within the scope of this project.
- The pathogenesis of VER from exposure to disease expression has not been well described in grouper.
- Synthetic plasmid controls have been prepared to support monitoring of the qR1T and qR2T assays towards both tracking the pathogenesis of VER and validation of both assays for application on grouper tissues.

Aims of this Chapter

- To test the efficacy of vaccine and dsRNA preparations to prevent infection and disease in juvenile *E. lanceolatus* following IM challenge with RGNNV viral extract.
- To assess the performance of the qR1T and qR2T assays to detect RGNNV from grouper derived brain and eye tissue following experimental challenge by IM injection with RGNNV.
- To track the pathogenesis of RGNNV from exposure to disease expression by applying analysis using RT-qPCR.

8.1 Introduction

In grouper aquaculture, stocking of ponds with NNV-free fingerlings does not prevent economically catastrophic VER outbreaks during grow-out stages of production. There is an urgent need to develop prophylactic measures to combat NNV infection in grouper grow-out systems. Despite numerous reports of successful vaccination against NNV in various experimental formats, there are few commercial transfers for application by industry.

The management of VER in grouper aquaculture is hampered by gaps in knowledge of both the development of the juvenile grouper immune system and the pathogenesis of VER. This study has prepared a vaccine and dsRNA as potential therapies to prevent RGNNV infection. Although monitoring of the juvenile grouper immune system is beyond the scope of this study, tracing the progression of the disease following IM challenge is possible with RT-qPCR. Despite the need to improve knowledge and the availability of validated, sensitive, specific, quantitative, rapid turnover and high throughput RT-qPCR assays, very few published reports have applied RT-qPCR to investigate the pathogenesis of VER (Jaramillo et al., 2017; Hodneland et al., 2011; Lopez-Jimena et al., 2011; Panzarin et al., 2010). Only one article details the application of RT-qPCR to grouper species (Kim et al., 2018).

8.2 Materials and Methods

8.2.1 Fish husbandry

Six hundred juvenile giant grouper, *E.lanceolatus* (abw 18g σ ~2 g, range 11.3 g to 23.6 g; aged 84 days post hatch) were provided by a commercial hatchery. Water temperature ranged between 24 to 28 ^{o}C and salinity ranged from 26 to 35 ppt. Fish were weighed, tagged with streamer tags to allow individual identification (Hallprint) (Refer to Figure 8.1) and fed *ad lib* twice daily with a commercial fish feed pellet (Ridley AgriProducts Pty Ltd). Fish were acclimated for two weeks prior to commencement of experimental procedures. Two weeks (98 dph) and six weeks (126 dph) after acquisition, fish were injected with the experimental treatments. All fish from the same experimental group were held in the same tank system until IM challenge. Fish were sedated for all treatments involving injection, according to JCU Animal Ethics requirements using AQUI-S® (AQUI-S).

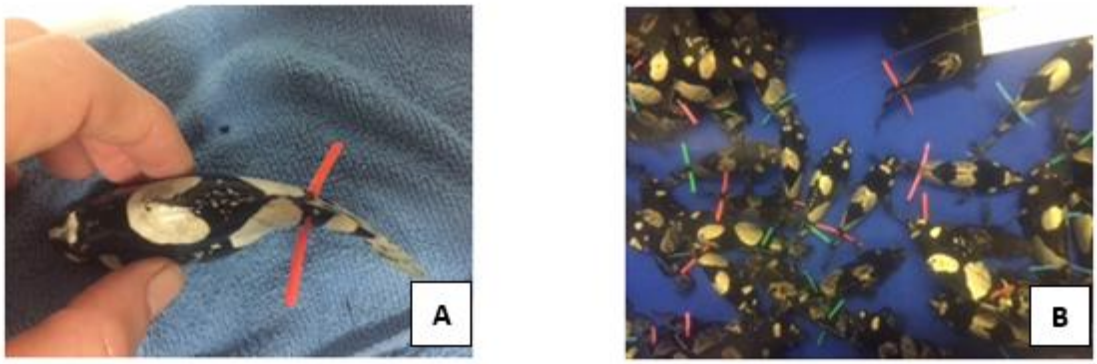


Figure 8-1. (A) Image of juvenile *E.lanceolatus* with tag and (B). multiple tagged fish within a recovery tank after injection with RGNNV extract

8.2.2 Experimental design and vaccination

Fish were distributed into ten experimental groups. The experimental groups were duplicates of five experimental treatments that were subsequently challenged with either PBS or RGNNV extract. The five groups consisted of treatments prepared as described in Chapter 6. In summary the experimental treatments were: (1) Placebo vaccinated with Adjuvant + PBS, (2) vaccinated with recombinant expressed capsid protein (500mg mL^{-1}) + adjuvant, (3) vaccinated with recombinant expressed capsid protein (500mg mL^{-1}) + adjuvant and a dsRNA (50pmol μl^{-1} dsRNA) construct with homology to RGNNV, (4) injected with a dsRNA (50pmol μl^{-1} dsRNA) construct with homology to RGNNV, (5) injected with a dsRNA (50pmol μl^{-1} dsRNA) construct with no homology to RGNNV. All injections were the same approximate body position as described in Chapter 2.2.3.

8.2.3 Experimental Challenge with RGNNV extract

Two weeks after the booster (140 dph) treatments or equivalent placebo treatments, fish were challenged with virus by either intramuscular injection with 50 µL RGNNV extract (calculated copy number 6.36 x 10^4 mL^{-1} of viral extract prepared in 2.1.6) or 50 µL sterile PBS. Fish were then redistributed into tanks with six fish from each of the five treatment groups were placed into replicated tank systems. The experimental groups are described in Table 8-1 and illustrated in Figure 8.2.

Table 8-1 Summary of the experimental design indicating description of prophylactic measures, IM challenge, number of fish in each group and number of fish sampled at morbidity or within planned schedules in the absence of clinical signs of VER.

Experimental Group	Description	Challenged 50 uL i.m. injection	Number of fish	Sampling schedule	
				At morbidity	Scheduled*
Placebo vaccine	IM injection of 100µL adjuvant/PBS 1:1	PBS	60	0	10
		RGNNV Extract	60	41	4
Vaccine & Anti-RGNNV dsRNA	IM injection of 100µL adjuvant/PBS:dsRNA (RGNNV)	PBS	60	0	10
		RGNNV Extract	60	32	9
Vaccine	IM injection of 100µL adjuvant/RGNNV capsid protein	PBS	60	0	10
		RGNNV Extract	60	26	8
Anti-RGNNV ds RNA	IM injection of 100µL PBS:ds RNA (RGNNV)	PBS	60	0	10
		RGNNV Extract	60	54	4
Non-specific ds-RNA	IM injection of 100µL PBS:ds RNA	PBS	60	0	10
		RGNNV Extract	60	54	4
* indicates at scheduled days in the absence of clinical signs of VER					

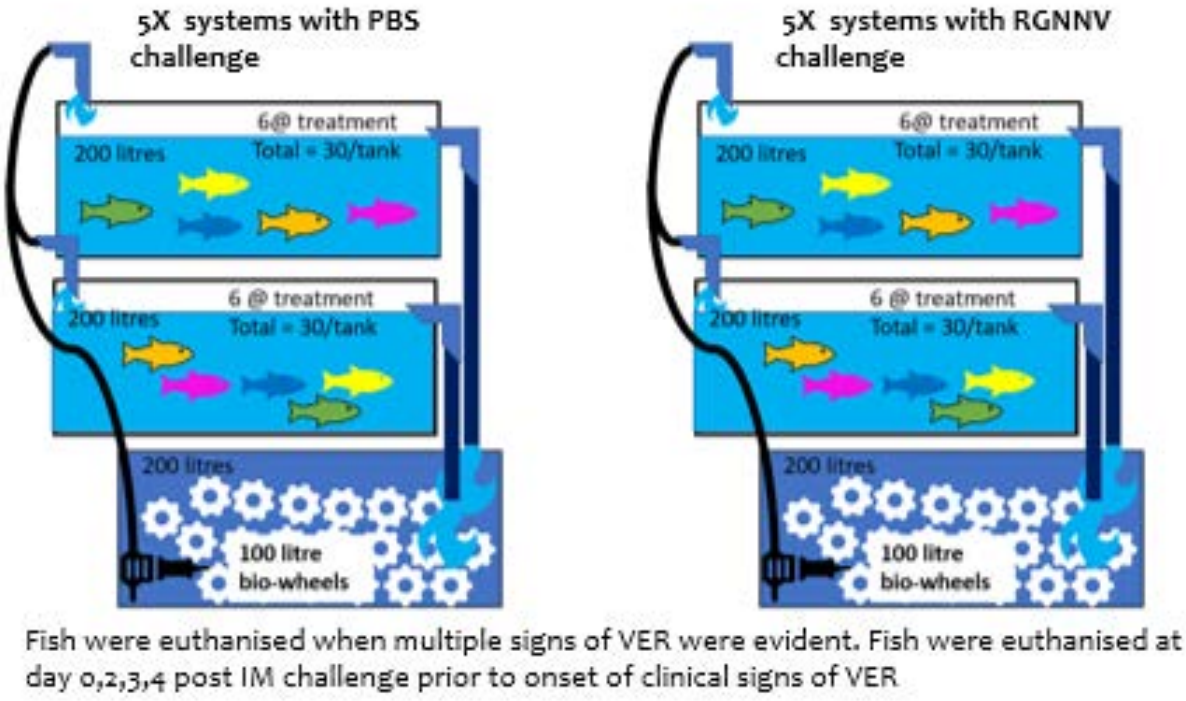


Figure 8-2: Graphic illustration of experimental tank design

Ten duplicate systems consisting of 2 x 300 litre tanks for housing fish, were equipped with 1 x 300 litre tank containing ~100 litres of bio-wheels with constant water flow 1200L hr^{-1} and constant aeration. Each tank contained 6 fish from each of 5 treatments. Fish were individually tagged with T bar anchor tags to indicate experimental treatments.

8.2.4 Monitoring of fish health and euthanasia

Fish were monitored twice daily for signs of adverse health. For the first six days post IM challenge, prior to the onset of clinic signs of VER, one fish from each treatment type was removed from each tank system and euthanased by Aqui-S overdose (Table 8.1). When fish

displayed more than one sign of VER they were euthanased. Euthanasia was conducted by Aqui-S overdose as described in 2.2.1. Euthanased fish were weighed, frozen whole in individually labelled plastic bags and stored at -20°C along with their identification tags. After 20 days post challenge, when morbidity had reached plateau phase, all surviving fish were collected from the RGNNV challenged tanks, weighed and consolidated into two of the experimental tank systems. Fish that displayed a clinical sign of VER, mostly darkened colour or inflated swim bladders, yet continued to feed were placed in the "clinical sign" system and fish that did not display signs of VER were placed in the "no clinical sign" system. The "clinical sign" fish were removed from the "no clinical sign" fish to avoid injury and stress. When held in the same tanks, the "clinical sign" fish were actively attacked and outcompeted for feed by the "no clinical sign" fish. The condition of fish in the consolidated systems was monitored for a further 80 days post IM challenge. During the 80-day period the streamer tags became damaged and were actively removed by the fish so accurate tracking of the individual treatments was, in many cases not possible. After 80 days all fish in the "clinical signs" tank were euthanased and half of the fish in the "no clinical signs" tank were euthanased. Euthanased fish were weighed and stored as previously described. Thirty-five fish with "no clinical signs", which had retained their tags were retained for longer term monitoring of growth. All of the retained fish were from one of the two vaccinated treatment groups. Tags were removed in an effort to reduce injury from fish trying to eat each other's tags. Grouping of the "no clinical signs" fish was required because the growth and subsequent aggression and territorial behaviour of the larger fish prevented retaining all of the survivors under conditions that would comply with Animal Ethics Permits. Throughout the remainder of the project, fish with "no clinical signs" were euthanased when fish growth caused overcrowding and the fish could not be maintained in conditions that complied with the ethical treatment of animals for scientific purposes. Ten fish from each of the experimental groups that were challenged with PBS were euthanased at scheduled intervals. One fish from each group was euthanased at days 0,2,3,4, and a further 6 fish from each group were euthanased at day 20 (Refer to Table 8-1).

8.2.5 Nucleic acid extraction

Total nucleic acid was extracted from eye as described in 2.1.2.

8.2.6 Analysis by RT-qPCR using qR1T and qR2T

Analysis by RT-qPCR using qR1T and qR2T was conducted on tissue extracts as described in 2.1.9.

8.3 Results

8.3.1 Expression of VER following experimental challenge of juvenile grouper with RGNNV extract and efficacy of protection of each preventative treatment.

There was 100% survival and no adverse reaction evident in any of the five groups of fish challenged with PBS injection for the duration of the trial period. Within the RGNNV challenged groups, fish fed and behaved normally for five days post challenge. Six days post challenge, a small number of fish displayed some typical signs of VER. Clinical signs included inflation of swim bladder, lethargy, erratic swimming behavior, darkened body color and flared opercula. The number of fish with multiple clinical signs increased markedly between days 8 to 10 post challenge after which morbidity plateaued (Table 8.2). Exposure to dsRNA (specific and non-specific design) was not effective in reducing morbidity in juvenile *E.lanceolatus* following IM challenge with viral extract. The groups that received the capsid protein within either vaccine formulation (vaccine alone, or vaccine + dsRNA) had reduced total numbers of fish displaying VER compared to the placebo vaccinated groups and the groups which received dsRNA in either form (Figure 8.3). By day 20 post viral challenge, the group that received vaccine and vaccine with anti-RGNNV dsRNA displayed 43% and 53% cumulative morbidity compared to the placebo vaccinated (68%) and either form of dsRNA injected (88% and 88%) (Figure 8.1). Observation of body mass of moribund and surviving fish from the RGNNV challenge indicates a trend whereby larger vaccinated fish tended to survive the RGNNV challenge. However, as there were small numbers of larger fish and body weight was not considered in the experimental design no statistical analysis was attempted.

Table 8-2: Number of fish sampled or euthanased during the experimental challenge including experimental group and day of sampling/euthanasia post challenge.

Experimental Group	Number of fish sampled and days post challenge sampled																
	0	2	3	4	6	7	8	9	10	11	12	13	14	15	16	19	20
Placebo vaccine	1	1	1	1	1	3	14	11	3	3	1	2	2	0	1	0	0
Vaccine & Anti-VNN dsRNA	1	1	1	1	2	3	9	8	3	0	1	4	0	1	1	0	0
Vaccine	1	1	1	1	1	3	14	3	2	0	1	1	1	0	0	0	0
Anti-VNN ds RNA	1	1	1	1	1	1	9	14	11	7	3	4	0	1	0	1	0
Non-specific ds-RNA	1	1	1	1	1	1	13	17	12	5	0	0	2	1	0	1	1
Day Total	*5*	*5*	*5*	*5*	*4*	*10*	*59*	*53*	*31*	*15*	*6*	*11*	*5*	*3*	*2*	*2*	*1*
Sub-clinical (S) or diseased (D)	S	S	S	S	D	D	D	D	D	D	D	D	D	D	D	D	D

Fish which were sampled within the project sampling plan with an absence of clinical signs of disease are indicated by S. Fish that were euthanased due to multiple clinical signs of VER are indicated by D.

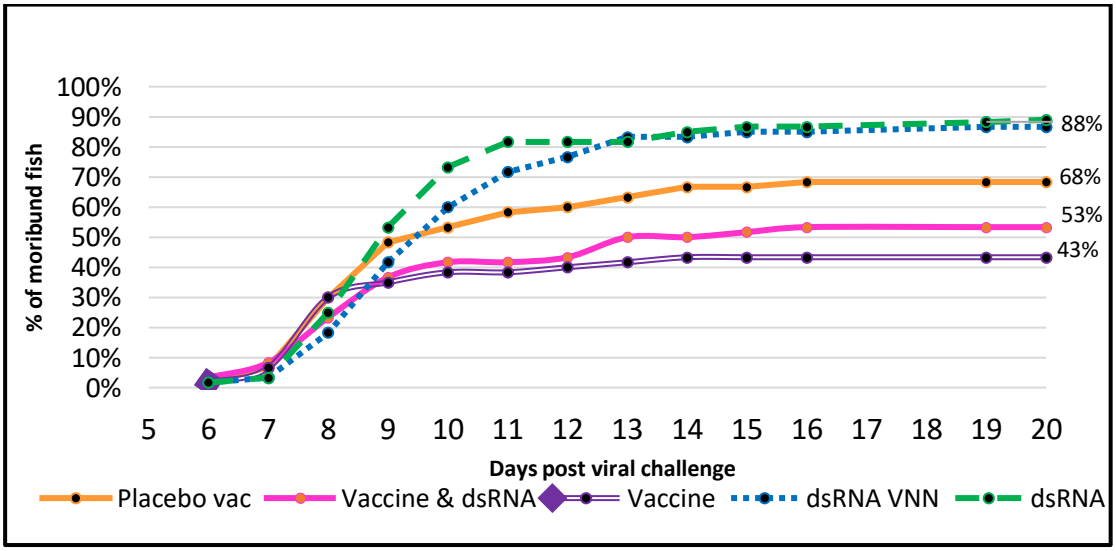


Figure 8-3: Cumulative morbidity of fish displaying signs of VER from each experimental group following IM challenge with RGNNV extract.

Total cumulative mortality (%) at day 20 post IM injection is indicated by data labels adjacent to each preventative treatment group. No fish from any of the experimental groups that were challenged with PBS injection displayed morbidity during the term of the trial (data not shown). Fish that were sampled prior to the onset of clinical signs were not included in the cumulative morbidity calculations.

8.3.2 Body weight of fish

The body weight of fish that were euthanased with clinical signs of VER were recorded (Figure 8.4).

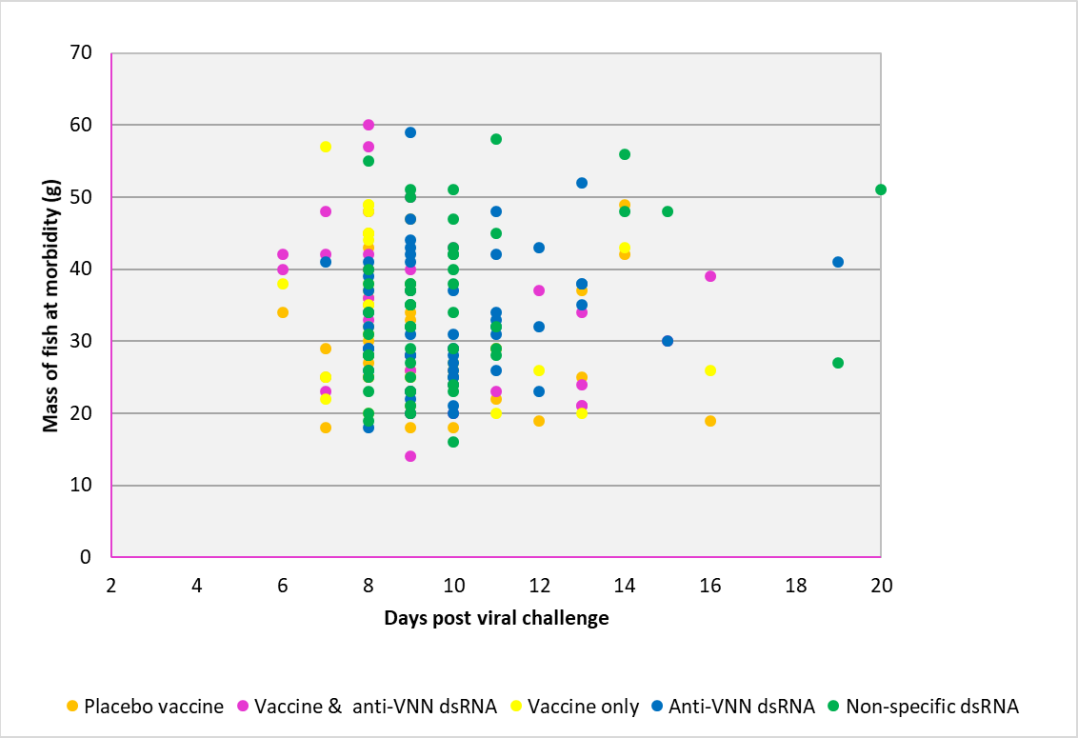


Figure 8-4: Scatter plot of mass of individual fish from each treatment group v days when euthanased due to morbidity for each day post challenge by IM injection of RGNNV.
Notably only 3 fish that received vaccine that were over 50 g in body weight were euthanased.

8.3.3 Detection and quantification of RGNNV by RT-qPCR assay

There was no detection of RGNNV genome by either assay from any fish challenged with PBS injection. RGNNV genome was detected with both assays from eye of viral challenged fish from day two post challenge, four days prior to the onset of signs of disease, until and beyond day 19 post challenge (Table 8-3). Assay qR1T, which targets the RNA-1 segment had higher Ct values than qR2T, which targets the RNA-2 segment (Table 8-3).

Table 8-3: Average Cycle threshold value of qR1T and qR2T from analysis on Eye tissue from viral challenged fish.

Experimental Group	Assay Target	Assay Name	Days post challenge										
			0	2	3	4	6*	7	8	9	11	13	15	19
Placebo vaccine	RNA 1	qR1T	nd	nd	37.7	26.7	19.7	16.1	16.2	17.2	15.5	16.7		
	RNA 2	qR2T	nd	45.0	30.0	29.5	15.3	19.0	11.6	12.9	11.3	11.9		
Vaccine & Anti-VNN dsRNA	RNA 1	qR1T	nd	31.8	nd	31.7	21.1	18.8	17.3	16.5	18.0	15.5	19.3	
	RNA 2	qR2T	nd	29.9	28.8	29.6	16.8	15.7	12.4	11.9	13.4	10.8	14.4	
Vaccine	RNA 1	qR1T	nd	36.8	34.9	34.5	nd	nd	16.0	15.8	15.5	16.4		
	RNA 2	qR2T	nd	30.8	30.4	31.4	30.7	30.9	11.5	11.4	10.8	11.8		
Anti-VNN ds RNA	RNA 1	qR1T	nd	nd	35.7	25.4	17.6	19.5	15.7	15.8	16.3	16.0	14.7	16.8
	RNA 2	qR2T	nd	45.0	29.7	22.6	14.8	15.2	11.1	11.4	11.7	11.4	10.8	11.8
Non-specific ds-RNA	RNA 1	qR1T	nd	nd	31.1	28.9	19.2	12.9		16.1	15.5		16.1	16.0
	RNA 2	qR2T	nd	35.8	27.3	34.9	14.9	16.4		11.6	10.6		11.0	11.3

* indicates day clinical signs of VER became evident

The calculated copy number of RGNNV genome detected per mg of eye tissue by each assay ranged from 0.02 which was detected at day 2 post challenge, to 8.3 $x10^5$ which was detected at day 11 post challenge. (Figure 8-5). Mass of eye tissue ranged from 125 mg to 347 mg (average 180.4 sd. 49.9 mg). Prior to onset of clinical signs, the calculated copy number was less than 10^3 mg^{-1}. From the onset of clinical signs at day 6, the calculated copy number of RGNNV mg^{-1} of eye tissue markedly increased in both assays. Detection by qR2T was consistently ten-fold higher than that of qR1T (Figure 8-5). Prior to the onset of clinical signs at day 6, the calculated copy number mg^{-1} was less in the two groups exposed to vaccine compared to the other three treatment groups. However, during the period of peak morbidity calculated copy number was comparable between the five treatment groups.

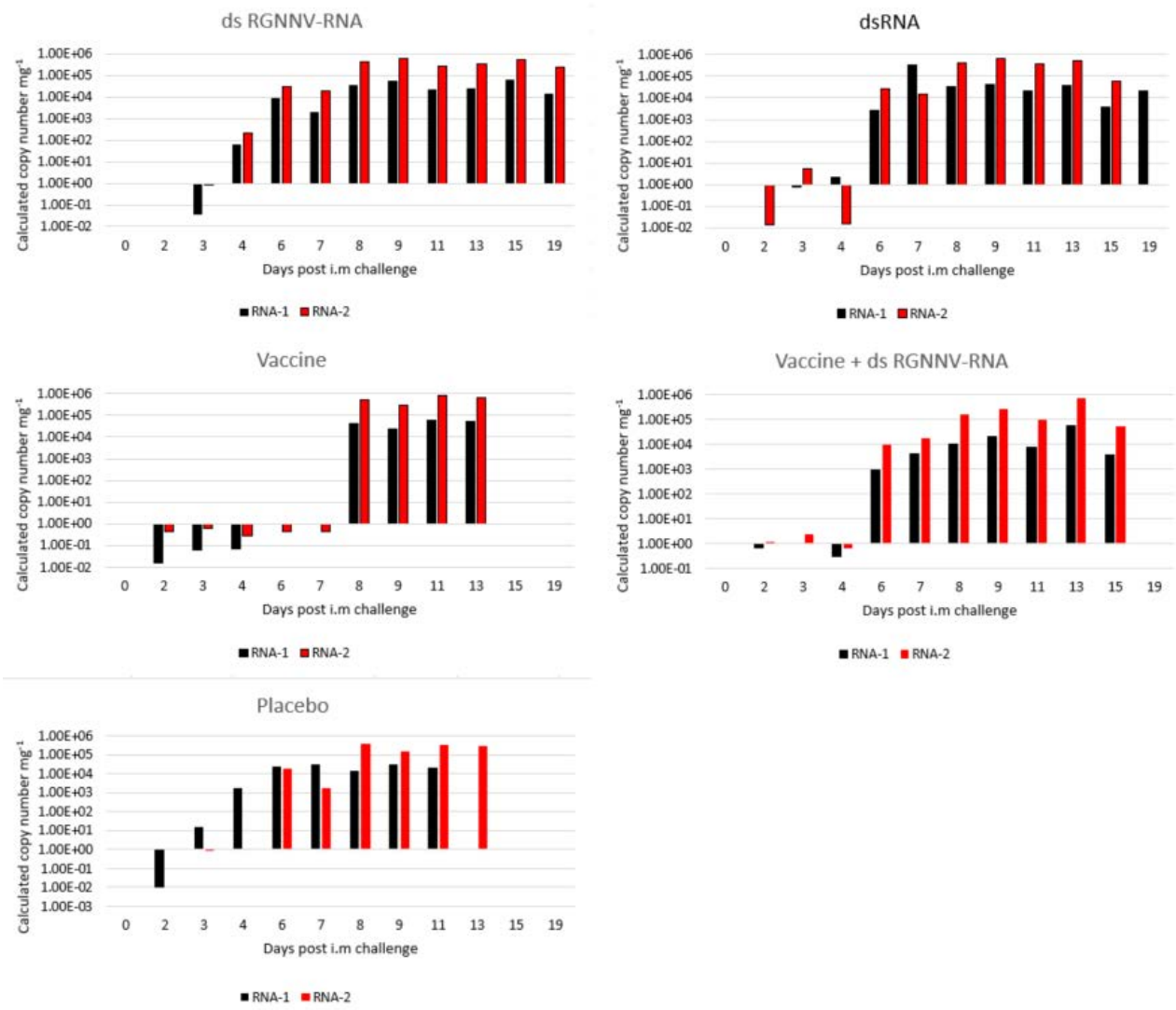


Figure 8-5: Calculated copy number of RNA 1 (Black columns) and RNA 2 (Red columns) segment per mg of tissue detected by qR1T and qR2T assay for each experimental treatment group (ds-RGNNV RNA; dsRNA, Vaccine, Vaccine +ds RGNNV RNA and Placebo) v days post challenge by IM injection.

8.4 Discussion

The application of RT-qPCR to quantify the detection of RGNNV genome segments RNA 1 and RNA 2 in response to IM challenge with RGNNV extract following exposure to experimental prophylactic measures was discussed within this chapter.

The two RT-qPCR assays, qR1t and qR2T displayed similar sensitivity to detect RGNNV genome. Both assays detected RGNNV genome segments from day 2 post-challenge albeit with a high cycle threshold value (Ct) and very low calculated copy number per mg tissue. The frequency of detection and calculated copy number per mg of eye tissue from both assays dramatically increased in all experimental groups from day 6 post challenge, where clinical signs became

evident. Detection of RGNNV before the clinical disease was reported previously from experimental challenges of juvenile sea bass after day one post-infection (Dalle Valle et al., 2005). Souto et al. (2018) reported calculated copy number of RNA 1 and RNA 2 segments by RT-qPCR *in vitro* of log $10^{5.5\text{-}8.5}$ copies mL^{-1} within 24 hours post-inoculation yet an absence of cellular pathological effect (CPE) until after 48hours.

Although RT-qPCR has significantly improved the ability to quantitatively determine viral replication and better understand the expression of VER, few publications have applied RT-qPCR for a purpose other than detection in disease outbreaks. Comparison of the levels of RGNNV detection associated with disease previous reports is difficult. Many reports that have applied RT-qPCR to experimental infection with NNV have presented results in different formats including cycle threshold (Ct) value (Hodneland et al., 2011 and Panzarin et al., 2010, Agnihotri et al., 2015), copies mL^{-1} (Kim et al., 2018), copies per 200ng total RNA (Dalle Valle et al., 2005), copies per 4 µg total RNA (Lopez-Jimena et al., 2011), log_{10} viral copies (Hick & Whittington, 2010) or log_{10} viral copies g^{-1} brain tissue (Souto et al., 2018). Only one of the prementioned studies were conducted on groupers (Kim et al., 2018).

In this experiment, average Ct values from eyes collected from the onset of clinical signs, days 6 to day 19 post challenge were 16.8 (s.d 1.67) (RNA 1) and 12.8 (s.d 2.14) (RNA 2) respectively. Brain samples from clinically diseased grouper had a Ct value of ca 10 (RNA 2) (Agnihotri et al., 2015), sea bass had a median Ct value of ca 15.0 (RNA 2) (Hodneland et al., 2011) and a range of Ct value from 9.91 to 18.6 (RNA 2) (Parnzarin et al., 2010). Other authors report calculated copy numbers of 10^9 mL^{-1} (RNA 1) to 10^6 mL^{-1} (RNA 2) (Kim et al., 2016) or 10^5 (RNA 1) and 10^7 (RNA 2) per 200ng total RNA (Dalla Valle et al., 2005).

In this investigation, although both assays detected the RGNNV genome, the calculated copy number of qR1T was slightly less than qR2T. An increased calculated copy number of RNA 2 compared to RNA 1 has been noted previously where the number of copies of RNA 1 was 100 times less than those of RNA 2 from the brain of sea bass from days 3 to 14 post experimental challenge (Dalle Valle et al., 2005). It is possible the differences in levels of detection are related to variable transcription of the RNA 1 and RNA 2 gene segments as infection progresses. As RNA 1 encodes the mRNA for the RNA-dependant RNA polymerase, the replication and translation of RNA 1 is required for transcription of RNA 2. The time course of sample collection in this investigation may not be sufficient to resolve shorter-term variation in transcription of RNA 1 and RNA 2 during the early stage of progression towards disease. The reduced efficiency in assays that detect RNA 1 may also relate to secondary structure interaction between RNA 1 and its translated form, Protein A, that contribute to the formation

of the viral replication complex, termed spherules, which are essential for nodavirus replication (Kopek, Settles, Friesen & Ahlquist 2010). The formation of the replication complex in the mitochondria, serves to protect the naked RNA 1 genome and facilitate viral replication. In contrast, RNA 1 was detected at much higher quantities (10^2-10^4) than RNA 2 in a variety of tissues from *H.septemfasticatus* following experimental challenge (Kim et al., 2016).

A range of criteria are applied to process of validation of an assay, one of which, the operating range of the assay, can be difficult to demonstrate beyond application of disease diagnosis. Specifically, determining the suitability of an assay to accurately and precisely detect NNV in sub-clinical carriers can be difficult because it requires access to experimental challenged animals where the infection status of the samples is pre-determined. In this study, NNV was detected by both RT-qPCR assays after IM challenge, prior to, and following the expression of disease symptoms. Similar results were reported by Dalle Valle et al. (2005) whereby RGNNV was detected within 1-day post challenge and clinical signs became evident 7 days post challenge. In this study NNV was detected at 2 days post challenge and clinical signs of VER were evident 6 days post challenge. NNV continued to be detected by RT-qPCR up to 80 days post IM challenge (data not shown). Groupers that were assigned to the non-clinical signs tank did not succumb to VER during an 18-month holding period (data not shown).

The rapid progression of disease observed in this report agrees with numerous experimental challenges in other fish species that indicate mortality from day ~5-10 post infection (Hodneland et al., 2011; Lopez-Jimena et al., 2011). Hodneland et al. (2011) reported 84% mortality of IM injected sea bass within 10 days post infection. Dalle Valle et al. (2005) reported initial lethargy in sea bass 7 days post infection, followed by death of fish at 9, 10- and 11-days post infection and an absence of disease in survivors at 13- and 14-days post-infection. Mortality in juvenile *E. septemfasciatus* (reclassified as *Hyporthodus septemfasciatus*) between days 6 to 9 were observed following challenge with a RGNNV titre approximately $10^{5\text{-}6}$ $TCID_{50}$ (Cho et al., 2017; Kim et al., 2018).

The prophylactic measures had varying effect on the cumulative morbidity of RGNNV challenged fish. Experimental groups that received vaccines containing recombinantly expressed RGNNV capsid protein displayed reduced levels of morbidity compared to fish that received placebo vaccine or dsRNA constructs. Modest reductions in the cumulative morbidity were observed in the vaccinated fish (43-53% morbidity) compared to the other experimental therapies (placebo 68% morbidity and dsRNA 88% morbidity). Although the presentation of dsRNA constructs provided no protection against the development of VER following exposure

to RGNNV extract in this experiment, the technology should not be discounted as a tool to better understand and manage VNN. The application of dsRNA as a therapeutic has been reported for the prevention of viral diseases of crustaceans (Owens et al., 2014). Although the technology faces a number of hurdles there is at least one US patent claiming successful application of dsRNA to prevent disease in an aquaculture species namely for the prevention of Infectious Myonecrosis Virus in shrimp and other invertebrates (Loy et al.US patent No. US8822427B2). This project attempted delivery of naked dsRNA, which was not protected from degradation. The delivery of unprotected dsRNA has been reported previously (Owens et al., 2014;). One of the main problems in the development of siRNA for therapeutic use is the need for a delivery method that prevents the degradation of siRNAs by cellular nucleases (Chernikov et al., 2019). Adjustments to the delivery, concentration, target sequence and duration of priming may lead to improvements in the efficacy of dsRNA prevent VER. Considering the range of variables that require refinement in dsRNA, a more ethical approach investigating dsRNA as a therapy would be to refine the target design in cell culture systems before further trials are commenced in fish. Approaches using dsRNA to knockdown fish viruses have been successfully applied to prevent the expression of the major capsid protein of the tiger frog Iridovirus in fathead minnow cells (Xie et al., 2005). However, further work with cell culture systems are beyond the resource and short time frame of this study.

The modest reduction in morbidity in vaccinated fish compared to unvaccinated fish was a promising result. The results suggest that some level protection is provided by the vaccine. However, the protection wasn't consistent across the vaccinated cohort.

Body mass was also noted to be variable within the experimental groups. Very few vaccinated fish with a body mass over 50g displayed morbidity following challenge with viral extract. There has been no reported effect of fish size on the efficacy of vaccination in grouper. There is a large range in body mass across the studies that have investigated vaccination against NNV in grouper (Table 8-4) but so far, no statistically analysed effect of body weight on vaccination. The design of this experiment prevents robust statistical analysis of effect of body weight on vaccination.

Table 8-4 Details of species, age and body weight from previous studies involving vaccination of grouper against NNV or bacteria.

Details of Groupers in the Studies			Reference
Species	Age (dph)	body mass (g)	
E.coioides	from 1 to 18	dnp	Lin et al. 2007
E.coioides	dnp	0.36	Lai et al., 2014
E.lanceolatus	dnp	20	Liu et al., 2006
E.malabaricus	dnp	20	Liu et al., 2006
E.septemfasciatus		28	Tanaka et al. 2001
E.coioides	dnp	33	Mo et al., 2019
E.coioides	dnp	32	Huang et al., 2014b
E.coioides	dnp	35	Atujona et al., 2019
E.septemfasciatus	dnp	75	Yamashita et al. 2009
E.septemfasciatus	dnp	80	Oh et al. 2012
dnp = detail not provided			

Within the literature there is very little information about the development of a competent humoral immune system in grouper in relation to age or body mass. Mao et al. (2012) reported with detectable antibodies to VNN and the oval shaped thymus of 4-month old (~112 dph) fish was clearly visible and there were amounts of T lymphocytes present. However, no protective functionality of the antibodies or T-lymphocytes was demonstrated. The differential morbidity of vaccinated fish may be related to factors other than body weight. However, few of those factors can be investigated with the available data.

8.5 Conclusion

The aims of this chapter were met in the following manner:

- The efficacy of vaccine and dsRNA preparations to prevent infection and disease in juvenile *E. lanceolatus* following IM challenge with RGNNV viral extract were assessed.
- The performance of the qR1T and qR2T assays to detect RGNNV from grouper derived brain and eye tissue following experimental challenge by IM injection with RGNNV.
- RT-qPCR analysis detected RNA 1 and RNA 2 NNV segments from grouper tissues prior to the onset of disease, during the period of peak morbidity and for a period when morbidity numbers had plateaued.

CHAPTER 9. ROLE OF PARENTAGE IN SURVIVAL FROM VIRAL ENCEPHALOPATHY AND RETINOPATHY IN *EPINEPHELUS LANCEOLATUS*

Background

- Initial attempts with vaccination led to a modest improvement in survival of *E. lanceolatus* following RGNNV challenge.
- Grouper are aggregative spawners. The juveniles used in the experimental challenge were produced in a mass spawning event with 7 potential parent contributions.
- Evidence of genetic resistance to NNV is reported in some fish species.
- There is very limited evidence of genetic resistance to NNV in grouper.
- Modest efficacy of the vaccine to protect against VER could be related to parentage rather than a protective effect of vaccination.

Aims of this Chapter

- Investigate if vaccinated fish that survived RGNNV (resistant) have a different parental assignment than fish that succumbed to VER disease (susceptible) following RGNNV challenge.

9.1 Introduction

The juvenile *E.lanceolatus* used in the trial described in Chapter 8 were derived from a mass spawning containing eight parent fish. It is possible the differential survival of vaccinated fish compared to the other experimental groupers was influenced an unknown parentage-linked genetic element.

Genetic selection for resistance to disease is often pursued as a management strategy to reduce the impact of disease outbreaks in aquaculture. Successful breeding of families with resistance to infectious pancreatic necrosis virus (IPNV), salmon alphavirus and infectious salmon anaemia virus (ISAV) have been reported in salmon aquaculture (Yanez et al., 2014). Previous studies with grouper species identified a relationship between major histocompatibility complex (MHC11) polymorphism and resistance to Singapore grouper Iridovirus (SGIV) in 40-60g *E. coioides* whereby allele (EPOC-DBB*1001) (NCBI JN980423) was significantly associated with resistance (Min et al., 2016).

There is limited evidence of genetic resistance to NNV in grouper species. Although there are rumoured reports of cross-species hybrid groupers being less susceptible to VNN, there are no published reports identifying genetic markers for resistance to VNN in hybrids or within a single grouper species. In *L.calcarifer*, a single study with larval fish identified five significant quantitative trait loci (QTL) for resistance to VNN which accounted for 2.2 to 4.1% reduced calculated copy number and 2.2-3.3 % survival time as phenotypic traits that indicated resistance (Liu et al., 2016). The authors proposed genetic resistance to NNV in *L.calcarifer* to be complex and controlled by the accumulation of small effects of many loci (Lui et al., 2016). A significant improvement in VNN resistance was noted in a cohort of 3-month-old *L.calcarifer* that possessed a variant (410_417) in the 3' untranslated region (UTR) of Receptor-transporting protein 3 (Rtp3) (Liu et al., 2017). Approximately 22% of fish that survived water bath challenge to RGNNV displayed the variant 410_417 compared to approximately 13% of the fish that succumbed to VER (Liu et al., 2017). Unfortunately, the samples were collected 5 days post challenge when mortality across the cohort reached 50%. In numerous studies, including that indicated in Chapter 8, mortality in susceptible cohorts typically exceeds 50% and consequently, a number of the fish included in the "survival group" may have succumbed to infection within a further 1- or 2-days post sampling. An analysis of survivors after the 80% mortality phase, or when the mortality curve reached a plateau stage, would have provided greater confidence in the significance of this allele.

Selective breeding programs for highly pathogenic diseases typically rely on binary assessment of susceptibility and resistance via disease-challenge testing (Houston 2017). The earliest

studies phenotypically determined “susceptible” as those individuals that succumbed to disease and “resistant” as those that survived challenge. More recently, determination of “resistance” has been refined to indicate a blocking of reproduction of the pathogen, namely survival and an absence of detection of the pathogen (Yanez et al., 2014). Survivors, where the pathogen can be detected are termed “tolerant” (Yanez et al., 2014).

Although it was not the intent of the challenge trial discussed in Chapter 8, the production of the juveniles used in this project from a mass spawning event may have inadvertently introduced a variable genetic element to the vaccine efficacy experiment. As a precautionary measure, prior to further assessment or attempted optimisation of the vaccination process, some assessment of the parentage of the experimental challenge fish is warranted. Fortunately, Bright et al. (2016) published multi locus sequencing analysis (MLSA) using microsatellite markers to determine parentage based on *E.lanceolatus* from The Company One broodstock. The aim of this investigation is to apply the markers of Bright et al. (2016) to determine the parentage of a selection of the vaccinated fish that were susceptible to RGNNV and those that survived RGNNV challenge.

9.2 Materials and Methods

9.2.1 Source of E.lanceolatus for family assignment

All the experimental challenge fish used in the family assignment were from the vaccinated group. Nine of the experimental challenge fish that were euthanased with signs of VER were included in the analysis as the susceptible group. Six of the experimental challenge fish, which did not display signs of VER, were euthanased at day 20 post challenge after the mortality curve had plateaued from the peak morbidity period between days 7 to 10 post challenge (Figure 7.2). As one of the aims of the efficacy investigation was to determine long term survival, only a small number of fish were euthanased for this investigation. Although parentage assignment could be conducted on fin clips, samples of brain or eye were required to assess the RGNNV status and determine if the survivors were resistant or tolerant to infection.

9.2.2 Nucleic acid extraction

Total nucleic acid was extracted from eye tissue of the experimental challenge fish as previously described in 2.1.2. Total nucleic acid was extracted from fin clips from the adult grouper that contributed to the experimental challenge cohort. Fin clips were extracted as previously described in 2.1.2.

9.2.3 Quantitative RT-PCR to detect RGNNV

Quantitative RT-PCR applying the qR2T assay to detect RGNNV was conducted as previously described in 8.2.6.

9.2.4 Confirmation of suitability of family assignment primers.

Eight primer pairs were selected for parentage assignment by polymorphic microsatellite analysis (Bright et al., 2016). Prior to submission to AGRF a preliminary assessment of primer suitability was conducted against the six nucleic acid extracts from fish that did not display signs of VER by applying touchdown PCR described by Bright et. al (2016). Briefly, PCR was conducted on a Maxygene thermal cycler (Axygen) in a 20 µL reaction with 2.5 µL of TNA extract and 0.5 µM each primer in a MyFi™ DNA polymerase Mix according to the manufacturer's instructions (BIO-21117 Bioline, NSW). The thermal cycle profile consisted of a 3 min incubation at 95^{o}C followed by 20 cycles of 30 s denaturation at 95^{o}C, 30 s of annealing temperature 62^{o}C (decreasing by 0.5 ^{o}C per cycle) and 45 s extension at 72^{o}C followed by 15 cycles of 30 s denaturation at 95^{o}C, 30 s of annealing temperature 50^{o}C and 45 s extension at 72^{o}C; and a final extension step of 10 min at 72^{o}C.

Table 9-0-1: Details of primer sequences applied to MLST analysis to determine family assignment

Set Id.	Locus	Orientation	Sequence (5'-3')	Size	Tm
H	ELMS007	F	TGT AAA ACG ACG GCC AGT TTT GCC TTT CCT AGA CTT AT	325-341	50
		R	CAT CAC ATG ATT CCT TTC TAT		
A	ELMS009	F	TTC CAC AGC AAT TAG CAG CA	257-300	56
		R	TTT CCT CCC ACA GTT CCA AAG		
F	ELMS019	F	TCA GCA AGC ACT TTT TGG AC	377-385	56
		R	TGC TTC CTT CAG TGC ATC AG		
E	An2	F	TGC CCC TCC GAC AAC TAA TA	226-250	61
		R	AAC GGG ACT TGT GGT TTT TG		
D	An4	F	TGT AAA ACG ACG GCC AGT GCT CGA AGA TGA GCT GGA AG	192-210	60
		R	AAG GTG CTG CTC CTG CTT T		
C	An8	F	TGT AAA ACG ACG GCC AGT ACC ATG CAT AAA TGC CCA CT	148-162	60
		R	GCT CTC TGT CTC GCA AGG AT		
B	An25	F	TGT AAA ACG ACG GCC AGT TCT GTG CTG ATG CCG ACT AC	146-200	58
		R	CCG TGT TTG CAC ACT CTC TG		
G	An31	F	TGT AAA ACG ACG GCC AGT TCA ATG TGT GCA AAC GCT GTA	184-218	61
		R	CAA CAT GGC CGA AAC CTA AT		
Source of All primer sequences Bright et. al. (2016)					
Tm, annealing temperature of Primer (°C)					

9.2.5 Gel electrophoresis

PCR amplification was confirmed by agarose gel electrophoresis as previously described in 2.5.3. The gels were loaded with 20 µL of PCR product and subjected to 60 min at 100 volts with a Hyper Ladder™ 50bp DNA ladder (BIO-33054, Bioline, NSW) (1 % gel). The gels were visualised and photographed on a UV trans illuminator.

9.2.6 Family assignment by microsatellite markers

Following confirmation of suitability of primers, total nucleic acid extracts from 7 parent and 15 experimental challenge fish, were submitted to the Australian Genome Research Facility (AGRF) for fine mapping and custom genotyping services applying the primers of Bright et al. (2016). Results of allele counts for each marker were provided by AGRF.

9.3 Results

9.3.1 Gel electrophoresis to confirm primer suitability

The primers of Bright et al. (2016) amplified products in the six extracts used in the pilot assessment. Refer to Figure 9.1 for image of gel electrophoresis.

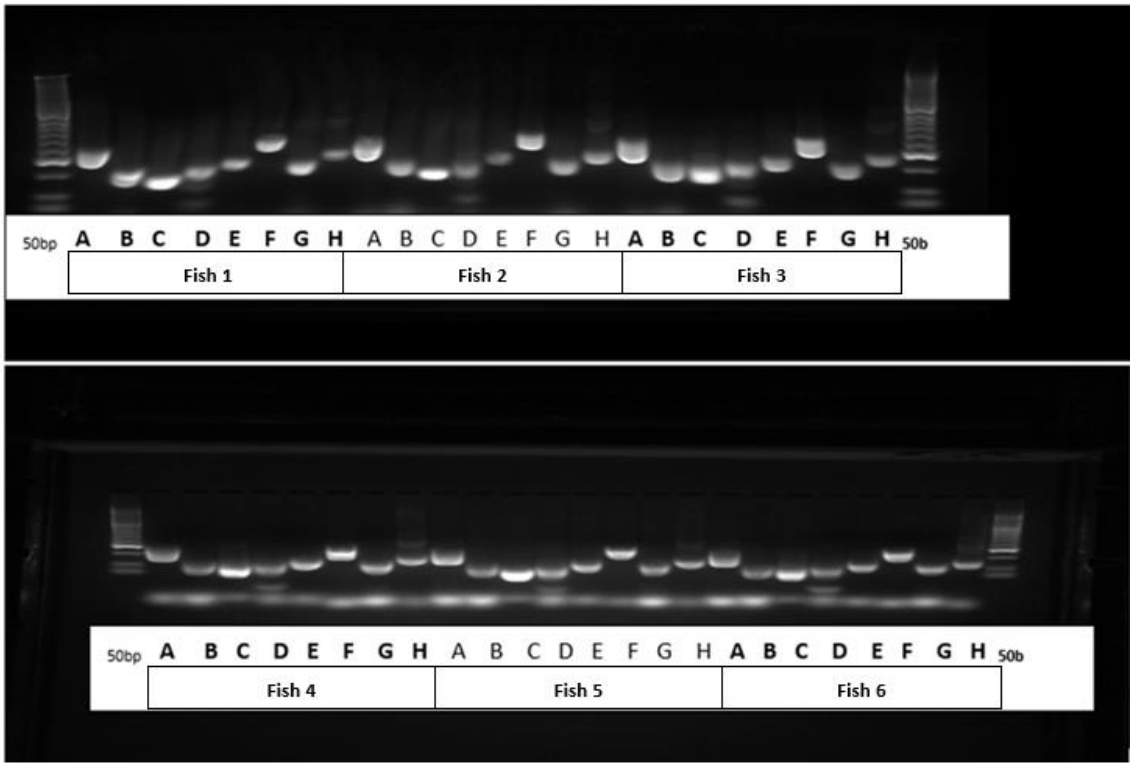


Figure 9-1: Gel electrophoresis image of amplicons

Produced by application of the eight primers A to H) of Bright et al. (2016) on extracts from the six fish (Fish 1 to 6) that did not display signs of VER to determine family assignment in *E.lanceolatus*. Refer to Table 8.1 for primer sequences.

9.3.2 Quantitative RT-PCR for the detection of RGNNV genome.

All samples collected from juvenile fish were positive for the detection of the RGNNV genome. Cycle threshold values ranged from 23 to 30 (Table 9.2). The RT-qPCR for detection of RGNNV RNA 2 was positive for all samples indicating, that the fish which did not succumb to VER should be considered tolerant rather than resistant to infection.

Table 9-2: Family assignment and qR2T Cycle threshold value (Ct) of vaccinated fish that displayed clinical signs or succumbed to VER following IM challenge to RGNNV extract.

Sample information		qR2T Ct	Parentage assignment	
Sample Id.	Clinical disease/No clinical signs		Female	Male
Fish-1	No clinical signs	27	F3	M2
Fish-2	No clinical signs	27	F2	M2
Fish-3	No clinical signs	27	F2	M2
Fish-4	No clinical signs	30	F2	M2
Fish-5	No clinical signs	27	F2	M2
Fish-6	No clinical signs	30	F4	M2
Fish 7	Clinical disease	24	F3	M2
Fish 8	Clinical disease	25	F2	M2
Fish 9	Clinical disease	23	F3	M2
Fish 10	Clinical disease	23	F2	M2
Fish 11	Clinical disease	23	F3	M3
Fish 12	Clinical disease	25	F3	M1
Fish 13	Clinical disease	22	F4	M2
Fish 14	Clinical disease	25	F2	M2
Fish 15	Clinical disease	24	F3	M2

9.3.3 Family assignment by microsatellite markers

Allele counts from each sample for each marker were provided by the Australian Genome Research Facility (Appendix 2). Five reactions out of 175 failed to amplify. Family assignment was performed based on allele sizes generated. The data provided by Markers An31 (primer set G) and Elms19 (primer set F) had very little resolution power and were not considered in the family assignment (Appendix 2). Family assignment analysis indicated 4 out of 6 fish that did not display clinical signs of VER were from Male #2 and Female #2. The remaining 2 fish that did not display clinical signs of VER were from Male #2 and Female #3 and Male #2 and Female #4. Within the susceptible fish 7 out of 9 fish had the same parentage assignment as those of the tolerant group.

9.4 Discussion

Although the experimental challenge study was not intended as an investigation into genetic selection for disease, due to the aggregate spawning behaviour of grouper, the modest survivals evident in the vaccinated groups of RGNNV challenged animals may have related to a different parentage rather than an efficacy of the vaccine i.e. a genetic trait. Although some correlative studies investigating genetic traits and survival to NNV have been reported in other species, no genetic resistance to NNV has been demonstrated in groupers. Studies linking genetic resistance to VER in other species have indicated the mechanisms of resistance are likely complex (Liu et al., 2016). This also applies generally to research reporting on genetic

resistance to viral disease in salmon (Yanez et al., 2014). Genetic resistance to disease typically involves detection of combinations of traits that are complicated to measure (Yanez et al., 2014). The interaction between multiple immune mechanisms, viral variants and environmental fluctuation make identification of traits that link to disease resistance very difficult (Yanez et al., 2014). Investigation of such traits was both beyond the scope of this project and restricted by a void in the understanding of mechanisms of the grouper immune system and its differential development with growth. Nonetheless, working within the limitations of knowledge and resources available at the time of this study, investigation comparing parentage of the vaccinated fish that were susceptible to RGNNV (euthanased due to clinical disease signs) against those animals that were tolerant of RGNNV (survivors) was conducted. Quantitative RT-PCR to detect RGNNV indicated the survivor were positive for the detection of NNV genome and were hence considered a tolerant phenotype for parentage analysis. Working with the MLST markers Bright et al. (2016) applied to successfully assign parentage to 574 offspring from a broodstock cohort from The Company One stock, this study investigated parentage of tolerant v susceptible individuals. The parentage assignment was comparable between both cohorts whereby two individuals from Male #2 and Female #2 dominated both the tolerant and susceptible groups. Within the restriction of the small number of samples analysed, the results of the parentage assignment were in agreement with those observed by Bright et al. (2016) whereby, a dominant male sired the majority of offspring and although polygamous mating was observed, the same dominant male predominantly spawned with a particular female. Whilst Bright et al. (2016) reported all of the markers except ELMS7 were useful for parentage assignment, this study noted that ELMS19 also had poor power in determining parentage assignment. This study demonstrates the modest improvements in survival following challenge with RGNNV in the vaccinated groups that were described in Chapter 8 were not simply caused by differential parentage within the experimental cohort. Further investigation into the optimisation of the vaccine and vaccination strategy to prevent VER in *E.lanceolatus* is therefore supported.

9.5 Conclusion

The following outcomes and conclusions were described in this chapter:

- RT-qPCR detected RGNNV in the eye of fish that survived RGNNV and those that succumbed to VER following IM challenge.
- Surviving fish are therefore considered tolerant rather than resistant to infection by RGNNV.
- Results of Multilocus sequence analysis indicate the fish that were tolerant to RGNNV following IM challenge were of the same parentage as fish that were susceptible to RGNNV following IM challenge.
- The modest improvements in survival observed in vaccinated fish were not due to variable parentage within the cohort produced to due to the grouped spawning production of the experimental cohort.
- Further investigation into the efficacy of vaccination to prevent VER following exposure to RGNNV is validated.

CHAPTER 10. TESTING EFFICACY OF EXPERIMENTAL VACCINE TO PREVENT VER FOLLOWING CHALLENGE WITH NERVOUS NECROSIS VIRUS IN LARGER FISH

Background

- Initial attempts with vaccination led to a modest improvement in survival of *E.lanceolatus* following RGNNV challenge.
- Some fish species display age related resistance to VER.
- Successful vaccination relies on the fish having attained a competent adaptive immune system.
- Many of the vaccinated fish that were tolerant of RGNNV challenge were a larger body weight than the fish that succumbed to disease.
- Delaying vaccination and challenge until fish are slightly larger (and therefore older) may improve the efficacy of vaccination as a strategy to prevent VER.
- The time frame for approval of a recombinant expressed capsid protein vaccine is likely to take a number of years.
- An emergency/research permit vaccine could be obtained in a shorter time frame based on heat-killed cell culture produced antigen.

Aims of this chapter

- Investigate if a refined vaccination strategy, on larger fish >50g, improves efficacy of vaccination to prevent VER.
- Investigate if heat-killed cell culture derived vaccine on larger fish is an effective measure to prevent VER.

10.1 Introduction

The Australian Pesticides and Veterinary Medicines Authority (APVMA) regulates the registration of veterinary vaccines in Australia (https://apvma.gov.au/node/1108). The registration process is lengthy, and in some cases, requires demonstration of compliance across multiple authorities. Vaccines containing cell-culture derived antigen must be prepared in compliance with Australian Government Department of Agriculture and APVMA (pers. comms from APVMA). Vaccines containing expressed recombinant antigen must address the standards regulated by Office of the Gene Technology Regulator (OGTR) and APVMA (pers. comms from APVMA). Although the path to registration for commercial production is long, there is provision for the registration of an experimental vaccine to provide interim or emergency protection (https://apvma.gov.au/node/1108).

Experimental vaccines are typically autogenous vaccines prepared from a pathogen isolated from a specific location and administered to other stock at risk within the same location (https://apvma.gov.au/node/1108). The several years that may be required to register a vaccine within the APVMA is not compatible with the immediate need for the current grouper aquaculture industry to prevent the economic losses caused by VER outbreaks in pond grow-out systems. For reasons of upscale and difficulty with biosecurity compliance requirements, a cell culture-derived vaccine is not the preferred vaccine format for future commercial preparations; however, if effective, the format provides an interim measure to prevent losses due to VER for the period required to meet compliance for commercial registration of expressed recombinant capsid protein vaccine. Therefore, this study investigated an autogenous cell culture-derived vaccine for effectiveness to prevent VER.

The observation of increased survival in larger vaccinated fish described in Chapter 8 may be due to several factors, one of which is the larger fish were in better condition and hence responded to vaccination more efficiently. The absence of improved survival in the non-vaccinated fish of similar body weight in the experimental trial (Chapter 8) indicates that survival following challenge was not a bodyweight-viral dose relationship. Although understanding the mechanism between bodyweight and vaccination efficacy is not within the scope or resource budget of this study, further investigation into a relationship is possible.

Researchers that apply experimental procedures to animals are obligated to ensure the ethical, humane and responsible use of animals in their care. One aspect of that obligation is to minimise the number of animals used (NHMRC, 2013). To minimise the number of fish used within experimental processes, testing the efficacy of the autogenous vaccine on juvenile *E.lanceolatus* was combined with testing the efficacy of the recombinant vaccine on larger

body weight fish. This chapter investigates the efficacy of vaccination, using a heat-killed viral-cell culture antigen or expressed recombinant capsid protein as antigen to prevent VER in juvenile grouper with a minimal body weight of 50g.

10.2 Materials and Methods

10.2.1 Experimental E.lanceolatus

Juvenile *E.lanceolatus,* (116 days post hatch), were provided by a commercial supplier, Fin Fish Enterprises (Australia). Two hundred fish, produced from a single mass spawn were used in the investigation. Fish were housed under the same conditions as described in section 2.3, including at a water temperature range of 24-28°C, until all fish had a body weight exceeding 50g.

10.2.2 Expressed recombinant viral capsid Vaccine production

The vaccine produced from expressed recombinant protein was prepared as described in section 6.2.7. For this trial the purified protein was retrieved after approximately 9 months storage at -80°C.

10.2.3 Heat-killed cell culture derived vaccine production

Heat-killed cell culture derived vaccine (HK-vaccine) was prepared from 2 x 15 ml E 11 striped Snakehead whole fry tissue (SSN) cell cultures (Nakai, European Collection of Authenticated cell cultures Sigma 01110916) inoculated with RGNNV extract (Iwamoto et al., 1999 and 2000). Briefly, one ml of the RGNNV extract was inoculated onto two confluent monolayers of SSN-E11cells that were prepared in 15cm^2 cell culture flasks (Corning), supplemented with Leibovitz L-15 media containing glutamine (Sigma L1518) and 10% Foetal Bovine Serum (Sigma F6178). The flasks were incubated at 28°C for 3-4 days, until CPE extended across the entire monolayers. The cultures were then frozen and thawed 3 times and filtered through 0.45µM and 0.22µM filters. The culture supernatant was stored in a sterile 50ml tube at -20°C. RT-qPCR analysis to detect NNV was conducted on a total nucleic acid extract prepared from a 400µl aliquot of supernatant as described in section 2.2.9. NNV was detected by the qR1T PCR with a Ct value of 10.38 which equates to a calculated copy number of 2.29 x 10^8 copies mL^{-1}. The viral culture supernatant was subjected to 40-minute incubation at 60°C (Labnet) to deactivate the virus (Frerichs et al., 2000). Vaccine was prepared by mixing 15ml of the deactivated viral culture with 15ml of Fruends' incomplete adjuvant and homogenising the mixture as previously described for the other vaccine formulations (Section 6.2.7). Prior to commencement of the experiment a pilot study was conducted to confirm no adverse impact of the heat-killed cell culture vaccine. Fourteen days after delivery of the grouper, ten

groupers were anaesthetised, injected with 100µl of HK-vaccine (as described in section 2.3.3 and observed for 14 days to monitor any adverse effect of vaccine. After 14 days of no notable adverse reaction, the experiment was commenced as described below. The ten fish were maintained throughout the duration of the experiment, however, no longer term effects of vaccine were observed.

10.2.4 Vaccination of Fish

One hundred and eighty fish were divided into 3 experimental treatment groups namely: (1) Placebo vaccinated with Adjuvant + PBS, (2) vaccinated rCP vaccine), (3) vaccinated with HK-vaccine. Fish were anaesthetised and injected with vaccines as described previously (section 2.3.2 and 2.3.3). A timeline of procedures and description of the experimental procedures is outlined in Tables 10.1 and 10.2. Briefly, fish were vaccinated four weeks after receipt, with 100µL vaccine by IM injection by the same protocol described in section 2.3.3, followed by a booster four weeks after vaccination (Table 10-1). A further ten fish were included as negative controls. The negative control fish were injected (IM) with placebo vaccine following the same protocol and schedule as the experimental vaccine group fishes.

Table 10-1: Timeline of age of fish days post hatch at acclimation, vaccination, booster and challenge with viral extract.

	Acclimation	Vaccination	Booster	Challenge
Days of this experiment	0	28	56	70
Age of fish (days post hatch)	116	144	172	186

10.2.5 Experimental Challenge with RGNNV extract

Two weeks after the booster (186 dph) vaccine or equivalent placebo vaccine, fish were anaesthetised, weighed and challenged with either intramuscular injection with 50 µL RGNNV extract (calculated copy number 6.36×10^4 mL^{-1} of viral extract, prepared in 2.2.6) or 50 µL sterile PBS. One hundred and eighty fish were challenged with the viral extract described in section 2.3.3. The ten negative control fish, that were vaccinated with placebo vaccine (adjuvant: PBS 1:1 ration), were challenged with IM injection of PBS following the same protocol as the NNV challenged fishes. Ten fish from each vaccine type that were challenged with viral extract were then distributed into tanks (n=30 fish per tank) (Table 10-2 & Figure 10-2). The negative control, PBS challenged fish were held in a separate recirculation system

within the same experimental room. The tank system of the negative control fish was a duplicate system of the fish challenged with viral extract (Refer to section 2.3.1).

Table 10-2: Summary of experimental design indicating experimental groups, description, number of fish in the trial and number of fish sampled at morbidity or at scheduled time points during the experimental trial

Experimental Group	Description	Challenged 50µL i.m. injection	Number of fish	Sampling schedule	
				At morbidity	Scheduled*
Placebo vaccine	IM injection of 100µL adjuvant/PBS 1:1	RGNNV Extract	60	57	3
Recombinant Capsid protein vaccine (rCP)	IM injection of 100µL adjuvant/ rCP-RGNNV 1:1	RGNNV Extract	60	6	54
Heat-killed cell culture vaccine (HK)	IM injection of 100µL adjuvant/heat-killed cell culture 1:1	RGNNV Extract	60	5	55
Placebo vaccine (Negative control)	IM injection of 100µL adjuvant/PBS 1:1	PBS	10	0	10
* indicates at scheduled days in the absence of clinical signs of VER					

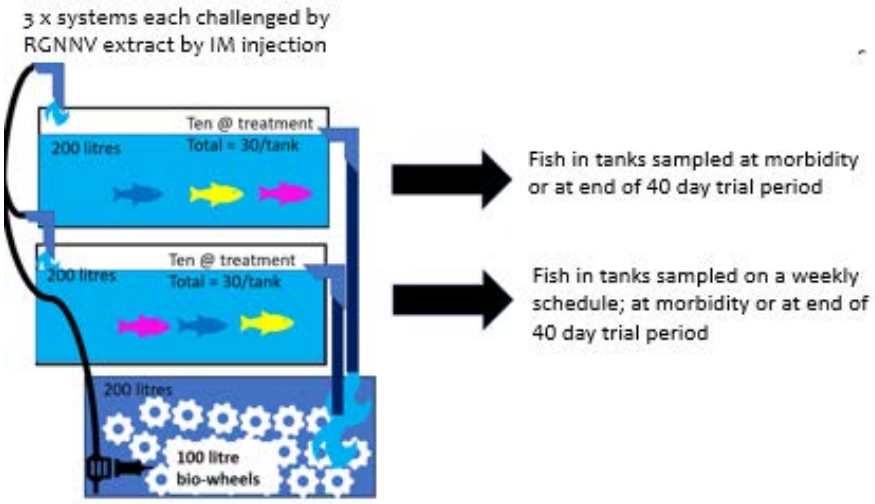


Figure 10-1: Diagram depicting experimental tank system. Three replicate systems each consisting of dual 200 litre tanks on a co-circulation system each containing 10 fish from each experimental group (placebo vaccine= blue fish; recombinant capsid protein vaccine= yellow fish; Heat-killed cell-culture vaccine= pink fish) making a total of 30 fish in each tank. The tanks on the top row were sampled at morbidity or at the end of the trial period and the tanks on the bottom row were sampled on schedule weekly, at morbidity or at the end of the trial period.

10.2.6 Monitoring of fish health and euthanasia

Fish were monitored twice daily for signs of adverse health. Fish were euthanased when more than one sign of VER was observed. In the absence of signs of disease, a fish from each of the experimental groups was euthanased every seven days for the first three weeks of the study. Euthanasia was conducted by Aqui-S overdose as described in 2.2.3. Euthanased fish were weighed, frozen whole in individually labelled plastic bags and stored at -20°C along with their identification tags. After 40 days post challenge all fish were euthanased with an overdose of

Aqui-S (as described section 2.2.3). The clinical state of all fish was recorded at the time of euthanasia.

10.2.7 Nucleic acid extraction

Total nucleic acid was extracted from a whole eye from each euthanased fish as described in sections 2.2.1 t0 2.2.3. Fish that were euthanased on schedule and at morbidity were subjected to nucleic acid extraction and quantitative RT-PCR.

10.2.8 Quantitative RT-PCR to detect RGNNV

Quantitative RT-PCR applying the qR1T and qR2T assay to detect RGNNV was conducted as previously described in section 8.2.6.

10.3 Results

10.3.1 Expression of VER following experimental challenge of juvenile grouper with RGNNV extract and efficacy of protection of each prophylactic measure.

The average body weight (abw) of groupers on the day of challenge was 103.5 g (σ 20.39 g) (data not shown). There was 100% survival and no adverse reaction evident in any of the groupers challenged with PBS injection. Within the NNV challenged groups, fish fed and behaved normally for six days post challenge. Seven days post challenge, a small number of fish displayed some typical signs of VER. Clinical signs included inflation of swim bladder, lethargy, erratic swimming behavior, darkened body color and flared opercula. The number of fish with multiple clinical signs increased markedly between days 8 to 10 post-challenge after which morbidity plateaued (Figure 10-2). The groups that received either vaccine formulation had reduced total numbers of fish displaying VER compared to the placebo vaccinated groups (Figure 10.2). By day 20 post viral challenge, 17% (HK-vaccine) and 20% (rCP-vaccine) of the vaccinated fish succumbed to VER compared to 93% of the placebo vaccinated (Figure 10.2). To remove any impact of scheduled euthanasia on measurements of survival or morbidity, the morbidity curves were only calculated from the tanks that were sampled in response to morbidity (n=30 each experimental group).

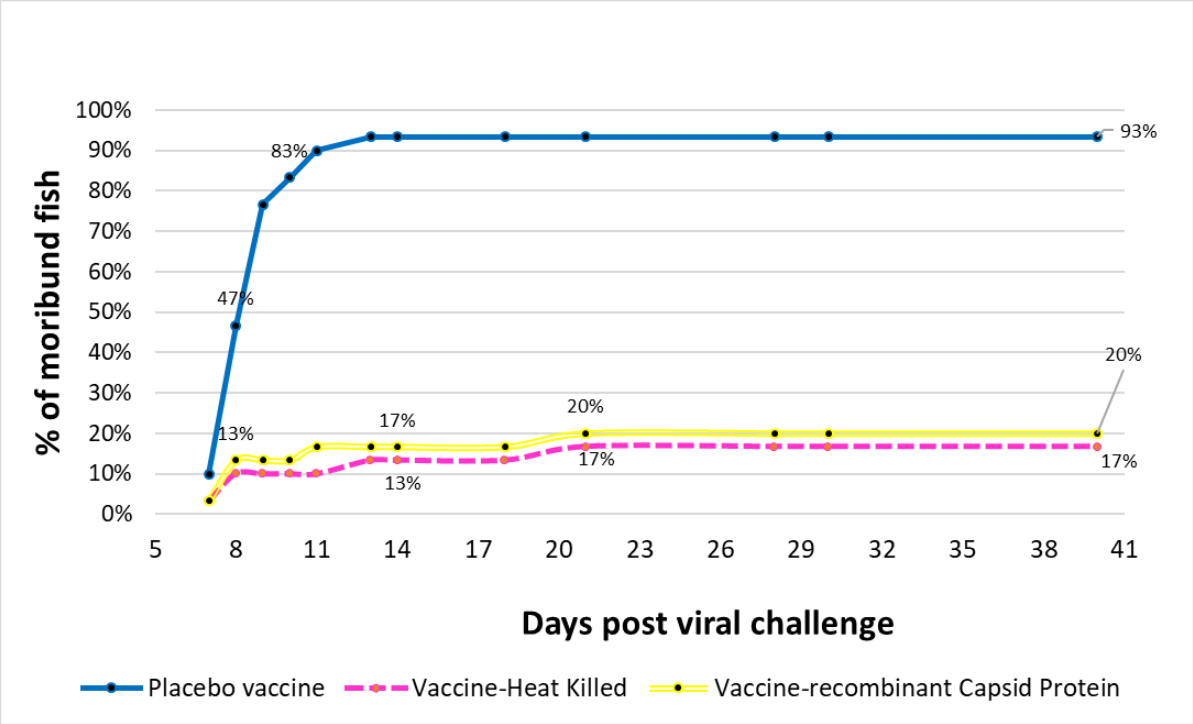


Figure 10-2: Cumulative morbidity (%) of fish with different vaccine formulations (placebo: blue line; heat-killed: pink line and recombinant capsid protein: yellow line) versus days post-challenge with NNV extract by IM injection.

10.3.2 Detection of RGNNV by RT-qPCR assay

There was no detection of NNV genome using qR1T or qR2T from any fish challenged with PBS injection. Within the groups that were challenged with RGNNV there were a variable number of positive detections of NNV by the RT-qPCRs (Table 10-3). There was 100% concordance between qR1T and qR2T assay results (Table 10-4). The number of detections of NNV by RT-qPCR was different to the number of groupers that displayed signs of VER (Table 10-3). Within the group that was vaccinated with placebo vaccine and challenged with RGNNV, fifty-seven (97%) fish were positive for the detection of NNV by both assays. Only three fish from the placebo vaccinated group survived challenge, did not display signs of VER and were also negative by both assays for the detection of RGNNV when euthanased at day 40 (Table 10-3; Figure 10-3).

Within the rCP-vaccinated group that was and challenged with RGNNV, twenty-three (38%) were positive for the detection of RGNNV by both assays (Table 10-3). Only six of the fish from the rCP-vaccine that were positive for the detection of RGNNV also displayed clinical signs of VER. A further 17 groupers were positive for the detection of RGNNV by RT-qPCR with an absence of clinical signs of disease (Table 10-3) (Figure 10-3). Thirty-seven fish that were rCP-

vaccinated and challenged with RGNNV were negative for the detection of RGNNV by RT-qPCR (Table 10-3; Figure 10-3).

Eleven of the groupers that received HK-vaccine, were positive for the detection of NNV by both RT-qPCR assays (n=60 fish each group; Table 10-3; Figure 10-3). Only five of the groupers from the HK-vaccine group had clinical signs of VER (Table 10-3; Figure 10-3). Forty-nine of the fish that received with HK-vaccine were negative for the detection of RGNNV by both RT-qPCR assays (Table 10-3; Figure 10-3).

Table 10-3: Number and percentage (%) of groupers positive and negative for the detection of RGNNV genome by RT-qPCR from each experimental group and number of groupers displaying clinical signs of VER or no clinical signs of VER at the time of euthanasia.

Experimental Group	RT-qPCR Result				VER clinical signs			
	Number Positive	% Positive	Number Negative	% Negative	Number Positive	% Positive	Number Negative	% Negative
Placebo vaccine	57	95%	3	5%	57	95%	3	5%
Heat-killed vaccine	11	18%	49	82%	5	8%	55	92%
Recombinant capsid protein vaccine	23	38%	37	62%	6	10%	56	93%

NB. Data calculated on all fish sampled including fish sampled according to schedule.

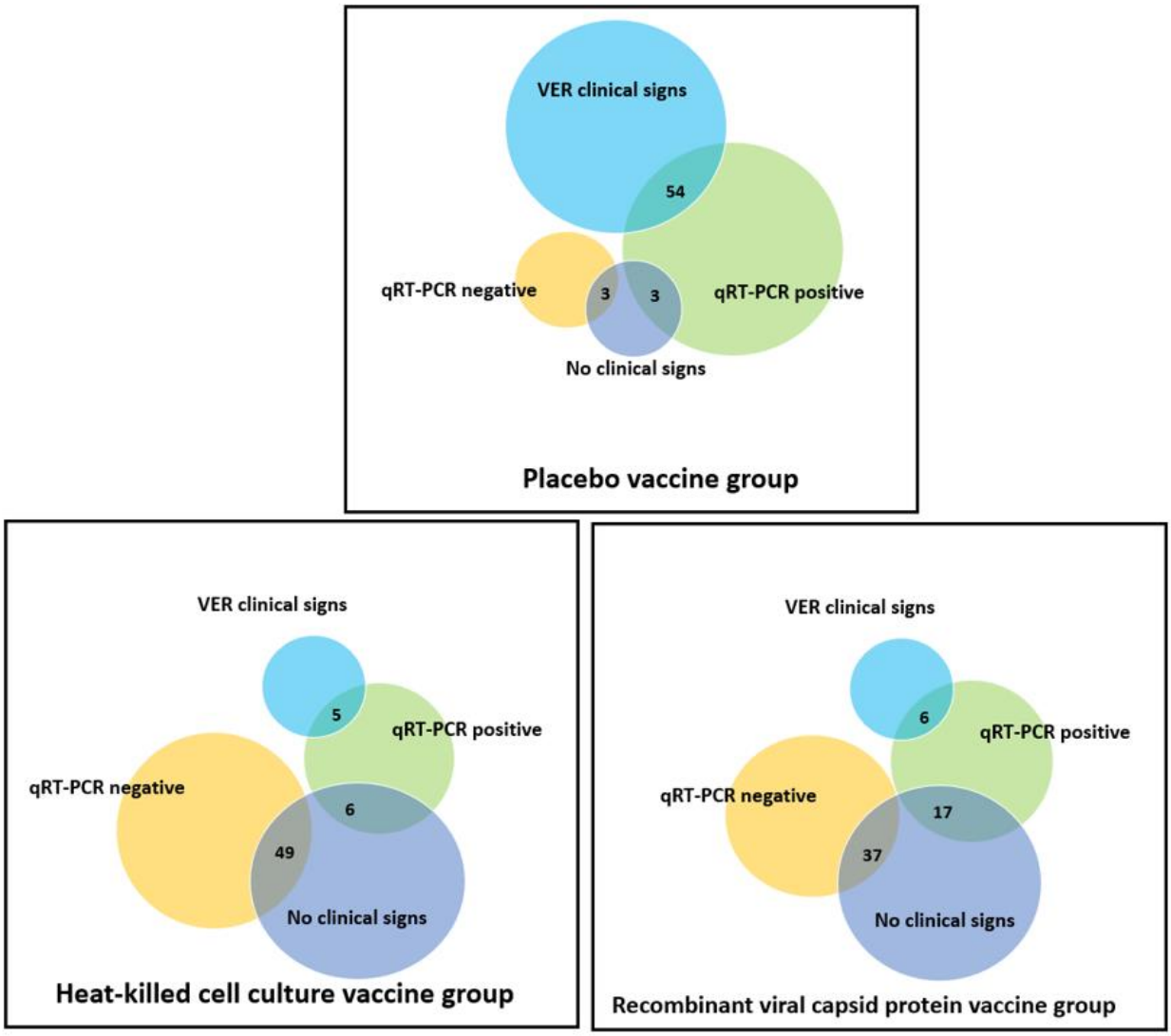


Figure 10-3: Number of groupers positive and negative for the detection of RGNNV by RT-qPCR from each experimental group with observation of presence (VER clinical signs) or absence of clinical signs (No clinical signs) of disease at the time of euthanasia. n=60 each group.

10.3.3 Quantification of RGNNV by RT-qPCR assay

RGNNV genome was detected with both RT-qPCR assays (qR1T and qR2T) from eye of virus-challenged fish from day 7 until day 40 when the trial was terminated (Table 10-4). Assay qR1T, which targets the RNA-1 segment had slightly higher Ct values than qR2T, which targets the RNA-2 segment (Table 10-4). RGNNV was detected by qR2T with lower Ct value in the fish that succumbed to VER than fish that did not display disease signs (Figure 10-4).

Table 10-4: Cycle threshold values of each RT-qPCR assay from eye of groupers from experimental group by days post viral challenge

Experimental Group	Assay Target	Assay Name	Days post challenge											
			0	**7***	8	10	11	13	**14**	18	20	**21**	**28**	40
Placebo vaccine	RNA 1	qR1T	nd	20.4	19.4	27.8	ns	ns	18.1	20.9	20.9	ns	ns	28.7
	RNA 2	qR2T	nd	19.8	18.2	25.3	ns	ns	16.5	19.0	ns	ns	ns	28.5
Recombinant Capsid protein vaccine (rCP)	RNA 1	qR1T	nd	37.5	ns	30.0	34.0	ns	34.0	ns	19.2	34.9	33.6	36.7
	RNA 2	qR2T	nd	37.2	ns	29.8	33.3	ns	33.7	ns	19.0	34.6	33.3	36.3
Heat-killed cell culture vaccine (HK)	RNA 1	qR1T	nd	38.9	ns	33.7	ns	26.8	nd	ns	33.6	nd	33.6	29.8
	RNA 2	qR2T	nd	38.6	ns	33.4	ns	26.5	nd	ns	28.3	nd	36.0	29.4
Placebo vaccine (Negative Control: PBS challenge)	RNA 1	qR1T	nd	nd	ns	ns	ns	ns	nd	ns	ns	nd	nd	nd
	RNA 2	qR2T	nd	nd	ns	ns	ns	ns	nd	ns	ns	nd	nd	nd

ns= no samples nd= not detected * indicates onset of clinical signs **Bold** indicates scheduled sample

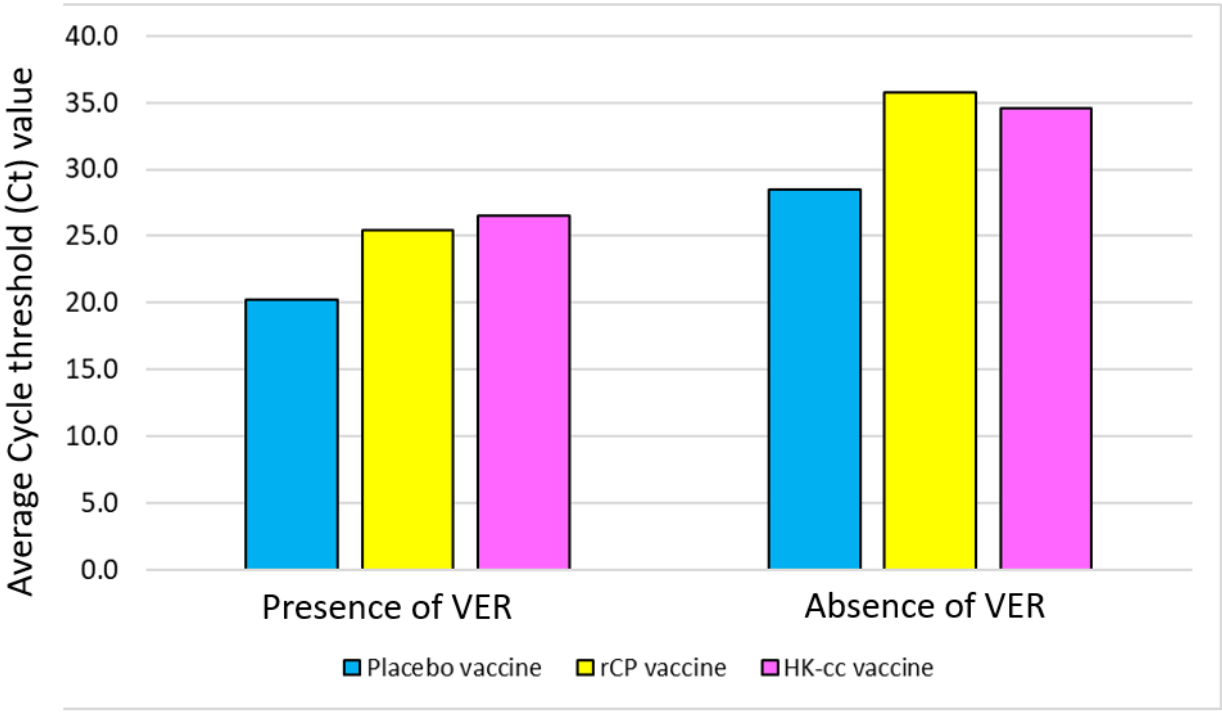


Figure 10-4: Average Cycle threshold (Ct) values of positive detections by qR2T from eyes collected from grouper from each experimental group according to the presence or absence of clinical signs of VER.

10.4 Discussion

In this study, vaccination of juvenile groupers with average body weight (abw) greater than 50g was associated with 17-20% morbidity (80-83% survival) following IM challenge with RGNNV (average body weight at challenge 103.5 g, (σ 20.39 g)). In contrast, 93% of the unvaccinated fish succumbed to VER following challenge with viral extract (7% survival). The survival of vaccinated fish in this trial was improved compared to the initial experimental trial discussed in Chapter 8. The mechanism that conferred improved efficacy of vaccination on the larger fish is unknown.

The acquisition of competence of the adaptive immune system is a prerequisite for successful vaccination. Excluding groupers, in many fish species, only the larval stages are highly susceptible to NNV (OIE, 2018). Despite the general acceptance of age-related susceptibility to NNV in juvenile barramundi, there is only a single report investigating the NNV susceptibility transition period (Jaramillo et al., 2017). In barramundi, juveniles older than 5 weeks of age develop a subclinical infection, whereas younger cohorts suffer clinical disease and mass mortality (Jaramillo et al., 2017). There is no characterisation of the mechanism that ensures resistance (Jaramillo et al., 2017) and a recent review of vaccination as a preventative strategy against VER did not refer to the development of the fish immune system (Hazreen et al., 2019).

Due to their typically low economic value, juvenile fish are often the subject of disease challenge and vaccine efficacy experiments. Although less expensive, and logistically more easily managed, challenges with juvenile fish may fail to consider conditions that affect the development and expression of adaptive antiviral immunity. Many studies are driven by a need for commercial outcomes and focussed on the production of a vaccine, rather than the basic science of grouper immunity. The FAO generally recognise that the lack of understanding of immune function in host species is one of the factors that reduce the ability to manage disease in aquaculture species (FAO 2018).

Foundational studies into vaccination of salmon identified the significant influence of water temperature on immune function in fish and discusses the term degree days (DD) as a unit to represent the interaction between number of days post-vaccination and water temperature (DD = water temperature (C^o) x number of days) (Holm et al., 2014 in Gudding et al., 2014). DD has also become an increasingly popular method for explaining variation in fish growth and development (Chezik et al., 2014). Recently, the body weight and water temperature have been demonstrated to be important in fish immunization (Soto et al., 2014).

The Biological Theory of Relativity (BTR) provides a more sophisticated framework for the study of a range of biological processes in marine ectotherms (Neuheimer 2019). The theory is

proposed to characterise biological phenomena, as a function of the variable interaction between water temperature and time with the biological variability of an organism (size of organism, age of organism, maturity of organism and environmental conditions of organisms' habitat amongst others). For example, in the context of vaccination: the biological process of expression of effective adaptive antiviral immune response following vaccination in grouper would be studied in relation to grouper age, species, size, nutritional status, (biological variations) stocking density, salinity, water quality (environmental variations) and water temperature and time (biological time). The recognition and study of the multitude of interactions between biological time and biological variability allow the identification of the critical factors for a prescribed biological function (Neuheimer 2019). Adoption of the BTR approach increases the ability of researchers to make predictions about biological functions and ultimately allow researchers to manipulate the interaction to achieve a desired biological outcome (Neuheimer 2019). Although using different terms, the BTR approach is similar to the concept proposed by Stentiford et al. (2017) that disease outbreaks in aquaculture are likely the result of a complex interaction between host genetics-environment-pathogen and likely requires a multidisciplinary approach. The Biological Theory of Relativity and the multidisciplinary approach (Stentiford et al., 2017) are recent concepts and there has been no concerted approach to record the range of factors that constitute "biological time" or "biological variation" or multitude of factors that may influence the development of immune competence or initiate VER disease outbreaks in juvenile groupers.

Although age and water temperature are considered important factors influencing the development of adaptive immunity in fish, few of the studies that investigate vaccination against NNV in grouper describe the age of fish or water temperature used in experimental studies (Table 10-5), although fish bodyweight is provided in the description of experimental groupers (Table 10-5). Review of the grouper vaccination studies indicates this project, amongst others, may have serendipitously been conducted across a transitional period of "biological time" required for the attainment of adaptive immune competence in juvenile cultured grouper (Table 10-5). Studies that report greater than 60% survival in groupers following vaccination and challenge, (Table 10-5) (Atujona et al., 2019; Yamashita et al., 2009; Oh et al., 2012 and this study: Chapter 10), involved larger bodyweight juveniles than the fish in less successful studies that report lower survival following vaccination and challenge (Mo et al., 2019 and this study Chapter 8)(Refer to Table 10-5).

Table 10-5: Summary of the age, weight, water temperature (°C) and reported survival of groupers from published studies that have investigated vaccination against pathogens in groupers.

Details of Groupers in the Studies					Reference
Species	Age (dph)	Temperature (°C)	Average body weight (g)	Survival after challenge	
E.coioides	from 1 to 18	dnp	dnp	*80-90%	Lin et al. 2007
E.coioides	dnp	dnp	0.36	no challenge	Lai et al., 2014
E.lanceolatus	dnp	25-30	20	no challenge	Liu et al., 2006
E.malabaricus	dnp	25-30	20	no challenge	Liu et al., 2006
E.lanceolatus	98	24-28	18	47-57%	this study (Chapter 8)
E.septemfasciatus	dnp	28	28	35%	Tanaka et al. 2001
E.coioides	dnp	28	33	17%	Mo et al., 2019
E.coioides	dnp	dnp	32	no challenge	Huang et al., 2014b
E.coioides	dnp	dnp	35	90%	Atujona et al., 2019
E.septemfasciatus	dnp	20	75	60+%	Yamashita et al. 2009
E.septemfasciatus	dnp	20-24	80	100%	Oh et al. 2012
E.lanceolatus	144	24-28	103.5	80-83%	this study (Chapter 10)
**this study reported survival of 44-69% in no-vaccine challenged fish. Virulence of the viral extract is questionable.*					
dnp = detail not provided					

However, within the concept of BTR or host-environment-pathogen model, it is unlikely that bodyweight is the sole factor influencing the effectiveness of vaccination to prevent VER. Water temperature has been demonstrated to affect *in vitro* and *in vivo* replication of RGNNV (Nishizawa et al., 2012). The bodyweight of groupers in the vaccination studies is influenced by other factors such as age, stocking density, nutritional status or other factors that could similarly impact on the ability of the grouper to respond to the vaccination. The slow release of any commercially produced vaccine against VER, despite the significant commercial value and reported successful application in experimental conditions since 2001 (Tanaka et al., 2001), suggests the prevention of VER disease in grouper grow out culture is more complicated than anticipated, and prevention of VER may rely on knowledge beyond vaccination.

In this study, although vaccination led to improved survival in *E.lanceolatus* following challenge with NNV extract, analysis by RT-qPCR assay detected NNV in the surviving and moribund fish. Although survival between the two vaccinated experimental groups was similar (87% and 80%), the detection of RGNNV varied between the two experimental groups. Namely, less fish in the HK-vaccinated group were positive for NNV (20%) compared to the rCP-RGNNV vaccinated group (41%) (Figure 10-5). Further interpretation of the effectiveness of each vaccine to prevent infection or the status of fish concerning RGNNV is not possible based on the results of RT-qPCR analysis. Detection of viral genome by RT-qPCR may be due to the presence of viable viral particles or alternately, be due to the detection of residual degraded

viral genome or non-infectious particles (Gu et al., 2014). Although RT-qPCR is useful for tracking the increase in the detection of viral genome from infection to disease expression, its ability to determine the carrier or recovery status of grouper following exposure to NNV is restricted. Viral isolation in a compatible cell culture system is the definitive proof of the presence or absence of viable viral isolation may have improved (Schnurr 2006). Analysis of tissue samples by virus isolation may have improved interpretation of the RT-qPCR results in this project. Inclusion of tissue analysis by viral isolation has been incorporated into subsequent research that extends beyond this project.

Regardless of the interpretation of the RT-qPCR results, this study demonstrated that increased survival of juvenile groupers, *E.lanceolatus* following experimental challenge with RGNNV viral extract, was markedly improved, with either HK-vaccine (83% survival) or rCP-vaccine (80% survival) antigens compared to placebo vaccinated groupers (7% survival). Survival in vaccinated grouper following NNV challenge was improved in this study compared to the modest survival of 43-53% of vaccinated grouper in the trial described in Chapter 8 that studied smaller bodyweight groupers. This study indicates the significant losses imposed on the grouper aquaculture by the endemic strains of RGNNV present in Northern Queensland may be preventable with vaccination. However, the efficacy of vaccination to protect against VER requires further optimisation, as within the conditions of this study, the efficacy of vaccination was influenced by grouper bodyweight. Although further research is required before commercialisation of the vaccine, the results of this study are very promising and provide confidence to support further investment in vaccine development.

10.5 Conclusion

The following outcomes were described in this chapter:

- Vaccination against RGNNV on larger fish >50g abw displayed improved protection against VER.
- Vaccination of *E.lanceolatus* with a vaccine derived from either recombinant expressed capsid protein or heat-killed cell culture antigen led to increased survival (80-83%)/ reduced morbidity (20-17%) compared to placebo vaccinated control fish (7% survival/ 93% morbidity) after 40 days post challenge.
- Bodyweight, or some factor that is reflected as bodyweight, appears to affect the efficacy of vaccination to prevent VER.

CHAPTER 11. SUMMARY OF FINDINGS AND DIRECTION FOR FUTURE WORK

11.1 Summary of Findings

This work improved understanding of the management of Betanodavirus infections in the culture of grouper in Australia. The project aimed to answer three broad research questions. The following summary presents the discoveries relating to the research questions:

Research Question 1: What strains of Betanodavirus are associated with VER outbreaks of tropical marine fish species in Northern Queensland?

To improve knowledge in the strains of Betanodavirus associated with VER outbreaks the following was investigated

- The species of NNV that were associated with three natural outbreaks of VER in marine aquaculture systems in Northern Queensland were determined
- The level of variation/conservation between strains that have been associated with VER in Northern Queensland and Australia was determined

The complete sequences of mRNA of RNA 1 and RNA 2 were obtained from three disease outbreaks of marine finfish aquaculture ventures in Northern Queensland (collectively referred to as NQAus NNV). The NQAus NNV strains obtained from outbreaks of VER in tropical marine fish species in Northern Queensland were all RGNNV species. Phylogenetic analysis of both the RNA 1 and RNA 2 segments did not detect any chimeric recombination with any other NNV species such as SJNNV or BFNNV. RGNNV continues to be the only species of Betanodavirus detected from Australian fish. Comparison with other Australian-collected RGNNV strains indicated highest homology to the strains collected from tropical marine species rather than temperate or freshwater species.

Comparative analysis with other strains in the NCBI database demonstrated the remarkable conservation of the RGNNV genome, both temporally and geographically. The RNA 1 and RNA 2 segments of the three NQAus NNV strains retained 97-98% homology to the original RGNNV strain isolated from Japan (SgWAk97) in 1997. The high level of conservation of the genome with other RGNNV strains had implications for vaccine design.

Research Question 2: Is qPCR a useful tool to assist in the management of VER in giant Queensland grouper, *Epinephelus lanceolatus* in Australia?

To assess the usefulness of qPCR as a management tool the following topics were investigated:

- The qR2T assay described by Hick & Whittington, which is also the current ANZSDP to detect NNV from grouper was applied to grouper derived tissue.
- The qR1T assay that targets RNA 1, designed by Hick & Whittington to detect NNV, was applied to grouper derived tissue.

Positive amplicons produced from RNA 1 and RNA 2 were cloned to produce standard control plasmids for the qR1T and qR2T RT-qPCR assays described by Hick & Whittington (2010). The RT-qPCRs were applied to trace the viral copy number of RGNNV during the progression of disease in the experimental challenge. Both RT-qPCR assays detected viral genome before the onset of clinical signs at a cycle threshold values ranging from 45 to 22.6. During the period of peak morbidity, viral genome was detected by both RT-qPCR assays with a cycle threshold range of 10.6 to 21.1. Both assays detected NNV genome prior to the onset of VER disease, during peak period of morbidity and also up to 80 days post IM challenge. There were no false negative results obtained from either assay in this investigation when samples were sourced from grouper displaying clinical signs of VER. Both assays detected NNV in every tissue sample derived from fish that displayed several signs of VER. Based on known challenge status, there were no false positives. Neither assay detected NNV in *E.lanceolatus* tissues from any of the PBS- challenged groups that were not exposed to NNV.

RT-qPCR was fit for purpose for application on brain and eye tissues of *E.lanceolatus* in situations that required rapid turnaround, high throughput, and quantitative detection of RGNNV genome such as during a during disease outbreaks or to demonstrate freedom from NNV infection. Both the qR1T and qR2T assays developed by Hick & Whittington (2010) are fit for purpose for the detection of RGNNV genome in eye and brain tissue collected from *E.lanceolatus.*

However, the interpretation of the results of RT-qPCR analysis must consider that detection of genomic material does not indicate viability of virus. As such, the RT-qPCR assay was not useful to assist in determining the nature of protection provided by the vaccines. In this study, vaccinated fish survived and did not display signs of VER. However, viral genome was detected from eye and brain tissues from the vaccinated fish that did not show signs of disease following IM challenge. It is not possible to determine if the viral genome that was detected by

the RT-qPCR assays was viable virus. Consequently, it is not possible to determine if vaccination lead to the production of fish that were immune and not-susceptible to the virus or were sub-clinical carriers with increased tolerance. RT-qPCR is a useful tool to assist in the management of VER in giant grouper. However, it is not the only analysis platform required to understand and improve management of the disease.

Research Question 3: How effective is vaccination or dsRNA designed against the endemic strains of NNV in preventing disease?

Vaccine and dsRNA were prepared that targeted the Ec2NQAus strain of NNV. The efficacy of the therapies to prevent VER were assessed by IM challenge of juvenile *E.lanceolatus* with viral extract.

In an initial trial, the IM delivery of naked dsRNA designed against Ec2NQAus strain of NNV did not reduce severity of morbidity following IM viral challenge and no further investigation into dsRNA was conducted. Although this trial was unsuccessful, a range of variables could be adjusted to improve the potential of dsRNA as a treatment or preventative. A more recent accessing of the IDT dsRNA design tool indicates the anti-NNV dsRNA will cross react with six transcripts from the human genome (Refer to Appendix 3). Notably four of the transcripts are members of the C-type lectin domain family 4 member A (CLEC4A) gene which is involved in the regulation of immune reactivity (uniport.org/uniprot/Q9UMR7) and speculatively, may also be an important component of the grouper immune system. Until the knowledge of the grouper immune system is improved, research seeking to apply dsRNA to prevent VER will be very difficult.

This study demonstrated vaccination is effective in preventing VER following IM challenge with viral extract. The effectiveness of vaccine appears to be affected by fish body weight. In an initial trial, involving fish with average body weight (~18g), the vaccinated groups of *E.lanceolatus* displayed between 43-53% cumulative morbidity after challenge with NNV compared to 88% morbidity in dsRNA exposed groupers. The modest reduction of morbidity by 35-45% indicated the vaccine formulation presented some potential as a preventative measure. There was a trend for vaccinated fish over 50g to have reduced morbidity.

In a subsequent study, *E.lanceolatus* with average body weight over 50g were vaccinated and challenged. The outcome was increased protection against VER in the slightly larger fish with the vaccinated groups experiencing 20-23% morbidity compared to the 93% morbidity in placebo vaccinated groupers.

Additional discoveries

During the term of the project additional research questions were posed as important considerations to achieve the project aims. The additional discoveries are summarised here:

Inclusion of leeches to improve waterborne transmission of RGNNV

Ideally, the efficacy of therapies to prevent VER is determined via a challenge model that is most representative of the natural infection route. Although waterborne transmission of RGNNV is the most likely path of transmission leading to natural VER outbreaks there is limited success applying waterborne transmission to experimental challenges. In a novel approach, waterborne challenge via co-circulation with diseased fish along with co-infection with the marine leech, *Zeylanicobdella arugamensis* was tested as an infection model. During a 40-day trial, despite habitation within a shared recirculation system containing 10 fish that displayed clinical signs of VER following exposure to RGNNV via IM challenge, none of juvenile groupers *E.lanceolatus* exposed/non-exposed to the marine leech *Z.arugamensis* succumbed to a VER disease outbreak. Furthermore, RGNNV was not detected by RT-qPCR from leeches collected from any of the tanks. The inability to induce VER via waterborne challenge despite the addition of leech infestation, lead to the adoption of intramuscular injection of RGNNV extract as a challenge model for all subsequent studies.

Role of parentage in survival to VER?

Recognising that the cohort of fish used in the experimental challenge were produced from a mass spawning event with eight potential parents, the influence of parentage on survival/mortality was investigated by applying Multi-loci sequencing analysis. Parentage did not coincide with improved survival or increased mortality within the studies conducted. Although other genetic factors may be involved, the variance in survival between vaccinated and non-vaccinated fish was not a direct reflection of different parentage between individuals.

Identification and conservation of the functional motifs of Betanodavirus

Understanding the replication characteristics of Betanodavirus may allow targeted approaches to prevent VER. This study completed a review of the functional motifs of the Betanodaviruses, mapped them to a schematic diagram of the mRNA segments and sought to confirm the presence or absence of the motifs in the NQAus NNV strains. Review and confirmation of the multiple motifs across an entire genome have not been reported previously from any strain of Betanodavirus.

The three strains of NQAus NNV obtained in this study retain the functional motifs of the Betanodavirus genome that are critical for viral replication and associated with most virulence. The confirmation of the presence of the essential motifs indicated that outcomes of this research based on the NNV strains obtained would likely be valid for translation to industry applications.

11.2 Future work beyond this study

Improved delivery of viral antigen

Along with previous reports, this project succeeded in demonstrating protective immunity against NNV using either expressed recombinant RGNNV capsid protein or heat-killed RGNNV grown in cell culture as a vaccine antigen. Whilst the results are encouraging, further refinement of the vaccination process is possible. Although vaccination is an effective measure to prevent disease in cultured fish species, the requirement of handling to complete injection, imposes a level of stress on the fish and increased costs to production. A vaccine that could be delivered as an oral antigen would avoid the risk of handling stress. A factor reducing the effectiveness of oral vaccination is the inability to deliver antigen of sufficient quantity and integrity to effectively stimulate immune memory. As the minimal epitope for immune stimulation has been identified to be quite small, the delivery of antigen via recombinant bacteria, virus or algae may provide an optimal vaccination strategy. Some experimental vaccination applying the improved delivery have been reported this year. Gonzalez-Silvera et al. (2019) reported oral vaccination of sea bass using direct delivery of *E.coli* expressing the capsid protein with no protein purification.

Improved understanding of the development of grouper immunity

Perhaps the most pressing issue to improve the management of VER in grouper is to identify the mechanisms of the immune system development in grouper that are involved in response to RGNNV and viruses generally. This would allow a more multidisciplinary and systematic approach to understanding VER expression and likely, other viral diseases. The need to better understand the immune function of aquaculture species is a factor limiting disease management across the multitude of tropical species (FAO 2018). There is an urgent need for research investment into understanding the mechanisms of immune function. Whilst such studies will not lead to production of commercialised products, they will provide the foundational knowledge that can be applied to adopt a more logical approach to improve health and reduce factors that impede fish immunity in aquaculture. For example, improved understanding of immune development may identify changes to husbandry practises or improve diet formulation that increase grouper health.

Investigate impact of environmental parameters on grouper immunity

Although the efficacy of vaccines can be accurately demonstrated in experimental challenge facilities, there are a multitude of other factors in grow out systems that may adversely affect fish immune function and ultimately contribute to VER outbreaks. Considering the metrological and agricultural conditions that dominate the locations prioritised for aquaculture development in Northern Australia, increased understanding of the following should be considered:

- Effect of temperature and salinity on grouper immune function.
- Effect of contamination of marine water ways with agricultural pesticides/herbicides on grouper immune function.
- Refinement of grouper grow out diet formulation to support optimal grouper immune function.

Investigate the roles of microRNA in the replication and management of NNV.

Whilst the presentation of dsRNA constructs provided no protective effect against the development of VER following exposure to RGNNV extract in this experiment, the technology should not be discounted as a tool to better understand NNV. The paradox of NNV is that one of the smallest viral genomes is capable of causing disease in such a range of fish species, with worldwide distribution, across temperate and tropical and marine and freshwater culture systems. Reverting to basic principles for how the Betanodavirus genome achieves such infectivity highlights further paradox in the nature of this virus. Namely why, considering the

requirement for the virus to be very efficient within the constraints of its genome size, would the 403 nt of UTR of RNA 2 (~28% of the RNA 2 strand) that is not translated to produce the viral capsid protein, be retained in the genome if it served no purpose? In the absence of protein translation, a potential role of non-translated genomic sequence is viral and/or host gene regulation. Whilst, I have not been able to investigate this particular topic, I believe it is an important question to raise and the knowledge gained by understanding any potential roles of v-miRNA in Betanodavirus may provide a useful model to improve knowledge in other RNA viruses such as Influenza. The improved efficiency and affordability of next generation sequences techniques provides an unprecedented potential for researchers to investigate the complexities of viral-host interactions.

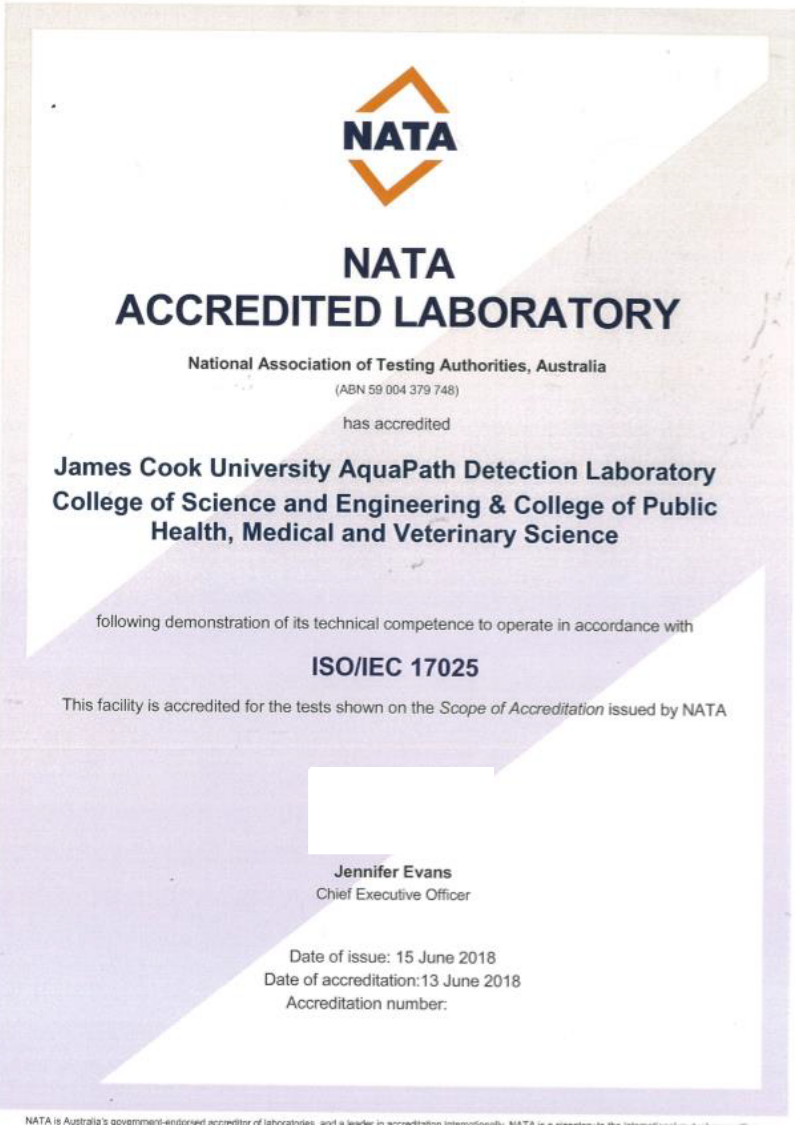
11.3 Implications of findings from this study

Although the viral aetiology of VER in Australian fish species was described by JCU researchers, Dr John Glazebrook and Steve de Beer (1990), research into the disease did not continue at JCU until this present study.

This study has provided a strong basis for future work in the management of VER in grouper and has been applied towards the fast tracking of an NNV vaccine under FRDC project 2018-098: "Vaccination for emergency and long-term control of nodavirus in Australian marine aquaculture". The project aims to apply the demonstrated application of recombinantly expressed capsid protein-vaccine within a commercial field study and acquire data to support both the commercialisation of a vaccine and registration for approval by the Australian Pesticides and Veterinary Medicines Authority.

The protocols to implement RT-qPCRs into a laboratory setting within this research project were audited against the international standard for testing and measurement (ISO 17025) within the field of animal health by the National Association of Testing Authorities (NATA). The laboratory, JCU AquaPATH, attained NATA accreditation in June 2018 at the James Cook University Townsville Campus. The specific scope of accreditation is the "detection of viruses by nucleic acid detection" specifically, the detection of Nervous Necrosis Virus. The facility is one of only three laboratories present in Northern Australia and one of only two universities nationally, that hold this scope of accreditation. The establishment of JCU AquaPATH on the James Cook University Campus provides core capability that will support future research in tropical aquatic animal health at James Cook University.

"From little things, big things grow" (Kelly and Carmody, 1991)



NATA

NATA
ACCREDITED LABORATORY

National Association of Testing Authorities, Australia

(ABN 59 004 379 748)

has accredited

James Cook University AquaPath Detection Laboratory
College of Science and Engineering & College of Public Health, Medical and Veterinary Science

following demonstration of its technical competence to operate in accordance with

ISO/IEC 17025

This facility is accredited for the tests shown on the *Scope of Accreditation* issued by NATA

Jennifer Evans
Chief Executive Officer

Date of issue: 15 June 2018
Date of accreditation: 13 June 2018
Accreditation number:

NATA is Australia's government-endorsed accreditor of laboratories, and a leader in accreditation internationally. NATA is a signatory to the

APPENDIX 1 MEDIA AND BUFFERS

Lysogeny broth (LB)

Component	Mass (grams litre^{-1})
Yeast	5
Tryptone	10
Sodium chloride	5

LB agar

Component	Mass (grams litre^{-1})
Yeast	5
Tryptone	10
Sodium chloride	5
Bacteriological agar	15

Blood agar

Component	Mass (grams litre^{-1})
Enzymatic digest of Casein	7.5
Enzymatic Digest of animal tissue	7.5
Liver digest	2.5
Yeast extract	5
Sodium chloride	5
Bacteriological agar	12g
Sheep's blood	100 mL

Preparation of stock solutions for cloning media

Component	Volume to prepare	Mass	Stock concentration
Isopropyl β-D-1-thiogalactopyranoside **(IPTG)**	5 mL	0.12 g	500 mM
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside **(X-Gal)**	2 mL (in dimethylformamide)	0.1 g	50 mg mL^{-1}

Additions of for LB media and Agar

Component	Stock concentration	Final required	Dilution of stock required
(IPTG)	500 mM	0.5 mM	1000
(X-Gal)	50 mg mL^{-1}	80 ug mL^{-1}	625
Ampicillin a	100000 ug mL^{-1}	100 ug mL^{-1}	1000
Ampicillin b	100000 ug mL^{-1}	50 ug mL^{-1}	2000

a pGEM T Easy; b TOPO TA Cloning

TAE Buffer (50x) (1 Litre)
242 g Tris base
57.1 ml glacial acetic acid
37.2 g $Na_2EDTA{\cdot}2H_2O$ (2 mM) H_2O to 1 litre.
For working solution add 200ml to 9800ml H_2O.

SDS PAGE 10× Tris glycine buffer
10 g SDS
30.3 g Tris
144.1g Glycine
Dissolve ingredient in 800 ml distilled water and adjust volume to 1 litre
Dilute 100ml in adjusted volume to 1 litre with distilled water.

SDS PAGE Silver Fixative
500ml Methanol
50ml Acetic Acid
400ml Distilled water
Mix solutions and adjust volume to 1 litre

SDS PAGE Development Solution
20 g Na_2CO_3
400µl Formalin
800ml^+ Distilled water
Dissolve ingredient in 800 ml distilled water and adjust volume to 1 litre

APPENDIX 2: FAMILY ASSIGNMENT RAW DATA

Counts of Alleles of microsatellite amplicons from primers of Bright et al. (2016) used in MLS analysis to determine parentage of vaccinated fish that displayed (clinical) or did not display (sub-clinical) signs of VER following IM challenged with Ec2NQAus viral extract. (Chapter 9)

					An2				An 25				An 31				An 4			
Group	Ct value	M	F	Sample Name	Allele 1	Allele 2	Size 1	Size 2	Allele 1	Allele 2	Size 1	Size 2	Allele 1	Allele 2	Size 1	Size 2	Allele 1	Allele 2	Size 1	Size 2
					An2	An2	An2	An2	An25	An25	An25	An25	An31	An31	An31	An31	An4	An4	An4	An4
Parent	not tested		F1	KCJCU-001	244	246	243.68	245.85	170	192	169.48	192.32	190	190	189.65	189.65	Failed	Failed	Failed	Failed
Parent	not tested	M1		KCJCU-002	232	246	231.34	245.78	174	192	173.68	192.44	192	202	191.81	202.17	195	201	195.12	200.78
Parent	not tested		F2	KCJCU-003	232	232	231.28	231.28	170	192	169.47	192.36	196	202	195.87	202.04	185	201	184.99	200.47
Parent	not tested		F3	KCJCU-004	226	232	225.07	231.22	168	168	167.35	167.35	190	192	189.64	191.72	195	201	194.75	200.63
Parent	not tested	M2		KCJCU-005	232	246	231.27	245.75	172	184	171.71	184.1	190	200	189.69	200	193	195	192.87	194.78
Parent	not tested		F4	KCJCU-006	232	232	231.31	231.31	170	170	169.47	169.47	192	200	191.72	200	193	193	192.84	192.84
Parent	not tested	M3	F5	KCJCU-007	232	244	231.23	243.64	186	194	186.03	194.3	190	196	189.54	195.73	195	201	194.78	200.63
Sub-clinical	27	M2	F3	KCJCU-008	232	246	231.31	245.79	168	172	167.36	171.59	190	192	189.64	191.72	193	201	192.84	200.63
Sub-clinical	27	M2	F2	KCJCU-009	232	246	231.33	245.76	**184**	192	184.18	192.44	Failed	Failed	Failed	Failed	193	201	192.91	200.47
Sub-clinical	27	M2	F2	KCJCU-010	232	232	231.29	231.29	170	172	169.61	171.56	196	200	195.89	200	185	193	184.91	192.88
Sub-clinical	30	M2	F2	KCJCU-011	232	246	231.44	245.87	170	172	169.72	171.78	200	202	200.15	202.15	185	193	185.13	193.06
Sub-clinical	27	M2	F2	KCJCU-012	232	232	231.39	231.39	170	184	169.72	184.2	196	200	196	200.15	193	201	193.06	200.61
Sub-clinical	30	M2	F4	KCJCU-013	232	232	231.31	231.31	170	184	169.61	184.11	Failed	Failed	Failed	Failed	185	193	184.91	192.88
Clinical	24	M2	F3	KCJCU-014	232	246	231.26	245.87	168	172	167.32	171.53	190	192	189.59	191.68	193	195	192.8	194.88
Clinical	25	M2	F2	KCJCU-015	232	232	231.28	231.28	172	192	171.62	192.32	**200**	202	200	202.05	193	201	192.8	200.47
Clinical	23	M2	F3	KCJCU-016	232	232	231.22	231.22	168	184	167.37	183.94	190	192	189.58	191.67	193	195	192.8	194.72
Clinical	23	M2	F2	KCJCU-017	232	232	231.25	231.25	172	192	171.64	192.32	196	200	195.78	199.84	195	201	194.83	200.47
Clinical	23	M3	F3	KCJCU-018	232	232	231.21	231.21	170	184	169.37	183.97	190	196	189.54	195.73	195	201	194.78	200.47
Clinical	25	M1	F3	KCJCU-019	226	246	225.17	245.79	168	192	167.36	192.2	192	192	191.72	191.72	195	201	194.75	200.63
Clinical	22	M2	F4	KCJCU-020	232	232	231.13	231.13	170	184	169.47	183.86	190	200	189.48	199.84	193	195	192.68	194.75
Clinical	25	M2	F2	KCJCU-021	232	232	231.39	231.39	**184**	192	184.19	192.44	196	200	195.91	200	185	195	184.98	194.97
Clinical	24	M2	F3	KCJCU-022	232	246	231.25	245.8	168	172	167.48	171.52	190	200	189.59	200	193	195	192.91	194.81

Group	Ct value	M	F	Sample Name	An 8				ELMS 7 poor resolution								ELMS 19 poor resolution			
					Allele 1	Allele 2	Size 1	Size 2	Allele 1	Allele 2	Size 1	Size 2	Allele 1	Allele 2	Size 1	Size 2	Allele 1	Allele 2	Size 1	Size 2
Parent	not tested		F1	KCJCU-001	An8	An8	An8	An8	ELMS7	ELMS7	ELMS7	ELMS7	ELMS9	ELMS9	ELMS9	ELMS9	ELMS19	ELMS19	ELMS19	ELMS19
Parent	not tested	M1		KCJCU-002	150	172	150.34	171.56	346	346	346.95	346.95	260	264	260.07	263.87	386	386	385.54	385.54
Parent	not tested		F2	KCJCU-003	144	150	143.81	150.34	345	345	345.38	345.38	254	264	254.63	263.94	382	384	381.8	383.64
Parent	not tested		F3	KCJCU-004	144	144	143.7	143.7	346	346	346.92	346.92	254	260	254.42	260.09	382	386	381.84	385.58
Parent	not tested	M2		KCJCU-005	144	166	143.89	165.89	346	346	346.92	346.92	260	264	260.12	263.92	382	386	381.77	385.52
Parent	not tested		F4	KCJCU-006	142	144	141.64	143.89	345	346	345.48	346.93	260	262	260.08	261.97	386	386	385.57	385.57
Parent	not tested	M3	F5	KCJCU-007	144	144	143.7	143.7	345	345	345.48	345.48	260	260	260.12	260.12	382	386	381.77	385.53
Sub-clinical	27	M2	F3	KCJCU-008	162	166	161.96	165.87	345	346	345.48	346.93	264	264	263.92	263.92	384	386	383.7	385.57
Sub-clinical	27	M2	F2	KCJCU-009	142	166	141.59	165.89	346	346	346.93	346.93	260	262	260.12	262.01	386	386	385.53	385.53
Sub-clinical	27	M2	F2	KCJCU-010	144	144	143.8	143.8	Failed	Failed	Failed	Failed	260	262	260.2	262.09	386	386	385.62	385.62
Sub-clinical	30	M2	F2	KCJCU-011	144	144	143.8	143.8	Failed	Failed	Failed	Failed	254	262	254.47	262	382	386	381.89	385.62
Sub-clinical	27	M2	F2	KCJCU-012	144	144	143.9	143.9	346	346	346.85	346.85	260	260	260.31	260.31	382	386	381.8	385.46
Sub-clinical	30	M2	F4	KCJCU-013	142	144	141.73	143.9	345	346	345.44	346.85	260	262	260.22	262.06	386	386	385.5	385.5
Clinical	24	M2	F3	KCJCU-014	144	144	143.8	143.8	346	346	346.94	346.94	260	262	260.14	262.02	382	386	381.88	385.62
Clinical	25	M2	F2	KCJCU-015	142	144	141.65	143.9	345	346	345.32	346.77	262	264	262.07	263.96	382	386	381.83	385.57
Clinical	23	M2	F3	KCJCU-016	142	144	141.64	143.89	346	346	346.91	346.91	260	262	260.07	261.97	382	386	381.88	385.65
Clinical	23	M2	F2	KCJCU-017	142	144	141.64	143.71	346	346	346.91	346.91	260	262	260.1	262	386	386	385.6	385.6
Clinical	23	M3	F3	KCJCU-018	142	144	141.59	143.62	346	346	346.8	346.8	254	260	254.4	260.19	382	386	381.78	385.51
Clinical	25	M1	F3	KCJCU-019	144	144	143.81	143.81	346	346	346.95	346.95	254	260	254.32	260.1	386	386	385.67	385.67
Clinical	22	M2	F4	KCJCU-020	144	150	143.8	150.17	345	346	345.48	346.77	264	264	263.9	263.9	382	382	381.77	381.77
Clinical	25	M2	F2	KCJCU-021	144	144	143.71	143.71	345	345	345.48	345.48	260	260	260.08	260.08	386	386	385.47	385.47
Clinical	24	M2	F3	KCJCU-022	142	144	141.59	143.62	346	346	346.94	346.94	254	260	254.54	260.17	386	386	385.62	385.62
					142	166	141.59	165.86	345	346	345.5	346.95	260	262	260.2	262.08	382	386	381.87	385.61

APPENDIX 3: UPDATED ANALYSIS OF DSRNA DESIGN BY IDT DESIGN ANALYSIS TOOL.

Updated output from IDT dsRNA design analysis tool. Accessed 29.9.19. Tool indicates dsRNA would target a number of genes of human (A image) and mouse (B image) origin. A lack of suitable knowledge in fish immune gene or microRNA prevents analysis against fish.

APPENDIX 4: PUBLICATIONS AND DISSEMINATION FROM THIS THESIS

Work published as part of this thesis:

Condon K., Bochow S., Ariel E., and Miller T., (2019) Complete sequence of Betanodavirus from Australian barramundi, *Lates calcarifer*. Microbiology Resource Announcements 8. https://doi.org./10.1128/MRA.00081-19

AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology Resource Announcements

GENO

Complete Genome Sequences of *Betanodavirus* from Australian Barramundi (*Lates calcarifer*)

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ABSTRACT The complete RNA-1 and RNA-2 genome sequences of *Betanodavirus* were obtained from Australian barramundi (*Lates calcarifer*). Phylogenetic analyses revealed that the sequences have closest homology to the red spotted grouper nervous necrosis virus (RGNNV) species and share between 91 and 98% homology with the other two published complete/near-complete sequences of isolates from Australian fish.

Members of the genus *Betanodavirus* cause the disease viral nervous necrosis (VNN), which is synonymous with vacuolating/viral encephalopathy and retinopathy (VER) (1). The disease has been reported from wild and cultured freshwater and marine fish from all continents except South America and Antarctica. The National Centre for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov) contains over 1,200 nucleotide accessions of betanodavirus sourced from over 220 fish species and over 30 countries. VNN has been recognized in Australia since 1988, when it first caused significant mortalities in hatchery-reared larval barramundi, *Lates calcarifer* (2). Of the 15 betanodavirus sequences from Australian fish species present in the NCBI database, there are only three complete or near-complete genome sequences (3, 4).

Diseased *L. calcarifer* juveniles were collected from an aquaculture hatchery in Queensland, Australia. The fish displayed mass mortality and clinical signs typical of VNN, including loss of appetite, darkened color, erratic and spiral swimming, and hyperinflation of the swim bladder. Betanodavirus was detected using reverse transcriptase quantitative PCR (RT-qPCR) analysis on eye and brain tissues (3). Further PCR, cloning, and sequence analysis were conducted. Eyes and brains were removed from clinically affected fish, homogenized, and subjected to RNA extraction (Roche High Pure viral nucleic acid extraction), and cDNA was synthesized using random hexamers (Bioline Tetro cDNA synthesis kit). PCR analysis using primers that targeted RNA-1 and RNA-2 were completed (3). PCR amplicons were purified and cloned using the pCR4-TOPO TA vector system. Plasmid DNA was extracted and submitted to Macrogen, Inc., and the Australian Genome Research Facility for analysis by Sanger sequencing. Sequencing data and alignments were analyzed using the default parameters in Geneious v.9.8.1 and the BLAST (5) in NCBI. Overlapping fragments from RNA-1 were aligned to form a contig (3,098 nucleotides [nt]) using Geneious and applying the red spotted grouper nervous necrosis virus (RGNNV) type species as the reference genome. The complete sequence of RNA-2 (1,036 nt) was obtained from direct cloning (3).

The RNA-1 sequence contained the mRNA encoding RNA-dependent RNA polymerase and B1 and B2 protein motifs characteristic of *Betanodavirus* RNA-1 genomes. The RNA-2 sequence contained the complete open reading frame (ORF) encoding the viral coat protein. Alignment of the sequence against type sequences of the four currently recognized *Betanodavirus* taxa indicated that the sequences have highest homology to the RGNNV type species.

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(*Lates calcarifer*). Microbiol
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Proposed publications from this thesis

Condon K, Ariel E and Jerry D (2020) The functional motifs of the Betanodavirus genome.

Condon K, Bochow S, Reynolds A, Knuckey R and Ariel E (2019) Real-time RT-PCR detection of Redspotted Grouper Nervous Necrosis Virus (RGNNV) in experimental challenge of *Epinephelus lanceolatus* following exposure to recombinant vaccine and dsRNA constructs.

Non-Peer Reviewed Additional Outputs of Doctor Studies

Appendix 3.1: The Management of Betanodavirus infections in QLD giant grouper *E.lanceolatus*. Presented to the 3rd Australasian Aquatic Animal Health FRDC Conference Cairns 2017.

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