Vaccination for emergency and long-term control of *Betanodavirus* in Australian marine aquaculture

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Contents

Tables

Figures

Acknowledgments

Abbreviations

AEC	Animal Ethics Committee
AGRF	Australian genome research facility
APVMA	Australian Pesticides and Veterinary Medicines Authority (APVMA)
ANZSDP	Australia/New Zealand Standard Diagnostic Procedures
BGH	Bovine growth hormone
BLASTn	Basic Local Alignment Search Tool (nucleotide)
BLI	Biolayer interferometry
BSA	Bovine serum albumin
BSL	Biosecurity Laboratory, Queensland Dept. Agriculture and Fisheries
cDNA	complementary deoxyribonucleic acid
C_p	Equilibrium oxygen concentration in mg/L
CPE	Cytopathic effect
Ct	RT-qPCR cycle threshold
CV	Coefficient of variation
DAF	Department of Agriculture and Fisheries, Queensland
DAFF	Department of Agriculture, Fisheries and Forestry, Commonwealth
DO	Dissolved oxygen
DpnI	Restriction enzyme DpnI
ELISA	Enyme linked immunosorbent assay
FPLC	Fast protein liquid chromatography
H&E	Haematoxylin and eosin
i.p.	Intraperitoneal
IgM	immunoglobulin M

IM	intramuscular
IMAC	immobilised metal-ion affinity chromatography
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
JCU	James Cook University
kDa	kilo Daltons
LB agar	Lysogeny broth agar (*syn.* Luria-Bertani agar)
MARF	Marine Aquaculture Research Facility, James Cook University
NEWMA	Native, Exotic, Marine and Wild Animals (Animal Ethics Committee)
NBF	Neutral buffered formalin
NBT/BCIP	Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate
NCBI	National Center for Biotechnology Information, USA
ng	nanogram
NNV	Nervous Necrosis Virus
Nt	Nucleotide
NQS	Network quality controls
OD	Optical Density
OIE	World Organization for Animal Health (Office International des Épizooties)
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PEF	Protein Expression Facility, The University of Queensland
pg	picogram
PIT	internal passive identification
pNPP	Para-nitrophenylphosphate
RAS	Recirculating Aquaculture System
RGNNV	Red-spotted Grouper Nervous Necrosis Virus
RPS	Relative percentage survival
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reactions
RT-qPCR	Reverse transcription quantitative real-time PCR
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SJNNV	Striped Jack nervous necrosis virus
SNT	Serum neutralisation test
SSN	Striped snake head
TBK	Terrific Broth with kanamycin

TBST	Tris buffered saline with Tween 20
TCO	The Company One
TSE	Transmissible spongiform encephalopathy
UQ	University of Queensland
VER	Viral Encephalopathy and Retinopathy
VI	Virus isolation
VLP	Virus like particles

1. Executive summary

Rocky Point Aquaculture in southeast Queensland experienced a disease outbreak in cage-reared giant grouper (*Epinephelus lanceolatus*) in late summer and autumn 2018 resulting is severe losses caused by a Betanodavirus. Following a request from the farm owner, Serena Zipf in July 2018, Dr Andrew Barnes from The University of Queensland, designed and built a recombinant protein subunit vaccine against the Betanodavirus and supplied the construct and production outline to Tréidlia BioVet Pty Ltd (Sydney) for manufacture in September 2018. Under direction from Dr Matt Landos and Dr James Fensham (Future Fisheries Veterinary Services) and with permission from Dr Richard Knuckey (General Manager, The Company One), juvenile grouper were vaccinated under a research permit from the Australian Pesticides and Veterinary Medicines Authority (APVMA) at The Company One (TCO) Cairns hatchery in October 2018, and shipped for grow-out at Rocky Point Aquaculture in December 2018, less than 6 months after concept discussion. Vaccination coincided with a reduction in the severity of outbreaks and from mid-2019 to 2022 the farm was free from nodavirus outbreaks. In summer 2022, a persistent low-level outbreak was recorded at the farm in vaccinated fish, although again well below the mortality levels of the 2018 outbreak. In controlled laboratory challenge studies by Dr Kelly Condon at James Cook University, the vaccine has been shown to be >90% effective. Vaccinated juveniles have also been purchased by Noosa Ecomarine and the vaccine has also coincided with substantially reduced mortalities by nodavirus in their recirculating aquaculture facility.

1.1. Background

The grouper aquaculture industry is relatively new to Australia but is growing, with recent new private sector investment in a hatchery in Cairns. A formidable threat to the success of the industry is mass mortalities caused by nervous necrosis virus (NNV) otherwise known as nodavirus, which is the cause of viral encephalopathy and retinopathy (VER) disease. Larvae and fry are typically affected, but large animals can also incur high infection rates. The losses are such that without disease control farming is unviable. NNV is an RNA virus capable of infecting a wide range of fish species and occurs worldwide. In warm marine waters, farmed grouper (*Epinephelus sp*), barramundi (*Lates calcarifer*), European sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) are particularly vulnerable. While strict biosecurity measures can keep hatcheries 'noda-free', open settings in grow-out are inevitably exposed. Moreover, stressors related to husbandry

practices, such as transport, high stocking density, water quality or dietary issues, can be particularly relevant in disease outbreak.

Two vaccines have recently been commercialized in Europe against nodavirus: ALPHA JECT micro®1Noda (Pharmaq) and ICTHIOVAC®VNN (Hipra). Both are formulated as inactivated virus in adjuvant for intra-peritoneal injection of European sea bass of minimum weight 12-15 g. No commercial NNV vaccine has been approved by the Australian Pesticides and Veterinary Medicines Authority (APVMA) for use in Australia.

1.2. Aims/Objectives

In response to the industry's need to control VER outbreaks, we aimed to develop and administer a safe vaccine against nodavirus on a farm where disease management was urgently required. Key considerations were to develop an effective NNV vaccine against the local isolate of the pathogen, but also to enable rapid scale-up and deployment by using a production platform already in commercial use and approved by the regulators, the APVMA, for use in other livestock. The vaccine would be tested in a pilot trial on a fully operational commercial farm, fish health regularly monitored and indicators of vaccine performance assessed.

1.3. Methods

We developed protein subunit vaccine by inserting the NNV capsid protein gene into an established manufacturing platform technology already in use for veterinary vaccines in Australia. The NNV gene sequence was obtained from infected giant grouper brain and eye tissue, collected during a 2018 outbreak on Rocky Point Aquaculture farm, southeast Queensland. The experimental vaccine was deployed under a research-use permit as a cold-formalin inactivated lysate of *E.coli* over-expressing the NNV capsid protein, as a water-in-oil-emulsion in the adjuvant Montanide ISA763A VG and delivered by intraperitoneal injection as a 100 µL dose.

Vaccine production scale-up was carried out by Tréidlia BioVet (Seven Hills, NSW). A safety trial was performed using a double volume dose of vaccine on 100 giant grouper juveniles at TCO hatchery in Cairns. As no deleterious effects were observed, juvenile fish, approximately 20-35 g in weight, were injected with a single dose of 100 µL of the vaccine at TCO hatchery in Cairns. Cohorts of vaccinated and unvaccinated control fish were transported by road to Rocky Point Aquaculture for grow-out.

Monthly sampling was conducted to monitor health and performance of fish as well as the response to vaccination. Blood and tissue samples were taken from representative fish and development of specific antibodies against NNV was determined as well as histology to assess any

abnormal changes that may have occurred. Water quality, stocking, feed, mortalities and incidents, along with harvesting data were supplied by the farm. An aquarium experiment was also set up at the University of Queensland (UQ) to determine the immunogenicity of the vaccine under controlled conditions and to see if immune memory could be established.

1.4.   Results/Key findings

1. **The vaccine was safe with no adverse side effects using a double dose**. In the safety trial no adverse side effects were observed. On farm we noted oil-emulsion associated inclusions in mesenteric fat in vaccinated juveniles. These became small and dark with time but did not adhere to or discolour muscle tissue. By point of sale, they had disintegrated and were not considered relevant by customers in uneaten viscra from the fish. Safety at 200 μL studies have now been conducted with each batch of vaccine since 2019 in accordance with APVMA requirements and no adverse reactions have been detected.

2. **The vaccine raises specific anti-NNV IgM antibodies and immune memory is established**. The raising of specific antibodies in vaccinated fish is a positive indicator of vaccine performance. We found vaccinated fish had higher levels of anti-NNV antibodies than unvaccinated fish especially in the early stages of grow-out. On re-exposure to purified antigen under controlled conditions in the aquarium, vaccinated fish had a significantly higher secondary response after 7 days at 28°C than unvaccinated fish.

3. **Since deployment of the vaccine on farm, the single VER outbreak which occurred was greatly reduced in severity.** There was one VER outbreak on the farm shortly after vaccine deployment, but severity was greatly reduced compared to previous outbreaks in unvaccinated fish. In 2017-18 the cumulative mortality was 86-100% in affected cages and fish were emergency harvested. In 2019 an outbreak occurred in vaccinated fish resulting in 9-20% cumulative mortality, but fish recovered. This outbreak was likely triggered by oxygen stress, as mortalities started during a prolonged period of low dissolved oxygen in the lake. We have shown that multiple factors such as diet and water quality contribute to outbreaks while vaccination is associated with survival. Improved husbandry measures were implemented on advice from the collaborating veterinarian Matt Landos.

4. **No outbreaks have occurred since mid- 2019**. Continuing with vaccination against NNV, the farm has been free of VER for two years to date.

5. **The virus isolated in southeast Queensland was very similar to NNV found in various grouper species in southeast Asia**. Sequencing of the NNV genome and comparison with other isolates

showed the Queensland isolate was highly similar to the virus found in farmed tiger grouper (*Ephinephelus fuscoguttatus*) and hybrid grouper (*E. fuscuguttatus* X *E. lancelotus*) in Asia.

6. **Vaccine dose is critical to protection**. A dose response trial indicated that a minimum dose size of vaccine is required for protection

7. **Duration of immunity is correlated with primary antibody response**. In a preliminary kinetics trial at 28 °C, protection against infection was only sustained for 4 weeks, with no protective effect evident at 6 weeks post-vaccination. It is unknown whether this is due to failure of natural virus to elicit a secondary antibody response, or whether the secondary response is too slow to protect the animals. Further investigation of alternative adjuvants or prime-boost strategies to extend protection is recommended.

1.5.   Implications for relevant stakeholders

The vaccine, in conjunction with improved husbandry measures, has reduced VER disease frequency and severity on farm. It is safe and the formulation meets permit requirements of the APVMA. Vaccine production is relatively cheap and has already been scaled up by a commercial producer. The vaccine in its present form is therefore a candidate for a minor permit to enable its wider use in Australian grouper farms. There may also be interest from southeast Asian grouper farmers, as the virus causing outbreaks in that region is almost identical to that which occurs in Australia.

1.6.   Recommendations

The duration of immunity provided in experimental challenges at the University of Queensland is restricted to the primary antibody response (about 4 weeks at 28 °C). This might be improved with a change of adjuvant or with a prime-boost vaccination strategy to extend the duration for which antibody is present in the serum. As barramundi are also highly susceptible to NNV, the current vaccine formula or a modified version for barramundi could be evaluated. Recombinant protein vaccines can be readily modified for different genotypes of NNV. This is a strength of the chosen vaccine format as the antigen component can be easily changed if deemed immunogenically relevant.

Keywords

Giant grouper, *Epinephelus lanceolatus,* nervous necrosis virus, *Betanodavirus,* subunit vaccine

2. Introduction

2.1. Background

In the wake of the white spot syndrome virus decimation of southeast Queensland's world class tiger prawn industry, innovative farmers turned prawn farm resources to finfish production. Rocky Point Aquaculture diversified and employed their infrastructure resource in the grow-out of giant grouper (*Epinephelus lanceolatus*). However, the transition from prawns was difficult with the occurrence of acute outbreaks of viral encephalopathy and retinopathy (VER) causing very high mortality in fish of 300-800 g, and lower but ongoing mortality in fish up to 1.5 kg. VER outbreaks challenge the economic sustainability of *E.lanceolatus* grow-out culture and control measures were deemed essential.

The virus causing VER disease, *Betanodavirus* or nervous necrosis virus (NNV), is capable of infecting a wide range of fish species worldwide, including grouper (Bandín and Souto 2020). The virus has a bi-segmented RNA genome with the family divided into four genotypes based on the hypervariable region of the capsid protein (Nishizawa et al. 1997), Red Grouper Nervous Necrosis Virus (RGNNV) is the major cause of infection in grouper species, and in barramundi, European sea bass and sea bream. RGNNV is the only genotype reported from Australian VER outbreaks. The virus can be transmitted vertically through the broodstock or shed from infected fish and transmitted through the waterbody, persisting at length without host (Doan et al. 2017).

Generally, VER is considered a disease of fry, and has caused major problems in hatcheries for barramundi in Australia (Hick et al. 2011), sea bass and sea bream in Europe (Toffan et al. 2017) and various species of grouper in Taiwan, Malaysia, Japan and now Australia (Chi et al. 2016). Once fish reach full immunocompetence in later juvenile stages they become more resistant, however, in several fish species larger animals remain vulnerable. In open settings where wild fish and invertebrates can act as reservoirs and there is likely reciprocal transmission between wild and cultured fish (Gomez et al. 2004, Bitchava et al. 2019). Moreover, stressors related to husbandry practices can be particularly relevant in disease outbreak such as transport, high stocking density, water quality or dietary issues (Hick et al. 2011). Elevated water temperature is also linked to the severity of outbreaks, as pathogenicity is greatest at the top of the optimal range for RGNNV replication 25-30 °C (Tanaka et al. 1998, Toffan et al. 2016).

Vaccination has the potential to increase fish resilience to viral infection associated with stress events or sub-optimal rearing conditions. In Japan, an inactivated NNV vaccine (OceanTect VNN, Nisseiken Co. Ltd) has been deployed for decades in seven-band grouper (*Epinephelus septemfasciatus)* before transfer to cages. This has mitigated heavy losses in cage grow-out where fish cannot dive to escape high surface water temperatures in summer (T. Nakai, pers. comm.; (Yamashita et al. 2009). Autogenous vaccines have been used to prevent VER in European aquaculture for many years (e.g. Ridgway Biologics). Since 2018, two vaccines have been commercialized in Europe against RGNNV for use in European sea bass (*Dicentrachus labrax)*: ALPHA JECT micro®1Noda (Pharmaq) and ICTHIOVAC®VNN (Hipra). Both are formulated as inactivated virus in adjuvant for i.p. injection to sea bass of minimum weight 12-15 g.

In Australia, Tréidlia BioVet have an established regulated autogenous vaccine production facility that has been employed successfully in aquaculture here for more than 15 years for a range of diseases. Recently, Tréidlia BioVet have developed a capability in viral culture (for the salmon pilchard orthomyxovirus vaccine) and have established recombinant vaccine production capacity for other animal species. Production of a nodavirus vaccine using established inactivated virus or recombinant technology is therefore feasible. The use of inactivated virus and recombinant NNV vaccines is well-established and in the public domain. However, there is protectable intellectual property (IP) around inactivation methods for killed NNV vaccines, and around the use of peptides that are immunoprotective for specific disease agents. However, use of the full capsid protein is not subject to restriction based on our thorough intellectual property priority searches.

2.2.   Need

The grouper aquaculture industry is new but growing in Australia with recent new private sector investment in a hatchery in Cairns. However, all current grow-out is affected by VER outbreaks which have been both acute and severe. Without a solution the industry will not be able to continue. Fortunately, nodavirus infection is preventable by vaccination and there is local capability to produce a recombinant-type vaccine. Whilst there is currently insufficient demand volume for a vaccine to make a fully privately financed vaccine initiative economic, now that an emergency vaccine under experimental permit has been put in place and a registration data pack developed, it is predicted that the industry will grow to a point where vaccine demand is sufficient for the private sector to finance the transition from experimental vaccine permit to an approved minor use permit. This project is to deliver an emergency vaccine under experimental permit to assist farmers through the 2018 and 2019 grow-out periods. It will build capability in the

vaccination of giant grouper in the tropical fish aquaculture farming industry. Most importantly it will provide the efficacy data, optimisation and formulation data with documentation appropriate for future permits for a vaccine for use in Australia under commercial farming conditions.

3. Objectives

The project was undertaken to achieve the following objectives:

1: An emergency vaccine to prevent nodavirus in the Queensland grouper cohort for stocking in spring/summer 2018

2: A data pack suitable for registration of an optimally formulated nodavirus vaccine for Australia

Changes and additional initiatives are listed in Table 1.

Table 1: Changes in activities

#	Activity	Changes
3	Immunogenicity test (new)	Aquarium antigen challenge experiment performed at UQ to ascertain the immunogenicity of the first vaccine formula, including testing for immune memory and the avidity of anti-NNV antibodies raised in fish.
4	TaqMan qPCR for viral load (new)	Diagnostics for NNV set up in-house at UQ, based on the method recommended by the World Organization for Animal Health (OIE) established by Hick and Whittington 2010.
5	Sequencing of NNV genome from Rocky Point isolate (new)	Both RNA1 and RNA2 were sequenced from NNV isolated from infected grouper at Rocky Point and uploaded to NCBI. This will allow comparison with other NNV isolates from outbreaks in the region and further afield, contributing to the knowledge base on the pathogen, variants and its spread.
6	Protein purification by FPLC (new)	Technique developed in house using fast liquid protein chromatography (FPLC) to obtain purified grouper IgM in order to raise a commercial polyclonal anti-grouper IgM. FPLC also used for purification of NNV capsid protein antigen to be used in ELISA and antigen challenge experiments.
7	Publications (new)	Four publications prepared that are connected to the project: 1) Li et al 2021; Purification and characterization of giant grouper IgM by mass spectrometry and genome sequencing (Published). 2) Li et al; A one-step dip-and-read Biolayer interferometry (BLI) assay to generate IgM real-time quantity and quality data directly from fish serum (Published). 3) Thwaite et al; A longitudinal field survey of the NNV vaccine deployment on farm, demonstrating multiple factors contribute to outbreaks and survival

		(published). 4) Oon et al. Anti-viral innate immune response in NNV infected fish on farm (In revision).
8	Establishment of optimum dose and protection kinetics	A dose response trial and kinetics trial were combined and completed at The University of Queensland. Optimum dose was established, and protection was established within 2 weeks of vaccination. But it was discovered that in challenges, vaccine protection was restricted to the duration of the primary antibody response – About 4 weeks at 28°C.

4. Methods

4.1. Protein subunit vaccine design and production

4.1.1. OGTR Approvals

Cloning of the nucleocapsid protein gene into pET28+, a bacterial vector for expression of N-terminally 6xHis-tagged proteins, and subsequent expression in *Escherichia coli* T7 Express were conducted in physical containment level 2 (PC2) premises certified by the Office of the Gene Technology Regulator (OGTR) at the University of Queensland, Building 60 rooms 327-330, certification number 2099 variation var-10184, expiry March 2025. Procedures were approved by the institutional biosafety committee under exempt dealing number IBC/923/SBS/2015.

4.1.2. RGNNV sequencing

Rocky Point Aquaculture suffered a major VER outbreak in *E. lanceolatus* from late March-May 2018. Five moribund fish ~ 300 g were euthanised in Aqui-S, and the brain was dissected into RNA-later (Ambion), transported on ice, then stored at -20°C. RNA was extracted from brains using RNeasy Mini Kit (Qiagen) following the manufacturer's protocol for tissue. RNA was reverse transcribed with Superscript ViLo (Life Technologies) according to the manufacturer's directions. cDNA was used as a template in specific PCR to amplify the Red-spotted Grouper Nervous Necrosis Virus (RGNNV) RNA2 gene with primers (Forward 22 5'-TCAMAATGGTACGCAARGG-3'; Reverse 23 5'-TCACTGCGCGGAGCTAACGGTAAC-3') and Q5 Hot Start High Fidelity Polymerase Mastermix (NEB). A single amplicon of approximately 1250 base pairs was sequenced with the amplification primers using Big Dye Terminator version 3.1 on a capillary sequencer (AGRF, Brisbane). Sequences were trimmed and assembled in Sequencer V5.2.4 (Gene Codes Corporation). Analysis by BLASTn of the resulting assembly resulted in 99% nucleotide identity with RGNNV from infected seven-band grouper (*Epinephelus septemfasciatus*) in Japan in 2008, GenBank accession number AB373029.1.

4.1.3. Cloning and expression of the capsid protein gene

All media employed were prepared from batches certified as TSE-free and pre-approved by the Australian Pesticides and Veterinary Medicines Authority (APVMA) for use in veterinary products. A recombinant protein vaccine vector was constructed in pET28a(+) based on the RNA2 sequence encoding the capsid of the local *Betanodavirus* strain isolated from *E. lanceolatus*. The viral sequence was first optimised for expression in *E. coli* BL21, synthesized and cloned into pMA-RQ

(AmpR) plasmid using certified TSE free reagents and procedures (GeneArt, Life Technologies). Then a Gibson assembly strategy (Silayeva and Barnes 2017) was designed *in silico* to insert the complete codon-optimised NNV nucleocapsid sequence in-frame directly between the 6xHis tags in the pET28a+ vector, removing the multiple cloning site, thrombin sequence and T7 tag. A stop codon was incorporated prior to the C terminal 6xHis tag, thus the expressed protein was tagged only at the N terminus. Primers are listed in Appendix A, Table A1. Gibson assembly was performed with the NEBuilder HiFi Cloning Kit (NEB), combining the optimised NNV insert (NNVEcoSTOP) and pET28a+ vector (Novagen) at a molar ratio of 2:1, proceeding according to manufacturer's instructions. Post incubation at 50°C for 60 min the assembly mixture was used to transform 5alpha high efficiency chemically competent *E. coli* included in the NEB kit, selecting via kanamycin resistance (50 µg/mL in LB agar). Assembly of the desired vaccine construct was confirmed by sequencing plasmid DNA isolated by miniprep (Qiagen) Appendix A, Table A2. The translated amino acid sequence was analysed by BLASTn and returned a 100% identity with the orange spotted grouper (*Epinephelus coioides*) NNV capsid protein (ALL27039.1) To obtain a vaccine expression strain, 1 ng of plasmid DNA was used to transform 50 µL aliquots of chemically competent T7 Express lysY/Iq *E.coli* (NEB) following the manufacturer's instructions.

Production of the recombinant nodavirus capsid protein from vaccine strain *E.coli* T7Express lysY/Iq pET28a+NNVEcoSTOP was confirmed by western blot. Briefly, single colonies selected on LB+kanamycin agar were cultured in Terrific broth containing 50 µg/mL kanamycin (TBK). Recombinant protein expression was induced with 0.4 mM dioxane-free IPTG (Thermo Fisher) when $OD_{600\,nm}$ reached 0.6 in a total culture volume of 5 mL. Cultures without IPTG were included as induction controls. After a further 2 h growth at 37°C with shaking at 200 rpm, cells were harvested by centrifugation at 3240 x g for 15 min at 4°C (Eppendorf 5810R). Cell pellets were resuspended in 100 µL 10mM Tris/HCl pH 8.0 and frozen overnight to initiate lysis. Once defrosted and vortexed, aliquots of 20 µL were mixed with an equal volume of denaturing buffer (Qiagen Ni-NTA Fast Start kit), incubated on ice for 30 min and prepared for SDS-PAGE (12%). This was followed by western blot with primary antibody anti 6xHis antibody (37-2900, Thermo Fisher) and secondary antibody (goat anti-mouse IgG whole molecule (A3562, Sigma) conjugated to alkaline phosphatase. Detection was via NBT/BCIP liquid phosphatase substrate (Sigma). A band of ~38 kDa was detected in IPTG induced cultures but not in non-induced controls. To confirm the result, a second gel and blot was performed, and probed with mouse anti-nodavirus monoclonal antibody (Mab P09, Aquatic Diagnostics), then secondary antibody and detection as described above. Again, a positive band of 38 kDa was detected in induced but not with non-induced cultures (Fig 1).
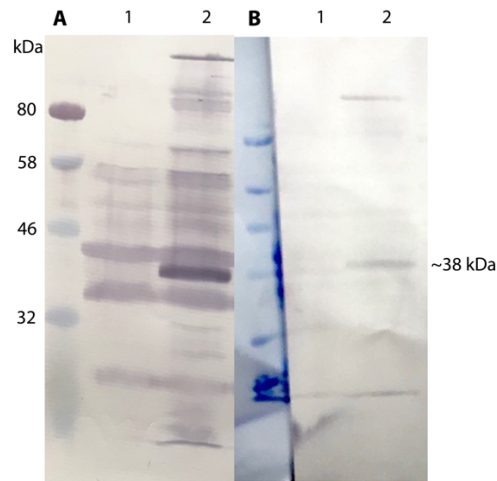
Figure 1. Verification of NNV capsid protein expression in cultured vaccine strain (*E. coli* T7Express lysY/Iq pET28a+NNVEcoSTOP).
Western blots of SDS PAGE (12%) gel of denatured whole cell extracts from control (lane 1) and 0.4 mM IPTG-induced (lane 2) cultures 2h post-induction. Size marker: Pre-stained broad range molecular weight markers (NEB P7712) (A) or unstained broad range markers (NEB) (B) A) Probed with Anti-6xHis monoclonal antibody 37-2900 (Thermo Fisher). B) Probed with mouse anti-nodavirus monoclonal antibody Mab P09 (Aquatic Diagnostics).

4.1.4. Reproducibility and creation of single-passage master seed stocks

Plasmid DNA from the vaccine strain, verified by both sequencing and protein expression in T7Express lysY/Iq to produce the protein of correct molecular mass, bearing the 6xHis tag and cross reacting with anti-nodavirus monoclonal antibody, was chosen as the vaccine construct. Five nanograms of plasmid was used to transform NEB *E. coli* 5alpha for storage and propagation. Glycerol stocks (LB+25% glycerol) were prepared and stored in triplicate in the UQ Aqualab strain collection as QMA0557 in separate, alarmed -80°C freezers. For expression, master seed cultures were prepared by transforming four independent aliquots of 50 µL T7 Express lysY/Iq cells (NEB) with 5 ng of plasmid. A single colony was collected from selective plates derived from 10-fold dilutions of the outgrowth of each transformation and patched onto new selective medium and incubated overnight at 37°C. From these patches, glycerol stocks were prepared as above and stored in triplicate in the collection as QMA0553, QMA0554, QMA0555, QMA0556. The patches were also used to inoculate 1 mL starter cultures to determine reproducibility of transformation/expression and optimise induction time. Starter cultures were grown for 2 h a 37°C with horizontal shaking at 200 rpm. Then, 2% v/v was used to inoculate 50 mL TBK in 250 mL Erlenmeyer flasks. At OD_{600} = 0.6, 2 x 1 mL samples were collected, cells harvested and stored for subsequent analysis. IPTG was added to 0.4mM and the culture continued at 37°C with shaking (200 rpm). Two 1 mL samples were taken every hour for 6 h and the cells collected and stored. After 6 h a 10 µL sample from each culture was analysed by phase contrast microscopy and 2 x 1

mL aliquots along with the entire culture were harvested and cells stored for subsequent analysis. Microscopy revealed refractive spore-like inclusion bodies in around 40%-50% of cells, with daughter cells free from inclusion bodies.

Pre-induction cells and cells collected from 1 h post-induction samples were resuspended in 20 µL of 10 mM Tris buffer pH 8.0. The remaining 1 mL cell samples were suspended in 100 µL 10 mM Tris pH 8.0. A 10 µL aliquot of each suspension was mixed with denaturing buffer (Qiagen), incubated for 30 min on ice, mixed with 5 µL SDS-PAGE reducing denaturing sample buffer, boiled for 6 min, centrifuged at full speed in a benchtop microfuge for 2 min and then 15 µL of supernatant loaded onto four (1 per culture) 12% acrylamide SDS-PAGE gels. Proteins were separated, blotted and gels were stained and blots probed with anti-6xHis antibody as described above. Resulting blots showed rapid and strong induction of the 38 KDa band, along with a potential trimer or complex at around 120 KDa. There was also some background, probably due to incomplete dissolution of inclusion bodies, supported by remnants in the well areas of the gel and an increase in background in later samples from the culture (Figure 2A-D). The independent cultures performed identically with the exception of culture 1, that was marginally slower to reach OD (Figure 2A). This was due, probably, to a slightly smaller colony fragment being used in the starter culture inoculation, and could be eliminated by longer (eg. overnight) growth of the starter culture. However, all four independent transformations induced effectively with high repeatability indicating the robustness of the system. Clones QMA0553, QMA0554, QMA0555 and QMA0556 were streaked from stock on to selective agar. After 4 h incubation at 37 °C, the plates were sealed, double wrapped and sent to Tréidlia BioVet Pty by overnight courier as passage 2 strains for creation of local master seeds by the manufacturer.
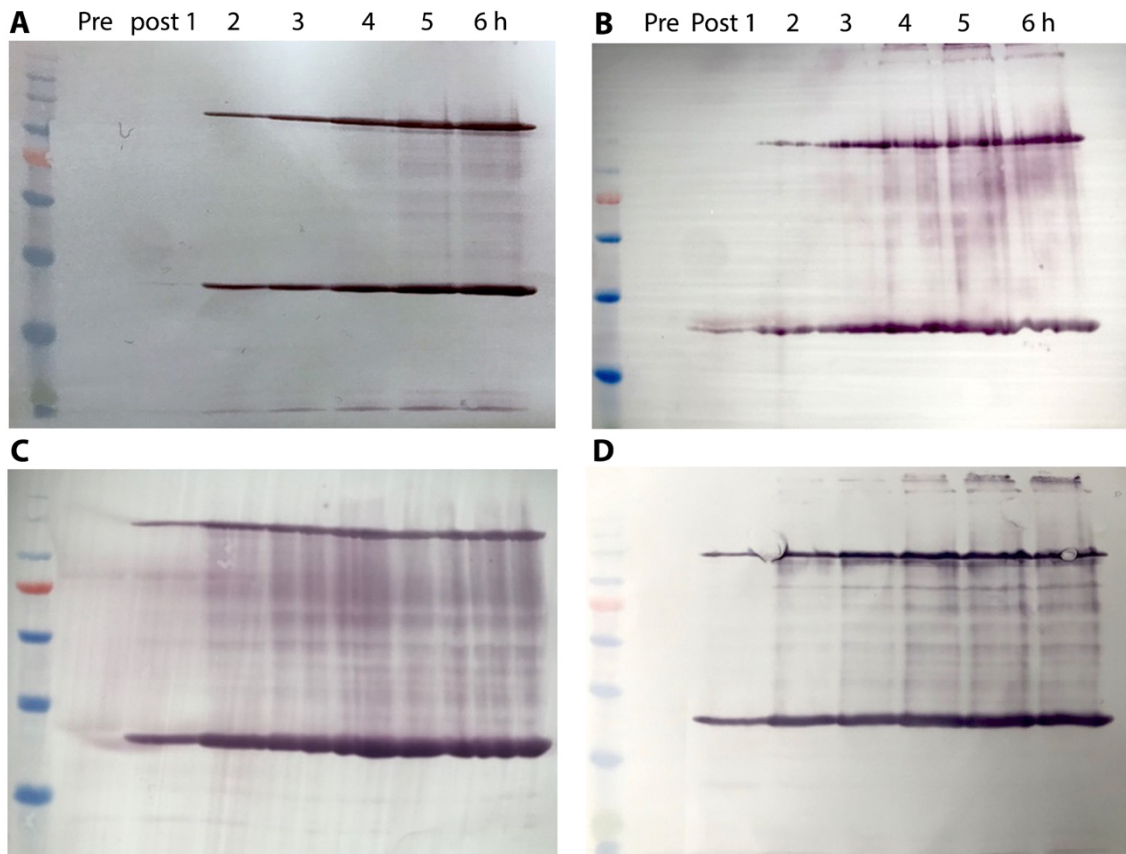
Figure 2. Western blots of SDS-PAGE (12%) gels of denatured whole cell extracts from *E.coli* T7 Express Lyse harvested at different times.
T0 (pre-induction) then 1, 2, 3, 4, 5 and 6 h (lanes 3-8) post-induction with 0.4 mM IPTG-induced TBK cultures of **A**) Clone QMA0553, **B**) Clone QMA0554, **C**) QMA0555 and **D**) QMA0556. Size markers: Pre-stained broad range protein molecular weight markers (NEB P7712). All blots probed with Anti-6xHis monoclonal antibody MA1-21315 (Thermo Fisher).

4.2. Commercial scale-up, emergency/research license application and safety trial

4.2.1. Vaccine formulation and licensing

The recombinant vaccine formulation was based on IPTG-induced TBK cultures of *E.coli* T7Express lysY/Iq pET28a+NNVEcoSTOP, grown at 37°C, lysed by freeze thaw and inactivated with 0.2% v/v formalin at 4°C, prepared as an emulsion with the adjuvant Montanide ISA 763A VG (Seppic, Tall Bennett Group) by Tréidlia BioVet Pty Ltd, Australia.

A research permit application was approved by the Australian Pesticides and Veterinary Medicines Authority (APVMA) for the trials in this report. A minor use permit is under application using the data generated from the trials and farm use documented herein. The recombinant vaccine has been designated by Tréidlia BioVet with the trade name Nodavax.

4.2.2. Safety trial and ethics approval

A safety trial of the vaccine was performed at TCO Cairns' hatchery, with Animal Ethics approval (SA2018/10/659) from the Department of Agriculture and Fisheries, Queensland (DAF) ethics committee, obtained on 17/10/2018. A single 100 µL dose (double the volume of the normal dose) of vaccine was administered by intraperitoneal (i.p.) injection to 100 giant grouper juveniles 20-35 g. No adverse reaction was detected in the test fish over 7 days of observation a nd assessment of gross internal pathology, thus vaccination of the spring cohort, November 2018, was approved by the supervising veterinarian.

4.3. Field application of emergency vaccine in Queensland grouper

4.3.1. Fish and farm setting.

Rocky Point Aquaculture is situated adjacent to an estuarine bay in southeast Queensland. The farm produces giant grouper and cobia, using indoor recirculating aquaculture systems (RAS) for smaller fish and for overwintering, and oudoor grow-out in cages in two connected saltwater lakes. Intake water for the RAS is pumped from an adjacent brackish river source. Prior to entry to the fish system the water is batch chlorinated, dechlorinated and settled. Settled water is pumped through an ozone contactor, solids filtration to 20 micron (µm) and carbon filtration. Within the RAS, UV filters are maintained on the loop and ozone is dosed by contactors. Drum screens perform solids filtration. The lakes that host the grow-out cages were created by sand mining excavation and dredging repurposed prawn ponds. The ponds were originally filled by pumping in marine water from the adjacent estuarine river. Since the completion of the lakes, there has been no further intake pumping of marine water. Inflow water for the lakes is solely via ground water intrusion, with salinity typically fluctuating between 21 and 28 g L^{-1}. The depth is determined by the natural saline groundwater level which rises and falls creating a depth ranging between 4 and 7 metres. A diverse number of endemic wild fish species have colonised the lakes and move freely around the cages. Farmed fish are raised in the lakes from spring to autumn (mid-September to June) during which time water temperatures are favourable for growth, ranging between ~20 – 30°C. For culture, juvenile fish are first raised in the RAS and transferred to the lake. An exception is the first cohort of vaccinated fish transported to the farm (batch 1) which went directly to lake grow-out. Water quality, stocking, feed, mortalities and incidents, and harvesting data were supplied by the farm. Farm commercial operating activities continued as usual throughout the study period, consequently some data are incomplete and husbandry practices changed over the

period of study in response to ongoing findings. Batches of fish vaccinated under the current APVMA research permit are listed in the appendix Table B1.

4.3.2. Vaccination of giant grouper

For batch 1, fifteen thousand fish of 20-35 g were vaccinated i.p. with a single dose of 100 µL fish^{-1} during the week beginning 12th November, 2018 (Figure 3), and transferred to Rocky Point Aquaculture by road in three trips beginning 4th December, three weeks later. A cohort of 5000 unvaccinated siblings were also transferred to the site as controls. In this first cohort, fish were larger when vaccinated (80-170 g), than in most subsequent cohorts. Half of the unvaccinated control cohort were used to stock an earthen pond (pond 6) as controls for 5000 vaccinated fish stocked into pond 9. Vaccinated fish were also distributed amongst three vacant cages in lake 2 (cages 7, 8 and 9). Due to the order in which fish were shipped, and stocking arrangements in cages for both cobia (*Rachycentron canadum*) and grouper, there was insufficient room to stock unvaccinated controls in the lake 2 cages. Consequently, the remaining unvaccinated controls were stocked in lake 1, cage 1. Subsequently i.p. vaccination of juvenile giant grouper continued throughout 2019 and 2020 to date, at a single dose of 100 µL fish^{-1} of typical weight 20-35 g. The batches of control and vaccinated fish were stocked in randomly assigned adjacent cages in the same waterbody, as originally stipulated in the trial design. Subsequently, intraperitoneal (i.p) vaccination of NNV-free juvenile giant grouper continued throughout 2019, 2020 and 2021 to date, at a single dose of 100 µL fish^{-1} of typical weight 20-35 g, with each batch safety tested *a priori* as above. A summary of the cohorts and vaccination is given in Appendix B Table B1.
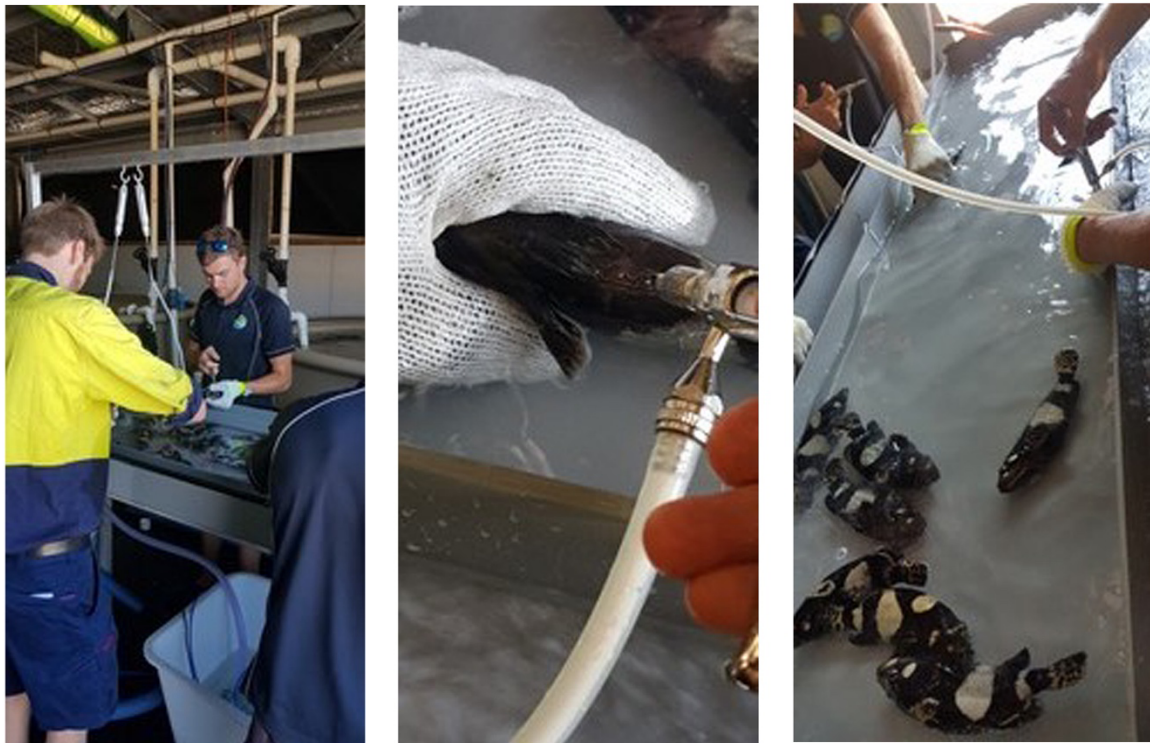
Figure 3. Vaccination of juvenile giant grouper with the NNV vaccine at The Company One, Nov. 2018.

4.3.3. On-site sampling

Animal Ethics approval for field pathology sampling was granted by the University of Queensland NEWMA Animal Ethics Committee under permit number SBS/506/18. Pathology sampling was conducted monthly from January 2019. Six fish were sampled per vaccinated and unvaccinated control group per time: Following euthanasia by overdose of anaesthetic (Aqui-S) under veterinary supervision, fish were weighed and a gill and skin mucus sample were collected on a glass slide for parasite screening by microscopic examination. Blood was then collected by caudal venepuncture into sterile 1.5 mL tubes for serum antibody determination by enzyme-linked immunosorbent assay (ELISA). The fish were then dissected, and the organs inspected visually for gross pathology prior to sampling for histology. Samples of gill (second arch), fore- and hind-gut, caudal kidney, spleen, liver, pancreas/stomach, heart and head kidney were collected in 10% neutral buffered formalin (NBF). The entire brain was removed from the brain case, divided in two and a portion placed with the histology samples in NBF while the other half was stored in 350 μL RNAlater for analysis by RT-PCR. Bloods were allowed to clot at 4°C overnight and then sera were collected by centrifugation at 10,000 x g for 5 min and stored frozen for detection of anti-nodavirus antibodies by ELISA. Fixed samples were transferred to 70% ethanol after 24h fixation in NBF. Brain samples

in RNAlater were stored at -20°C. A spreadsheet record of all fish sampled from December 2018 to July 2021 is available on the UQ Research Data Manager.

4.3.4. Histology

Fixed tissue was cut into cassettes and then processed overnight in a Miles Scientific Tissue Tek VIP1000 through a series of dehydrating and clearing agent baths comprising of an ethanol gradient (70-100%) and xylene (Hurst Scientific). After embedding in paraffin, 5 μm sections were cut, mounted onto slides, tained with Hematoxylin and Eosin (H&E) stain or Periodic acid-Schiff (PAS) stain and cover-slipped. Images were captured using an Olympus DP-22 SAL camera fitted to an Olympus BX43 microscope .

4.4.   Detection by ELISA of specific anti-NNV antibodies from on-farm samples

4.4.1. Recombinant protein production for ELISA

Soluble capsid protein was prepared from the recombinant vaccine construct (see section 4.8) to coat ELISA plates with antigen. Briefly, *E. coli* T7Express lysY/Iq pET28a+NNVEco was grown in Terrific broth at 37°C until OD_{600} reached 0.6, then protein expression was induced with IPTG at 0.4mM and culturing continued at 16°C overnight to favour soluble protein production. Expression of the 38 kDa NNV capsid protein was confirmed by Western blot using an anti-nodavirus monoclonal antibody (Mab P09, Aquatic Diagnostics). For ELISA, highly purified capsid protein was required to avoid detection of potential cross-reactive antibodies produced in the fish against *E. coli* components in the vaccine formulation. Purification of the protein was performed by fast protein liquid chromatography (FPLC), using the ÄKTAxpress (GE Healthcare), with IMAC purification followed by size exclusion chromatography (SEC) at the Protein Expression Facility (PEF), University of Queensland. Purified NNV capsid protein fractions were pooled for ELISAs (yield 2.47 mg from 1 litre of bacterial culture).

4.4.2. Indirect ELISA

An indirect ELISA was developed and optimised to detect anti NNV capsid antibodies in grouper serum, and then used to assay the serum antibody response through time in batches 1 and 3. Briefly, 96 well microlon® high binding plates (Greiner) were coated with 1 μg well[-1] of purified soluble NNV coat protein in 50 μl well[-1] carbonate bicarbonate buffer pH 9.6 (Sigma C3041), overnight at 4 °C. Washes between steps were performed in triplicate with TBST (20mM Tris, 0.14 M NaCl, 0.003M KCl, pH 7.4 with 0.05% v/v Tween 20). Blocking was done with TBST + 2% BSA

(Sigma) for 1.5 h at room temperature. All remaining incubation steps were done at 28 °C for 1 h, followed by washes. Serial 1:2 serum dilutions and antibody dilutions were prepared in TBST. Antibodies were anti-barramundi IgM polyclonal raised in sheep (provided by Prof. R. Whittington, University of Sydney) at 1:5000 and then alkaline phosphatase conjugated anti-sheep IgG (A5187, Sigma) at 1:20000. Final washes were performed in duplicate, first in TBST and then in TBS. Detection was via enzymatic reaction with *p*-nitrophenyl phosphate substrate (Sigma) at 28 °C, with absorbance at 405 nm measured on a BMG FLUOstar OPTIMA microplate reader every 10 mins for 70 minutes. A pool of sera from high responders and a low "naïve sera" control from young (120 days old) unvaccinated fish were used to determine signal consistency between plates. A baseline naïve sera absorbance was established across plates for each batch as the mean +3 standard deviations (SD) from all naïve sera readings at a 1:32 dilution. Specific antibody titre was defined as the inverse of the greatest dilution which gave a signal higher than the naïve baseline.

4.5. Indicators of vaccine performance

In addition to monitoring fish health and survival during farm deployment of the vaccine, a number of molecular analyses were performed related to vaccine performance. Experiments were conducted under approval by the University of Queensland Native Exotic Marine and Wild Animals (NEWMA) committee, permit number SBS/506/2018 and subsequent amendments, in the approved aquatic animal holding facility at UQ Ritchie Building 64A rooms 103-105.

4.5.1. Determination of vaccine-induced immune memory by prime-antigen challenge.
To determine whether immune memory was elicited by the vaccine, a prime-challenge experiment was conducted using commercially formulated vaccine (with adjuvant) and vaccine antigen (no adjuvant) in a full factorial design. Vaccination with the commercial vaccine was performed at the hatchery and vaccinated and unvaccinated sibling fish were transported to the experimental facility at the University of Queensland 90 days post-vaccination. The grouper were acclimatised for 7 days at 28 °C in a 1,800 L RAS. The fish were fed 5% of body weight per day with a commercial 3 mm extruded diet. Fish were anaesthetised with Aqui-S and weighed (average 85.6 g). Six vaccinated and six unvaccinated fish were injected i.p. with 200 µl recombinant NNV antigen. This was the cultured vaccine recombinant *E. coli* strain induced with IPTG for 6 h, frozen and then deactivated with 0.2% v/v active formalin and administered without adjuvant, to elicit a secondary response in the pre-vaccinated fish. A further six vaccinated and six unvaccinated fish were injected with 200 µl PBS as unchallenged controls. Seven days after the injection, all 24 fish were euthanised with an overdose of Aqui-S and bled from the caudal vein. Blood samples were

kept at 4 °C overnight to allow clotting and sera collected the next day by centrifugation (3800 x g, 5 mins). Serum samples were stored at -20 °C for subsequent analysis.

4.5.2. *Detection of primary and secondary antibody responses and avidity*

NNV-specific antibody in each treatment group was detected by indirect ELISA as described in section 4.4.2. Chaotrope elution ELISA was performed to measure the avidity of the specific antibodies. The ELISA protocol (section 4.4.2) was followed with the following modifications: Each fish serum sample was added into five sets of triplicate wells on the ELISA plates. After the fish serum incubation and washing steps, 0, 0.05, 0.1, 0.3, and 0.5 g mL^{-1} NH$_4$SCN (in TBST, pH 6) was added separately to the triplicate wells of each serum sample (50 µL well^{-1}) for exactly 15 mins at room temperature. The plates were washed three times with TBST. A secondary polyclonal antibody, rabbit antiserum raised against giant grouper IgM antigen purified via protein A affinity chromatography (Li et al. 2021) (1:8000 in TBST), was added to the plates (100 µL well^{-1}) for 1 h at room temperature. Tertiary antibody (1:15000 in TBST), goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma, A9919), was then added to the plates (100 µL well^{-1}). Alkaline phosphatase yellow (pNPP) liquid substrate system (50 µL well^{-1}, Sigma) was added to develop absorbances. The OD was plotted against the chaotrope concentrations per sample and the resulting curve was fitted with a log-decay model using Prism 9 (GraphPad). The concentration of ammonium thiocyanate (NH$_4$SCN) required to reduce 50% of the original OD (0 g mL^{-1} of NH$_4$SCN) was calculated from the model and was defined as the ELISA avidity index.

4.6. Diagnostics: viral load assay

4.6.1. *Choice of genomic RNA target and detection method*

The World Organization for Animal Health (OIE) Manual of Diagnostic Tests for Aquatic Animals, 2019 provides a number of options for the diagnosis of viral encephalopathy and retinopathy (VER) (OIE 2022). However, to diagnose both clinically and sub-clinically infected fish reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) to detect NNV viral genomes is necessary. Of the options provided, we used the method which is prescribed in the Australia/New Zealand Standard Diagnostic Protocols (ANZSDP) to detect the RGNNV RNA2 via TaqMan RT- qPCR (Hick and Whittington 2010) .The assay, termed, qR2T was designed based on a RGNNV RNA2 sequence isolated from infected barramundi in northern Australia (isolate LcNNV_09/07, NCBI GenBank (GQ402011.1). The sequences for the hydrolysis probe, and sense and anti-sense primers were present with 100% identity to the sequence of RGNNV RNA2 from grouper in the Rocky Point VER outbreak in May 2018 (MW590702).

4.6.2. Construction of plasmid for standards

A 698 bp fragment of the RNA2 genome segment, encompassing the genome target region of the qR2T assay, was amplified, gel purified and cloned into plasmid pcDNA3.1. All PCR reactions were performed with Q5 hot start High-Fidelity 2x Master Mix (NEB) according to the manufacturer's instructions. The purified amplicon was used as template for PCR to incorporate overhangs that were complementary to the pCDNA3.1 plasmid sequence flanking the multiple cloning site.

A previously constructed plasmid pCDNA3.1_MCherry vector, conferring purple coloration of *E. coli* colonies, was used as a template to amplify the pCDNA3.1 backbone for cloning (excluding MCherry sequence) (Oleksandra Rudenko unpublished). The vector DNA was amplified with primers complementary to the overhang sequence. All primers are listed in Appendix C Table C1. Post amplification, the PCR reaction mix was treated with DpnI restriction enzyme to cleave circular plasmid DNA according to manufacturer's instructions (Thermo Fisher Scientific ref ER1705), followed by column clean up (IBI Gel/PCR DNA fragments Extraction kit). Ligation of the vector to the NNV2 amplicon was performed using NEBuilder HiFi DNA Assembly Cloning Kit (E5520S), aiming for a molar ratio of vector: insert between 1:3--1:5. The assembly reaction was performed at 50°C for 55 min. The ligation mix was used to transform competent cells (NEB@ 5-alpha). Ampicillin resistant clones containing the NNV insert were distinguished from clones transformed with plasmid pCDNA3.1-SD-MCherry by the loss of purple coloration. Six candidate clones were tested for the presence of the insert by colony PCR using the T7 forward and BGH (Bovine growth hormone) reverse universal primers which flank the insertion site. PCR reactions were performed with OneTaq Quick-load 2x Master Mix (NEB M0486S) using cycling conditions according to the manufacturer. All colonies had the same amplicon at the expected size ~ 750 bp as single bright bands. Plasmid DNA was also Sanger sequenced (AGRF, Brisbane), to confirm achievement of the desired construct, which we call NNVRNA2_pCDNA3.1. We have stored glycerol stocks in our strain collection, coded QMA0644.

4.6.3. RNA extraction from on-farm fish samples

Samples were collected from farm grow-out fish from batch 1 (2018-19) and batch 3 (2019-20) were analysed since outbreaks of VER had occurred during the grow-out of the former and both cohorts were in the lake over the summer season. Homogenization of grouper brains stored in RNAlater at -20°C was performed in a 2 cm tube by milling on a Magnalyzer for 90 sec at 7000 rpm with 100-130 mg of sterilized acid washed glass beads (Sigma G1145 size 150-121 µM). RNA was extracted using Qiagen RNeasy Plus mini-kit following the manufacturer's instructions. RNA was eluted in 30-50 µl RNase free water and quantified on a Qubit fluorometer with hsRNA reagents

(Thermo Fisher). A typical yield was 5 µg of total RNA obtained from ~17 mg of brain tissue with a final concentration of 168 ng/µl. Several samples were checked for purity on a NanoPhotometer (Implen) via the A260/280 and A260/230 ratio. Results for A260/280 were 2.00-2.20, evidence of satisfactory purity RNA with respect to DNA contamination; A260/230 was typically 1.70-2.00, with occasional lower ratios.

4.6.4. TaqMan RT-qPCR

RT-qPCR analysis was conducted using a QuantStudio6 (Applied Biosystems), following the fast-cycling reaction recommended by the manufacturer for the Fast Virus 1-Step Master Mix, viz. Reverse transcription 50°C, 5 min; RT inactivation/initial denaturation 95°C, 20 s; 40 cycles: denature 95°C, 3 s; anneal extend 60°C, 30 s. For the grouper brain samples from the field trials, 50 ng of total RNA was used per reaction in duplicate. RNA from the brains of two infected fish from the January 2019 outbreak were used as positive controls. As negative controls, brains from small fish in the RAS were used (sampling S19, 30.6.20, average weight 77g). Controls performed as expected for all 5 runs of analysis.

To obtain a standard curve for quantification, using the number of base pairs in NNVRNA2_pCDNA3.1, the number of plasmid copies was computed and related to quantity in nanograms using the URI genomics and sequencing centre calculator http://cels.uri.edu/gsc/cndna.html. Amounts of plasmid A serial dilution from (10^8 to 10^1) of known plasmid copies were analysedto obtain a standard curve, plotting log of copy number versus Ct values.

4.7. Sequencing of NNV genome from the Rocky Point isolate

NNV RNA1 and RNA2 genome was sequenced from o*E. lanceolatus* brain and eye tissue collected during the VER outbreak March-May 2018. RNA extraction was as described in section 5.3. cDNA was synthesized with QuantiTect® Reverse Transcription kit (Qiagen) following the manufacturer's quick start protocol, using random primers (0.25 µM) provided in the kit and specific primers at the 5' and 3' end (0.5 µM forward and 0.5 µM reverse) for each genome segment, RNA1 and RNA2. Routine PCR reactions were performed to amplify DNA usingQ5 Hot Start High-Fidelity 2x Master mix (NEB) following the manufacturer's instructions using annealing temperatures according to the NEB Tm calculator for each primer pair. Bands were column or gel purified using the Gel/PCR DNA Fragments Extraction Kit (IBI), following the manufacturer's instructions, and quantified on Qubit (BrDNA). Primers used for genome sequencing are provided in Appendix C Table C1 for RNA2 and Table C2 for RNA1. Sanger sequencing was performed at the

Australian Genome Research Facility (Brisbane node). Sequence data were quality-checked, trimmed and assembled using Geneious Prime 2020.1.1

4.8. Protein purification by Fast Protein Liquid Chromatography

4.8.1. NNV capsid protein purification by FPLC

To coat ELISA plates with NNV antigen, we initially produced the NNV capsid protein from clone QMA0553 (6-His-NNV-C) and purification was done by the Protein Expression Facility (PEF) at UQ. We then acquired an ÄKTA start system (GE Biosciences, Uppsala, Sweden) to perform liquid chromatography in-house. Recombinant 6-His-NNV-C was cultured in Terrific broth with kanamycin 50 µg mL^{-1} at 37 °C until the OD$_{600}$ reached 0.6, then protein expression was induced with IPTG at 0.4 mM and culturing continued at 16°C overnight to favour soluble protein production. The culture was centrifuged on an Eppendorf 5810R at 3200 g for 40 min at 4°C. The pellet, 2.4 g wet weight, corresponding to ~250 mL of culture, was sonicated (Branson 250) on ice in 12 mL of lysis buffer (20 mM sodium phosphate, 500 mM NaCl, 20mM imidazole, 1% Triton x-100, 2mM MgCl$_2$, pH 8.0) plus 5 µl benzonase (25 U µl^{-1}) and 125 µl *E. coli* protease inhibitor cocktail (Sigma 8465) as follows: 5 min at 20% amplitude, 0.5 s ON/0.5 s OFF, 5 min cool down and 5 min repeat. The mixture was centrifuged in an Eppendorf 5810R, 4°C at 12000 x *g*, for 20 min, the supernatant filtered (0.45 µM) and degassed under vacuum.

A 5 mL HiTrap IMAC HP Sepharose (GE Healthcare) column, loaded with 0.1M NiSO$_4$ was equilibrated with wash/binding buffer (20 M sodium phosphate, 500 mM NaCl, 20 mM imidazole pH 8, at room temperature (18-20°C) on the ÄKTA start. Samples were loaded at a flow rate of 2 mL/min and washed in 20 column volumes of wash/binding buffer. Bound protein was eluted in a 2-step gradient with elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 8, RT) as follows: Step 1. 30 % elution buffer at flow rate 4 mL min^{-1} until peak 1 resolved. Step 2. 100 % elution buffer until peak 2 resolved. Two mL fractions were collected from flow-through and throughout elution (Appendix C Fig C1). 15 µl of a selection of fractions were run on 12% SDS-PAGE gels to identify those with the most concentrated and purified NNV capsid protein (~38 kDa monomer and ~ 120 kDa trimer). Fractions 17 to 21 from peak 2 were pooled and dialysed in a 10000 MWCO Slide-A-Lyser 10K dialysis cassette G2 (Thermo scientific) against 20 mM Tris, 200mM NaCl pH 8.0 at 4 °C, overnight with stirring. Post dialysis, to enhance solubility, an equal volume of buffer was added to the dialysed sample. Representative samples from the IMAC purification and dialysis were run on a 12% SDS-PAGE gel (Appendix C Fig C2). Protein was

quantified on QUBIT: 370 ng μ^{-1}, total yield 6.48 mg from 250 mL of culture; and stored in aliquots at -80 °C.

4.8.2. Purification and characterization of giant grouper IgM

Giant grouper IgM was purified by Protein A affinity chromatography on the in-house FPLC system. This was to obtain purified immunoglobulin to inject into rabbits in order to raise high affinity anti-IgM polyclonal antibodies, with potential to detect a broad range of IgM epitopes for high throughput methods such as ELISA. During the process, giant grouper IgM was also characterized by mass spectrometry and genome sequencing. This work has been published (see "Project Materials Developed" section), and a full description of the methods is given (Li et al. 2021). The paper can be found via the publisher using the document identifier https://doi.org/10.1016/j.fsi.2021.03.014, but Elsevier copyright conditions prevent a full text from being included with this report. Full text can, however, be obtained legally and free of charge by emailing the author at a.barnes@uq.edu.au

4.9.   Laboratory vaccination trial at JCU: Antigen comparison

4.9.1.   Quantification of NNV at JCU

NNV was detected and quantified by RT-qPCR analysis using the ANZSDP-Betanodavirus (Moody and Crane 2012). The assay targets the RNA-2 strand, mRNA for the capsid protein (Moody and Crane 2012). The ANZSDP is the method validated by Hick and Whittington (2009).   Although the assay had been previously validated on barramundi, *Lates calcarifer*, the assay has been validated within JCU AquaPATH for application to *E. lanceolatus* sourced tissues within the JCU NATA scope of accreditation protocols (data not shown). During this project JCU AquaPATH participated with satisfactory results for all of the Australian laboratory proficiency testing program for aquatic animal diseases (Department of Agriculture, Fisheries and Forestry) for the detection of NNV.

Table 2. Description of the primer and probe sequences used in the qPCR. Including qPCR format, primer name, primer nt sequence, RNA segment target, nt binding position and melt temperature of primer and probe.

PCR format	Primer Name	Sequence (5'-3')	RNA segment	Position~	Tm (°C)
RT-qPCR	qR2T-F	CTT CCT GCC TGA TCC AAC TG	2	401	62
	qR2T-R	GTT CTG CTT TCC CAC CAT TTG	2	476	61
	qR2T-Probe	CAA CGA CTG CAC CAC GAG TTG	2	454	

Source of all primer and probe sequences Hick &Whittington (2019). Position is with reference to NCBI records GQ904199 (RNA 2).

Analysis to detect RGNNV RNA-2 was conducted using the primer and probes described in Table 2. Analysis of eye and brain, wherever possible for the same fish, were conducted on the same

analysis run. Analysis was conducted using a Quanti-Studio-5 real-time PCR machine using a 384 well plate. Cycling conditions consisted of: 45°C for 10mins, followed by 85°C for 5 min; 95°C for 3minutes followed by 40 cycles of 95°C for 15s and 60°C for 30s.

The qPCR amplification results were downloaded in eds format and analysed using the QuantStudio Design and Analysis software v 1.5.1. Cycle threshold values were generated using the relative threshold option with a 0.05 setting. Only samples with efficient amplification curves were accepted within the results analysis. The results of analysis were downloaded in Excel format and transferred to experimental charting files. The Ct value of Network Quality Controls (NQC) were within +/- 1 Ct of the accepted NQC Ct. Across all analysis runs spanning this investigation, the NQC was detected with an average Ct value of 25.24 (sd 0.39; n=36 runs).

4.9.2. Preparation of NNV challenge extract.

Viral extract was prepared from *E.coioides* tissue collected from a farm VER disease outbreak. The affected fish, of average body mass of ~600 g were cultured in cages in commercial earth ponds in north Queensland. Fish were confirmed to be suffering VER by Biosecurity Queensland (*pers communication from farmer*). 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. Viral extract was confirmed to be negative for Infectious spleen and kidney virus by qPCR (Mohr et al. 2015). Separate filtered supernatants from each tissue, herein termed RGNNV viral extract, was stored in sterile 50 mL centrifuge tubes at -20°C. The viral sequence had been obtained during the PhD studies of Condon (2019). The RNA 1 and RNA 2 gene segments displayed 97-99% nucleotide homology with the RGNNV sequences in the NCBI database. The sequence encoded for the amino acid serotype C determinant motif PDG described for RGNNV. In previous experimental challenges (Condon,2019), the viral extract diluted 1:1000 in sterile PBS had an with an approximate copy

number of 6.36×10^4 mL^{-1} and induced morbidity with clinical signs of VER in approximately 8-12 days post challenge.

4.9.3. Animal ethics and biosecurity statement

Experiments were conducted under Animal Ethics Approval Number A2709. AQIS controlled materials (biologicals), including cell lines and some components of culture media were used in accordance with the biosecurity pathway proscribed in the import permit *viz*: For use *in vitro* and *in vivo* in laboratory animals only. No such materials were used in the vaccination of fish for grow-out on farm. All laboratory animals were destroyed at the end of the experiments and disposed of via the clinical waste stream in line with the biosecurity pathway documented in the import permits.

4.9.4. Animal husbandry

For experimental challenges, 98-day old juvenile *E. lanceolatus*, (average weight 47.1g) were provided by TCO. On receipt, five fish were euthanised by overdose with Aqui-S for analysis to confirm the absence of RGNNV by RT-qPCR. Fish were held under the conditions approved within the JCU institutional animal ethics permits and fed twice daily *ad libitum* on commercial fish feed pellet appropriate to the size of the fish (Otohime, BM Aqua). All RAS at JCU were supplied with saltwater obtained from the Australian Institute of Marine Science. Water was collected from the ocean and subjected to five treatments which included 5mm screen within a high-density polyethylene basket; a TIMEX hydrocyclone 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 28 to 32 g L^{-1}. Fish were distributed approximately 80-85 per tank into 6 x 1000L tanks that were held on Aqua system 1 in the Marine Aquaculture Research Facility (MARF). There was only 1 incidental mortality observed in the experimental fish cohort prior to the RGNNV challenge. There was no RGNNV detected in the incidental mortality.

4.9.5. Preparation of vaccine formulations for challenge trial

In preparation for the aquarium challenge trial at JCU, four vaccine formulations were prepared and tow adjuvant controls. Two different antigens were to be used: the current recombinant NNV-C as *E. coli* lysate (Nodavax), already being used in farm trials, and an in-house antigen from JCU. In addition, two adjuvants from Seppic were tested: Montanide ISA 763A VG (in the Nodavax formulation) and Montanide ISA 660 VG, which is a faster release adjuvant than the former. For simplicity, these are referred to as Adj 7 and Adj 6 respectively. Adj 7 requires a 30:70 mix antigen:adjuvant, vol:vol; Adj 6 requires 40:60. Tréidlia BioVet provided the NNV vaccine Nodavax

(recombinant protein formulated with adj 7) and antigen NNV-004-02 (the lysate containing RGNNV recombinant protein)t o prepare emulsions with the alternative adjuvant (Adj 6). Six preparations were formulated (Table 3), vortexed, then passed through a 23-gauge needle 18 times to emulsify. Emulsions were held for 7 days at 4°C and examined for emulsion separation. All emulsion preparations remained stable. Final preparations were stored at 4°C and shipped to JCU. The table refers to quantities for a final volume of 10 mL

For preparation of the JCU antigen, a frozen 1.5ml aliquot of RGNNV cell culture, previously prepared (Condon 2019) was retrieved from liquid nitrogen storage. The cell culture was a second-generation passage from the viral extract prepared from *E. coioides* (orange spotted grouper) tissue homogenates collected during an outbreak of disease in Queensland. The cell culture was prepared as described by Condon (2019). Briefly, heat-inactivated 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 following the approach of Iwamoto et al. (1999 and 2000). One ml of the RGNNV extract was inoculated onto two confluent monolayers of SSN-E11 cells 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. The Ct value of the pooled cell culture was 14.69 (calculated viral copy number of 2.1 $x10^9$ ml^{-1}) using the RT-qPCR protocols described in 4.9.1 (Condon 2019).

For the current study, 10 mL of the frozen culture supernatant was aseptically removed from the frozen RGNNV tissue culture supernatant. The viral culture supernatant was subjected to 30 min incubation at 54°C in a water bath (Labnet) to deactivate the virus (Frerichs et al., 2000). Vaccine was prepared by mixing deactivated viral culture each adjuvant, Adj 6 and Adj 7 at the ratios provided in Table 2, according to the manufacturer's instructions (Seppic). Adjuvant and vaccine antigen was emulsified by repeated rapid intake and expulsion into a 1 ml syringe in a 10 ml sterile tube. Vaccine was considered mixed when adjuvant and antigen appeared as a white emulsified solution that did not separate when left at 2-4°C for approximately 2 days (JCU) prior to use and Tréidlia BioVet, NNV subunit vaccine Nodavax (4.*2.1 Vaccine formulation and licensing*) and adjuvant only vaccines were prepared on the 16/2/21 and then stored at 2-4°C for 70 days prior to use in experimental animals. Prior to delivery to experimental fish, the vaccine was confirmed to be complete white emulsion and had not separated into phases.

To confirm the heat killed step rendered the RGNNV non-infectious, five fish were each intramuscularly injected with 100μL of the heat-killed RGNNV extract planned for vaccine formulation, held for one month and then euthanised by overdose of anaesthetic (Aqui-S) prior to analysis of pooled brain and eye tissue for NNV by RT-qPCR.

Table 3: Composition of vaccine formulae for challenge trial

Preparation	Antigen	Adjuvant	Comment
#1	Recombinant NNV vaccine (Nodavax)	Adj 7 already added	Vaccine in use on farm trial
#2	3 mL recombinant NNV antigen + 1 mL PBS	6 mL Adj 6	alternative adjuvant to #1
#3	3 mL PBS	7 mL Adj 7	control
#4	4 mL PBS	6 mL Adj 6	control
#5	3 mL of JCU antigen (whole virus, heat inactivated) to be added (HK-vaccine)	7 mL Adj 7	alternative antigen to #1
#6	1 mL PBS + 3 mL JCU (whole virus, heat inactivated) antigen to be added (HK-vaccine)	6 mL Adj 6	alternative antigen & alternative adjuvant to #1

4.9.6. *Vaccination of fish*

Two weeks post receipt, between 77-80 fish were injected with each vaccine candidate (Table 3). Fish were anaesthetised with 15-20 mg L^{-1} Aqui-S and injected with 100 μL vaccine or respective adjuvant control by intraperitoneal injection. Following vaccination, fish were returned to 6 x 1000L tanks and held for 22 days at 26-28°C or ~594-degree days before being transferred to the Veterinary Precinct for RGNNV challenge testing. No adverse reaction was recorded and fish resumed feeding within 2 h.

4.9.7. *Veterinary Science Pathogen Challenge Aquarium Facilities*

For experimental challenge to test vaccine efficacy, fish were transferred to the infectious housing Aquarium facilities of the Veterinary Science Precinct of JCU (Building 35). Within the Veterinary Precinct, experimental animals were held in 3 x 5000L recirculation systems that each consisted of

a UV unit, 2 x cannister bag filters equipped with 50UM bag units, a 300L biological filter equipped with 150L biowheels and a variable drive pump. Water was pumped at a rate that ensured a 100% exchange approximately every 70 minutes. Aeration was supplied from a closed-circuit room airline fed by a an airpump. Two airstones were supplied to each housing tank and each biological filter. The entire system was housed in an experimental room with 12/12h controlled light/dark cycle and water temperature was maintained between 25-28C˚C for the duration of the viral challenge. The biological filter was seeded with commercial "NitroFix" twice daily for 5 days when the fish were introduced. Fish were acclimated for two weeks prior to commencement of any experimental procedures. Nitrogenous species (nitrate, nitrite and ammonia) and pH were checked daily for the first week post stocking and weekly thereafter. All discharge water from the rooms in Building 85 are directed to 10,000L storage tank. Prior to release, water in the storage tank is chlorinated at a dose 100ppm for a minimum of 24 hours before discharge into the Townsville city council sewerage line (i.e. not to storm water drainage)

4.9.8.   Challenge dose calibration

Because the viral extract had been stored at -20°C for three years prior to this experiment, three pilot studies were conducted to calibrate the challenge dose. Thirty-five fish were retained non-vaccinated for pre-trial pilot experiments. Pathogenicity of the viral extract was confirmed by 100µl IM injection of RGNNV extract (which had been stored for 3 years at - 20°C, Condon 2019) at different doses; 4 x 10^5, 4 x 10^4 and 8 x 10^3 approximate viral copy number per mL (ten fish each dose). The dose that induced 50% of fish to display clinical signs of VER in approximately 9-10 days post IM challenge was determined. Fish were monitored twice daily for signs of adverse health and euthanised by overdose with 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. Fish were fed a commercial grouper feed twice daily *ad libitum* (Otohime, BM Aqua).

4.9.9.   Challenge experiment and sampling

Vaccinated fish from each experimental group were distributed into three replicate tanks with six cages within each tank, one per vaccine (Table 3) treatment or control, 20 fish per cage. Fish were challenged by IM injection of 100 µL diluted infected homogenate of RGNNV (see section 8.1 and Condon 2019) containing ~8 x 10^3 virus copies per mL. When fish were observed to display more than one clinical sign of VER they were removed from the experimental tanks and humanely euthanised. For euthanasia, 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 30 ppt seawater containing a working solution of 15-20mg/L of Aqui-S. For euthanasia, fish were held until death as indicated by cessation of movement and respiration. Aeration of water was provided throughout the procedure. The last fish to display multiple clinical signs of VNN was euthanised on day 31 post challenge. After 32 days, all surviving fish in Tank 1 were humanely euthanised to collect tissue and blood samples for analysis to detect RGNNV by virus isolation and RT-qPCR. All surviving fish in Tank 2 were terminated after 14 weeks. All surviving fish in Tank 3 were terminated after 17 weeks.

4.9.10. Tissue sampling for NNV detection and quantification

For sample processing, fish were weighed and the brain and one eye were aseptically dissected using a sterile scalpel per fish. Eye and brain were placed in 700 µL sterile PBS in a tube of Lysing Matrix D (MP Biomedical) and homogenised at 5000 rpm in bursts of 30 sec using a Precellys 25 tissue homogeniser (at room temperature). Samples were homogenized until tissue was fully dispersed,(maximum of 3 bursts). Nucleic acid was extracted from 350 µL of the homogenates using the Kingfisher 96 DW protocol with the heat step (Thermo Fisher Scientific). Nucleic acid was eluted in 100 µL RNAse-free water and stored at -20°C pending analysis. RNA2 from RGNNV was quantified by TaqMan RT-qPCR following the ANZSDP method of Hick and Whittington (2009) (qR2T).

Virus isolation was conducted using the remaining 350 µL of the homogenised tissue sample. Homogenate was diluted 1:10 and 1ml was transferred to a sterile 1.7ml microcentrifuge tube (Axygen), frozen (-20°C) and thawed three times, then clarified by centrifugation for 5 min at 12,000 rpm. The supernatant was filtered through a 0.22um syringe filter and frozen in a 1.7ml microcentrifuge tube (Axygen MCT-175) and stored at -20°C. The remaining 2.5ml of the diluted tissue homogenate was stored frozen at -20°C. Virus was cultured from the extract on snakehead whole fry tissue cell E11 clone line cultures (SSN-E11 Nakai, European Collection of Authenticated cell cultures 01110916; Sigma) in Corning 24-well flat bottom tissue culture plates (Sigma Aldrich CLS3527). Briefly, the clarified, filtered tissue homogenate prepared above was diluted 1/10 in L15 medium + glutamine (Sigma L1518) and 100µL inoculated into a well of a 24-well flat bottom tissue culture plate containing a 24 h culture of SSN-1 E-11 cells. Plates were incubated at 28°C for 1 h before 1.5mL of L-15 culture medium containing glutamine (Sigma L1518) (with 10% FBS Australian origin, Sigma F9423) was added to each well of the culture plate and the plate incubated at 26°C. Plates were checked daily for 5 days to observe any CPE evident by destruction

of the monolayer. After 5 days, each plate was frozen at -20°C and stored until analysis by by RT-qPCR. Cell wells with positive CPE were recorded and aligned with RT- qPCR results. Prior to processing for RT-qPCR, culture plates were frozen and thawed 3 times. In the absence of CPE, virus isolation was indicated as positive if the Ct value on the cell culture supernatant was approximately equal or lower than the Ct value of the tissue sample (within 2 Cts if higher).

Attempts to conduct serum neutralisation test (SNT) assays were not successful. Initial attempts to culture cells in 96 well round bottom plates (Sigma M9311) were not successful as uniform growth across the 96 wells prior to the addition of test serum could not be achieved. After numerous attempts at cell culture the SNT assays were abandoned. The limited amount of serum collected from the relatively small fish limited the number of attempts at SNT. Delays in delivery of 96 well plates, due to high COVID-related demand also impacted attempts to troubleshoot the SNT.

4.10. Laboratory challenge trial at UQ: dose titration and onset of protection

4.10.1. Trial context and trial design compromise

This trial was conducted at the University of Queensland (UQ) and was performed at the end of the project as an approved extension. The UQ vaccine group generally employ blind tag-and-mix challenge models, in which all treatment groups are mixed within the challenge system and identified post-hoc by means of internal passive identification tags (PIT). This eliminates conscious or unconscious bias and tank-effect and is generally performed at UQ in a purpose built 8000 L recirculating aquaculture system (RAS) with 3 x 1000 L holding tanks across which the model is replicated either in duplicate or triplicate. It is general policy to perform independent repeated trials to ensure robust evaluation of the vaccine candidates. Due to the unscheduled nature of the present experiment, the vaccine trial system was unavailable as it was in-use in a yellowtail kingfish (*Seriola lalandi*) vaccine development trial FRDC2018-101. A system designed for feed trials within the same facility became available, but this system comprises a matched pair of RAS comprising 10 x 113 L tanks each. This required a different model for the challenge. Fortunately, through a collaboration with Department of Primary Industries Western Australia (DPIRD) on FRDC project 2018-101, a challenge model that retains the advantages of tag-and-mix, but can be performed in systems comprising many small tanks, had been designed for the Waterman's Bay Facility in Western Australia by Prof. Alan Lymbery of Murdoch University. In this model, a single PIT-tagged animal from each treatment group is placed in every tank in the systems, such that all treatment groups are represented in every tank. The challenge model is operated, and the system is treated as a whole unit. The model is effectively a blind tag-and-mix and eliminates any tank

effect. Outcomes in trials at Waterman's Bay are robust statistically when using Mantel-Cox log rank tests and were demonstrated to be repeatable in FRDC project 2018-101, albeit against a different disease agent. For the present challenge, the UQ feed trials system was employed using the Waterman's Bay model. Vaccinated fish were held in one of the matched systems, one treatment per tank (10 tanks, 10 treatments). Periodically, cohorts of 10 fish per treatment were taken from the holding system and challenged with one fish from each treatment distributed to every tank in the second system. Moribund fish were identified via their PIT and censored from the group for statistical analysis post-hoc. The challenge was repeated on a fresh batch of fish weekly, with survivors of the previous weeks challenges remaining in the system with the new cohort. This enabled detection of the onset of protection and simultaneous analysis of antigen dose response within the short window of time and the systems available. Several compromises were made. Due to constraints on time, the systems could not be emptied, cleaned, repaired, refilled and the biofilters re-established after a 23-week thermal stress trial in seawater Atlantic salmon. Consequently, the systems were kept running after the salmon trial, the water was changed (~80%) with clean filtered seawater, but the biofilters retained and the temperature increased to suit grouper. This enabled the introduction of 700 juvenile animals into one of the systems without problems of water quality as the biofilter was already mature. The major compromise was in the size of the RAS which are only 2000 L total volume each. Given the rapid growth and high feeding rates of grouper, the RAS became very heavily stocked (>60 kg m^{-3}) later in the trials and several fish were removed from the trial and euthanised due to ulcerative lesions on the skin presumed to be caused by *Tenacibaculum* species. Removal of these fish compromised the statistical power of the experiment in week 6 post-vaccination.

4.10.2. Animal Ethics approval

Pre-challenge, vaccination and final challenge experiments were performed under approval of the Native Exotic Wildlife and Marine Animals (NEWMA) animal ethics committee at the University of Queensland under permit number 2022/SBS/000386. Animals were held in the University of Queensland approved aquatic animal holding facility Ritchie Building 64A rooms 103-105.

4.10.3. Experimental animals and husbandry

Under a translocation permit approved by the Department of Agriculture and Fisheries Queensland, approximately seven hundred juvenile giant grouper (*E. lanceolatus*) of approx. 5 g weight were shipped by air from TCO, Cairns and acclimatised in two identical 2000 L marine recirculating systems, each comprising an aerated 750 L biofilter sump with in-line bag filters, a

300 L clean sump with a 75 W UV steriliser (Pentair), 3 kW heater chiller (Teco) and 10 L foam fractionator (Aquasonic). Filtered (200 μm) natural seawater was recirculated through 10 x 113 L circular tanks via a centrifugal pump delivering 23000 L/h with water flow controlled by means of tank valves and a bypass return to the sump to reduce backpressure. Flow was maintained per tank to exchange water 10-12 times per h. Tanks were supplied with air via air stones and compressor. Dissolved oxygen, temperature, pH and salinity were monitored continuously and logged every 5 min with an Oxyguard Pacific System (Technolab). Ammonia, nitrite and nitrate were tested daily with a kit (API saltwater master). Tanks and bag filters were cleaned daily and water was exchanged with clean seawater at a rate of approximately 10% per day. Fish were acclimatised and ongrown for three weeks, fed twice daily with commercial feed (Biomar cobia 3mm).

4.10.4. Tagging, vaccine and vaccination of fish

When fish reached 10-15 g, all fish were captured, anaesthetised with Aqui-S (Primo Aquaculture, Brisbane) and tagged with 1.2 x 8 mm Bio-glass passive identification tags (PIT, Swiss+ ID, Brisbane) by injection into the dorsal muscle immediately posterior to the head using the supplied sterile applicators. Fish were allowed to recover in clean aquarium water and then returned to their tanks, approximately 70 fish per tank in one of the recirculating systems. After one week to permit full healing of the tagging injection pore and to account for any tag loss (no tags were lost), fish were once again netted from their tanks, anaesthetised in Aqui-S, scanned with a PIT scanner (AEG, Germany), the code recorded, and 60 fish per treatment were vaccinated by intraperitoneal injection with 50 μL of vaccine or control (Table 4). To prevent excessive handling of the fish (sorting) for subsequent challenges, each vaccine or control was held in a separate tank in the RAS for the holding period. The antigen for the vaccine is the recombinant subunit protein as described in section 4.3.1. Cloning and expression of the capsid protein gene. All vaccines and controls were prepared under commercial conditions by Tréidlia Biovet, Sydney, with the exception of vaccine 8 (ISA660 Adjuvant control, Table 4), which was prepared at UQ. Spare fish were retained in a separate identical system for pre-challenge calibration.

Table 4. Vaccines employed in dose response/onset kinetics trial. All vaccines delivered IP in 50 μL volume

Vaccine No	Antigen dose (μg delivered)	Adjuvant	Ratio (adj:ant)
1	PBS	Montanide ISA763A VG	70:30
2	x	Montanide ISA763A VG	70:30
3	2x	Montanide ISA763A VG	70:30
4	4x	Montanide ISA763A VG	70:30
5	6x	Montanide ISA763A VG	70:30
6	8x	Montanide ISA763A VG	70:30
7	10x	Montanide ISA763A VG	70:30
8	2x	Montanide ISA660	60:40
9	8x	Montanide ISA660	60:40
10	PBS	Montanide ISA660	60:40

4.10.5. Pre-challenge dose titration

Eyes and brain collected from moribund grouper, exhibiting clinical signs consistent with NNV, at Rocky Point Aquaculture during routine field sampling on 23rd February 2022 were snap frozen in a dry ice ethanol slurry and stored at -80°C until required. To prepare an infectious filtrate, eyes and brain from two grouper (fish 2 and fish 6) samples were pooled and weighed in a sterile 50 mL centrifuge tube. Sterile ice-cold phosphate buffered saline was added a rate of 3.5 mL per gram of tissue and the tissue was homogenised on ice at 20000 rpm using an IKA Ultra Turrax T25 homogeniser fitted with a S25N 18G emulsion head (IKA) for 5 min. The resulting homogenate was divided into 2 tubes, and centrifuged at 10,000 x g for 15 min, 4°C in an Eppendorf 5810R refrigerated centrifuge. The supernatant was carefully aspirated through the fat layer with a 10 mL pipette and pump while the pellet was frozen at -80°C in case further extraction was required. The retained liquid fraction was transferred to 15 mL centrifuge tubes and further centrifuged at 18,000 x g for 15 min, 4°C. The supernatant was diluted threefold with ice cold PBS then filtered through a 5 μm filter. Then, 3 mL aliquots were filtered through 0.45 μm and 0.22 μm syringe filters and the infectious filtrate (hereafter called "filtrate") containing Betanodavirus was stored in aliquots at -80°C. Subsamples collected from each aliquot prior to freezing were checked for bacterial contamination by spreading 100 μL onto tryptone soya agar containing lysed horse blood and incubating the plates for one week, checking for bacterial growth each day. No bacterial contamination was detected.

For the challenge, on 7th October 2022, an aliquot of Betanodavirus-containing filtrate was defrosted on ice and diluted 10-fold and 100-fold in ice cold sterile PBS and mixed by inversion. Aliquots (200 µL) of each dilution were retained at -80°C for TaqMan RT-qPCR quantification of viral copies (to be done retrospectively). Each dilution was loaded into 500 µL insulin syringes fitted with a 29-gauge needle. Juvenile grouper (60) from the tagged cohort and weighing approximately 35 g were anaesthetised in Aqui-S and injected intramuscularly (IM) with 50 µL of neat, 10 x or 100 x dilution of filtrate, 2 x 10 fish per dose, and then transferred to 113 L holding tanks, 10 per tank, in the second 2000 L RAS. Fish recovered and resumed feeding the same day. On the 4th and 5th day post-injection, fish ceased feeding and some fish became sluggish and disoriented. Late on day 6 and on day 7 fish became moribund in all challenged tanks. Morbidity continued for a further 9 days with no clear dose response between the dilutions. Consequently, a further challenge was conducted in the same manner, but employing filtrate dilutions of 1000 x, 10,000 x and 100,000 x. As previously, fish ceased feeding in the tank challenged with 1000 x filtrate on day 4 post-challenge but feeding continued for two further days in tanks containing fish challenged with 10,000 and 100,000 x dilutions of filtrate. Morbidity commenced in a dose-dependent manner at these dilutions.

4.10.6. Challenge of vaccinated and control fish
For the challenge of the vaccinated cohorts, a filtrate dilution of 500 x was chosen to provide 70-100% morbidity in control fish, accounting for the growth of the animals in the period between the pilot challenge calibration and the challenges of the vaccinated fish. A fresh aliquot of filtrate from -80°C storage was defrosted on ice then diluted in ice-cold PBS. Aliquots (200 µL) were retained and stored at -80°C from the neat and diluted filtrate for subsequent virus quantification by RT-qPCR (RT-qPCR not performed on these samples at time of writing). Vaccinated fish and their respective adjuvant controls, 10 per treatment, were anaesthetised in Aqui-S, the PIT scanned and the barcode recorded. Each fish was challenged by intramuscular injection of 50 µL diluted filtrate 1 week, 2 weeks, 3 weeks, 4 weeks and 6 weeks post-vaccination. The complete trial was terminated 10 weeks post-vaccination and surviving fish, euthanised by overdose of anaesthetic and scanned to cross-check survivors against the morbidity data.

4.11. Data analysis

4.11.1. On-farm dissolved oxygen analysis

Raw longitudinal farm data providing dissolved oxygen (DO) in mg/L and temperature in degrees Celsius (°C) was converted into % oxygen saturation using the formula % saturation= (100 x DO mg L^{-1})/C_p; where C_p is the equilibrium oxygen concentration in mg/L, composed of non-standard and standard pressure (Mortimer 1956). As the farm flanks the coast, the atmospheric pressure was considered 1 atm, and the equilibrium oxygen concentration at standard pressure (C*) was calculated using the formula: $C* = \exp[(7.7117-1.31403) \times \ln(t + 45.93)]$, where t is temperature in °C. Including non-standard pressure in the calculations contributed negligibly under these conditions and therefore we used C* as a proxy for Cp. Salinity remained stable over the periods directly compared: December to April for both 2017-18 (batch 0) and 2018-19 (batch 1). Over these periods the average salinity was 27.32 g L^{-1}, with SD 0.89 for batch 0 and 28.33 g L^{-1}, with SD 0.70 for batch 1, respectively.

Values for oxygen tolerance of groupers are not fixed since salinity, gill disease status, xenobiotic exposures and water temperature all have significant impacts. For nursery culture of *E. lanceolatus*, *E. fuscoguttatus* and *E. coioides*, the Australian Centre for International Agricultural Research best practice manual recommends the DO should range from 4-8 mg/L (ACIAR 2012). However larger fish tend to be more susceptible to oxygen stress. For grouper in grow-out, we considered ranges of percentage oxygen saturation to be indicative of hypoxia as follows: < 30% severe hypoxia; 30- 50% moderate hypoxia and 51-70% mild hypoxia. As a minimum, the DO should be >4 mg L^{-1}.

4.11.2. Indirect ELISAs farm trial data

Prior to any analysis, data were tested for normality using the Shapiro-Wilk test. When the test was passed at alpha=0.05, (batch 1), multiple t-tests (including discovery test) were applied over the whole batch to compare antibody levels in controlled and vaccinated fish. When on few occasions some groups were non-normal (batch 3), the data were analysed separately at each sample point. An unpaired, 2-tailed t-test was performed for normal data, otherwise a Mann-Whitney non-parametric test was used. Analyses were done using Prism 9 (Graphpad). *P* < 0.05 was considered statistically significant in all analyses.

4.11.3. Indirect ELISAs and elution ELISA with data from aquarium NNV challenge

Prior to analyses, data were tested for normality using the Shapiro-Wilk test. As all data were normally distributed, a one-way ANOVA with Tukey's multiple comparisons test was performed using Prism 9 (Graphpad). $P < 0.05$ was considered statistically significant.

4.11.4. Challenge survival analysis

Laboratory challenge data from JCU and UQ were entered as morbidity events against time, plotted as survival curves and analysed by log rank Mantel-Cox test in Prism 9 (GraphPad).

5. Results

5.1. The inactivated recombinant NNV vaccine is safe, has negligible side-effects and elicits immune memory

The safety trial in which 100 juvenile grouper were vaccinated with 200 µL dose of vaccine resulted in no adverse effects. During extended sampling on farm, we noted oil-emulsion associated inclusions in mesenteric fat in vaccinated juveniles. The inclusions became small and dark with time but at no stage during grow-out did they adhere to or transfer melanisation to muscle tissue (Fig 4). As the fish grew these inclusions disaggregated and were not considered relevant by customers at point of sale and no fish were rejected by customers as a consequence of pigmentation in the viscera, according to reports from the farm owner. No other vaccine-related side-effects were observed.
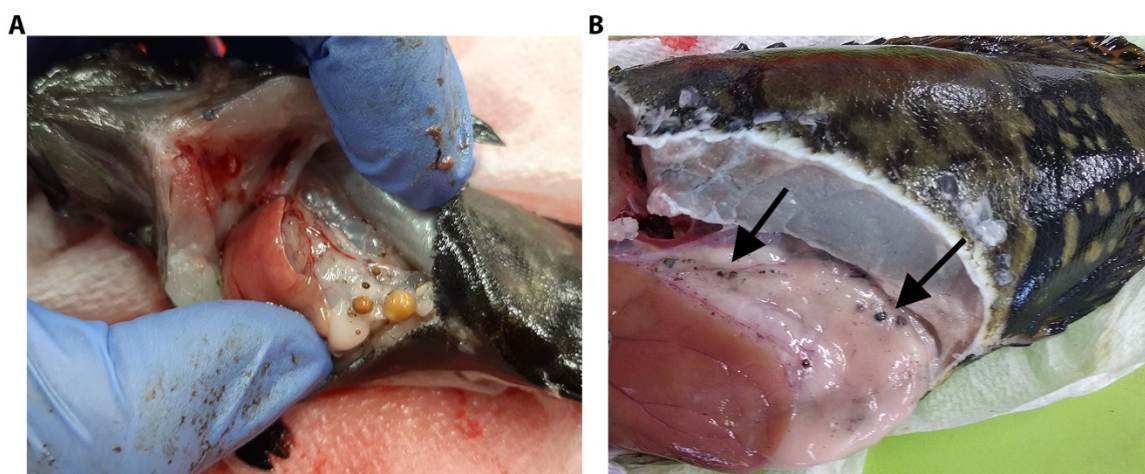
Figure 4. Oil emulsion associated inclusions in mesenteric fat of vaccinated giant grouper juveniles.
A: Fish ~55 g, 6 weeks post vaccination. **B**: Fish ~600 g, 6 months post vaccination. Arrows indicate small dark granules that disintegrated with time.

A prime-antigen challenge experiment was conducted under controlled conditions in an aquarium facility to determine whether or not the vaccine elicited immune memory. Batches of fish primed with the vaccine or unvaccinated, were subsequently challenged with recombinant protein antigen alone, or PBS as a handling control, to measure the magnitude and quality of the secondary response versus any residual primary antibody response. On re-exposure to antigen alone, vaccinated fish had significantly higher secondary response after 7 days at 28°C than unvaccinated fish (Fig 5A). Challenging unvaccinated fish with antigen elicited a primary response that was detectable compared to handling controls even after 7 days. When vaccinated fish were

injected with PBS as an innocuous challenge, 3 months after primary immunization, antibody

quantity was similar to unvaccinated fish (Fig 5A). Avidity of antibodies elicited by exposure to the

unadjuvanted antigen was higher in vaccinated fish than in unvaccinated fish (Fig 5B).
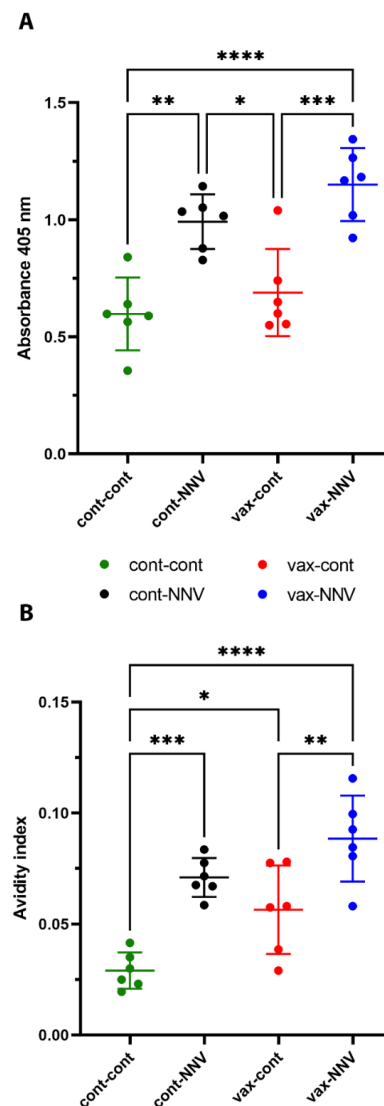
Figure 5. Anti-NNV antibodies raised in giant grouper after vaccination and antigen challenge.
~90 days post initial vaccination or unvaccinated, fish (~86 g) were subject to an antigen challenge with recombinant NNV capsid protein or with PBS. Four resulting treatment groups (n=6) were: no vaccine control-PBS control (cont-cont), no vaccine control-NNV antigen (cont-NNV), vaccine-PBS control (vax-cont) and vaccine-NNV antigen (vax-NNV). One week post challenge sera were tested by **A. Indirect ELISA** to determine anti-NNV IgM antibody levels and **B. Elution ELISA** to determine avidity of the anti-NNV IgM antibodies. All data were normally distributed (Shapiro-Wilk test) and analysed with a one-way ANOVA with Tukey's multiple comparisons test. Points are individual fish, lines: 5-95th percentile with mean. Significance levels * $p < 0.05$, ** $p < 0.01$, ***$p<0.001$, ****$p < 0.0001$

5.2. Outbreaks of VER pre and post vaccination

For the purposes of comparing VER outbreak scenarios, we used farm data from grow-out

December to April for batch 0 (no vaccination, lake 1, 2017-18) and from batch 1 vaccinated fish

(lake 2, 2018-19). Batch 0 was stocked prior to commencement of the vaccine project, thus no fish were vaccinated against VER or systematically sampled. Batch 1 was the first group of fish in the field trial which were sampled monthly as described in the methods section, 3.2. In both batches, approximately 8000 fish were stocked in the two lakes.

5.2.1. Outbreak kinetics

An acute outbreak of VER occurred in batch 0 in late-March 2018 and NNV infection was confirmed by histological analysis and PCR at Queensland Biosecurity Sciences Laboratory (BSL). Cumulative mortality of 86.8%-100% occurred in five adjacent cages containing grouper of mean weight 840 g and stocked at 40-60 kg m^{-3}(Fig 6A). The last cage in which mortality started contained the largest animals (Cage 6, ~1000 g) and an emergency harvest was instigated to mitigate losses. Cumulative mortality in that cage was 36% at point of emergency harvest (Fig 6A). Approximately 8 days after the first dead fish was reported in Cage 4, mortality started to increase rapidly and within a few days spread to adjacent cages (Fig 6A). Peak daily mortality rate was observed approximately 10-12 days after mortalities commenced in each cage. With a combination of rapid harvesting and deaths, cages were completely de-stocked five weeks after the first mortality was noted. Approximately 90% of fish harvested showed clinical signs of VER, especially impairment of vision as assessed by non-response to visual stimuli, corneal opacity and hyper-buoyancy. During this batch grow-out, water temperature and dissolved oxygen (DO) mg L^{-1} were only monitored in the morning and is incomplete (Fig 6A). One week prior, and during three weeks of the outbreak, the water temperature at 1 m ranged from 24.0 to 26.7 °C.

The following year, an outbreak of VER occurred in the first batch of vaccinated fish during January 2019 and the presence of NNV was confirmed by BSL. The outbreak occurred in in three adjacent cages of grouper, average weight 350 g, stocked at 4.5-7.5 kg m^{-3} in cages in Lake 2. While a few mortalities had occurred in December, rapid mortality started on January the 8th when temperatures reached 28.6 °C (Fig 6B). One week prior, and during three weeks of the outbreak, the water temperature at 1 m ranged from 28.2-30.1 °C. In fact, towards the end of January the water temperature remained consistently high, over 29 °C for 10 days. However, the outbreak was far less severe, with cumulative mortalities from 9 to 20%. The duration of infection peak was shorter, and fish resumed feeding shortly after. Daily mortality rate peaked around day 8 after onset and the outbreak lasted approximately three weeks (Fig 6B). The highest mortality occurred in Cage 9 (around 600 fish, ~20%) and this cage had the highest stocking density, estimated at 7.5 kg m^{-3} at the time of outbreak, and was the cage where sporadic mortalities had begun during

December 2018. Total mortality for the 3 cages was 14.5%. Morning oxygen saturation remained less than 50% (and DO < 4 mg L^{-1}) for 10 days, indicative of chronic moderate hypoxia up to January the 8th, when the mortality rate increased (Fig 6B). To improve water quality, a current mover was installed near the cages within days of the outbreak, and the percentage oxygen saturation improved, though was still considered mildly hypoxic (Fig 6B).
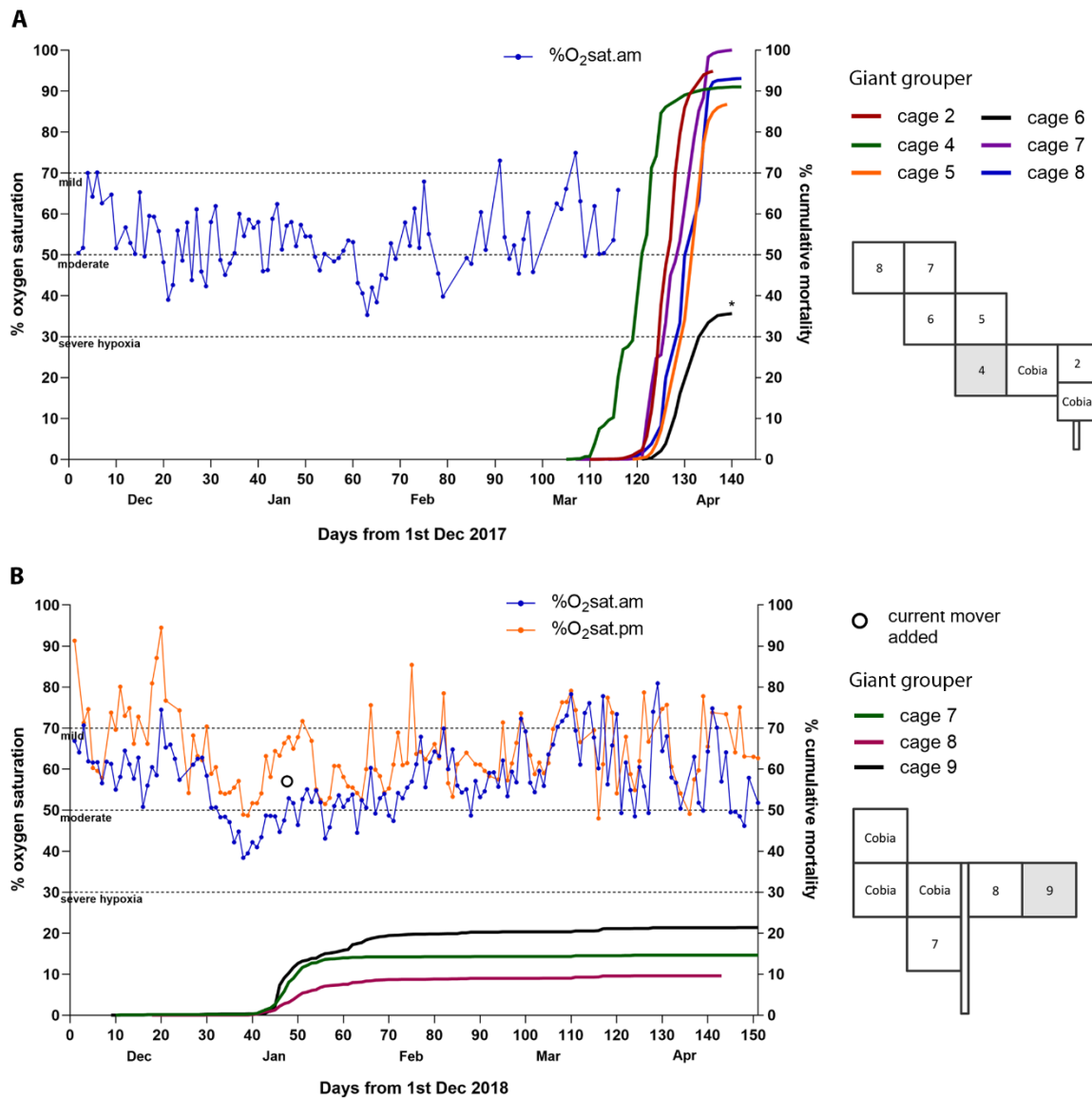
Figure 6. Oxygen saturation and mortalities during VER outbreaks
A: Unvaccinated fish, batch 0, summer 2017-18. In this grow out temperature and DO mg/L were only measured in the morning. Cage 6 (*) was harvested rapidly before a greater level of mortality was reached. B: Vaccinated fish, batch 1, summer 2018-19. For giant grouper, oxygen saturation <30% is considered to indicate severe hypoxia; 30- 50% moderate hypoxia and 51-70% mild hypoxia. A current mover was introduced in B (o) after 10 days of continued moderate hypoxia (am). Note: In the cage layouts (right), grey filled cages are where first mortalities occurred in each scenario.

Monthly blood and histology samples were collected from batch 1 in mid-December 2018, late January and February 2019 before and after the outbreak occurred. Pathologies in January 2019

included brain and retina inflammation, and moderate necrosis and vacuolation (Fig 7). One month later, no brain or retinal pathology was detected in any samples suggesting that the surviving fish had either not been infected or had recovered.
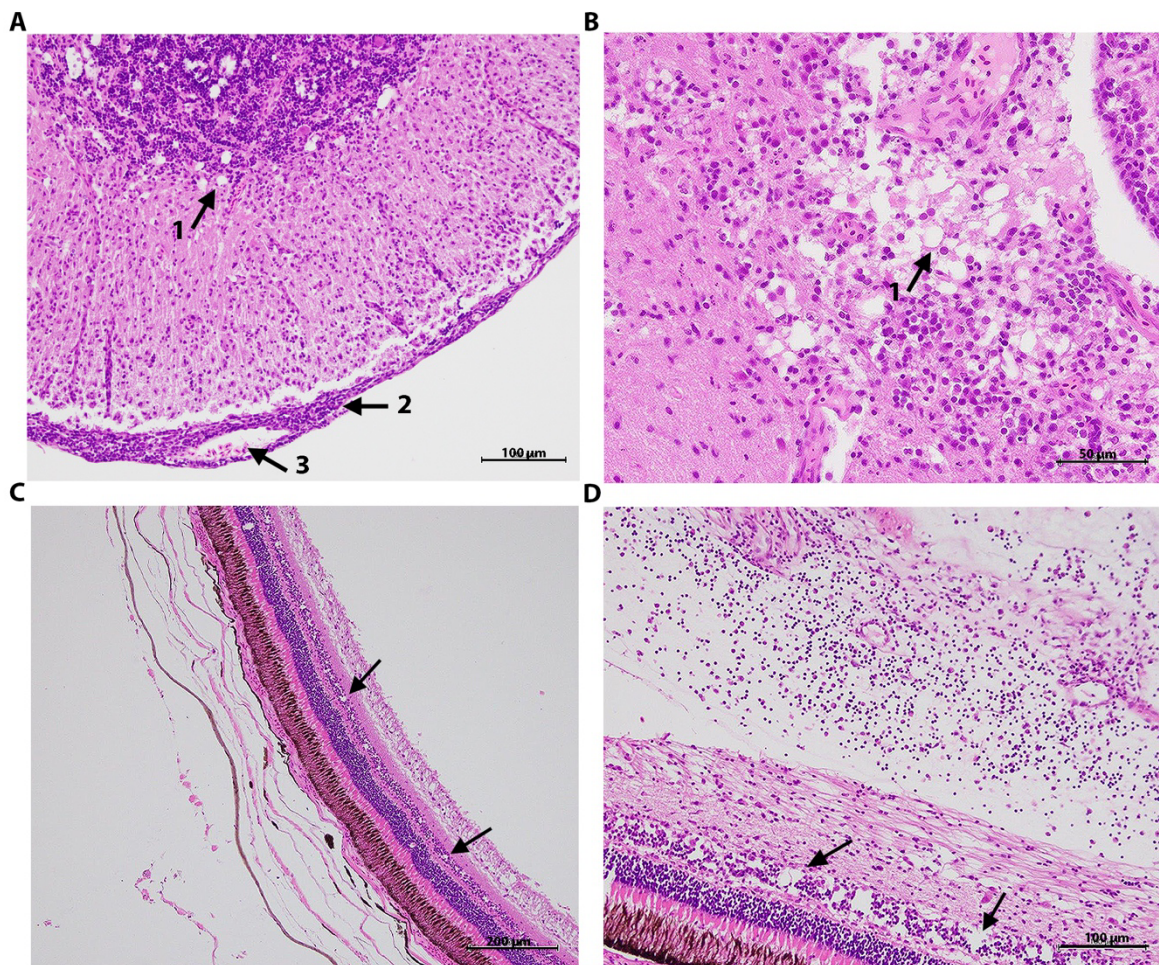
Figure 7. Vacuolation and necrosis in *Betanodavirus* infected moribund giant grouper
A & B: brain and C & D: retina. Images are from three individuals in batch 1 (vaccinated), January 2019. **A.** Vacuoles (1) within the inner granule cell layer of the cerebellum. Meningitis (2) with lymphocytes extending from the meninges into the outer molecular layer via pial reflections. (3) Erythrocytes within a vein in the meninges. **B.** Inflammation and vacuolation (1) within the brain. **C**. In retina, arrows highlight examples of vacuoles and necrosis in the inner nuclear layer. **D**. Vacuolation in the retina. The optic nerve is surrounded by mixed inflammatory cells where it entered the retina and these had detached during processing and become lodged in the vitreous humor (upper part of image). Staining with hematoxylin and eosin.

Histopathology from mid-December 2018 revealed liver pathology in vaccinated (Lake 2) and non-vaccinated fish (Lake 1), showing inflammation, the presence of melanomacrophages, bile duct proliferation and oedema around the bile duct (Fig 8A). Glycogen and lipid is prominent in the cytoplasm of hepatocytes. Hepatocytes were hypertrophied and there was severe hepatomegaly (Fig 8B and 8C). The intestines of control and vaccinated fish also showed moderate to severe oedema and a thickened lamina propria and oedema in the submucosa (Fig 8D). Gross

pathological observations were enlarged pale livers with occasional green patches (Fig 9A). Diets were changed by the farm to successively reduce carbohydrate and increase taurine inclusion over the course of the trial and improved the liver pathology (Fig 9B and 9C). There have been no further outbreaks of VER in any subsequent batches of fish, since July 2019.
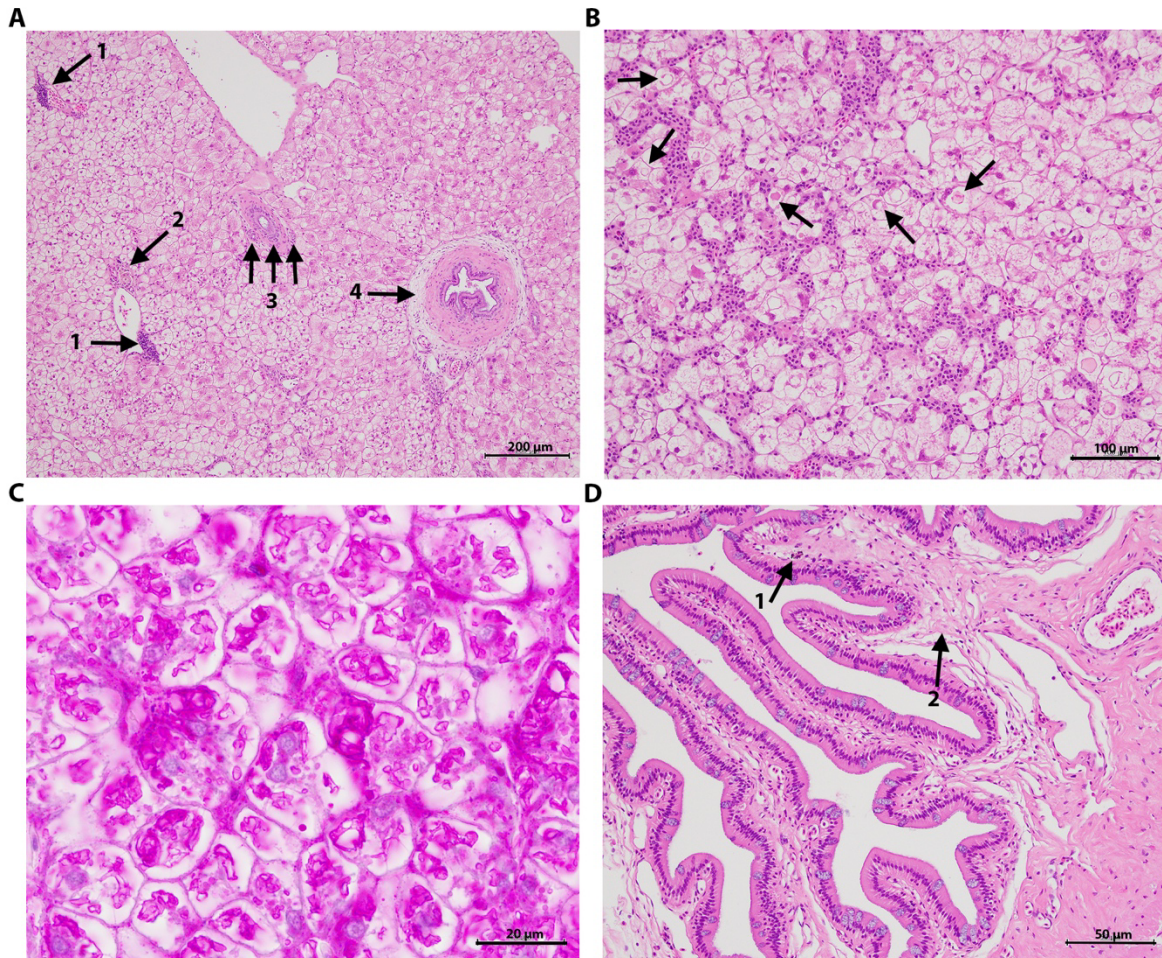
Figure 8. Liver and intestine pathology in giant grouper prior to VER outbreak.
A: Liver inflammation. Arrows indicate 1. Lymphocytes cuffing of veins 2. Melanomacrophages 3. Bile duct proliferation 4. Oedema around a bile duct. **B:** Liver inflammation. Aggregations of mononuclear cells can be seen throughout the parenchyma. Arrows indicate unusual eosinophilic material within hepatocytes, possibly glycogen. The colourless regions in cytoplasm are lipid. **C:** Glycogen accumulation (bright pink) in hepatocytes. **D**: Intestine. Arrows indicate 1. melanomacrophages in the lamina propria. 2. Oedema of the lamina propria and submucosa. Images are from 4 individuals in December 2018, batch 1. Staining hematoxylin and eosin in A, B & D. Periodic acid-Schiff stain in C.
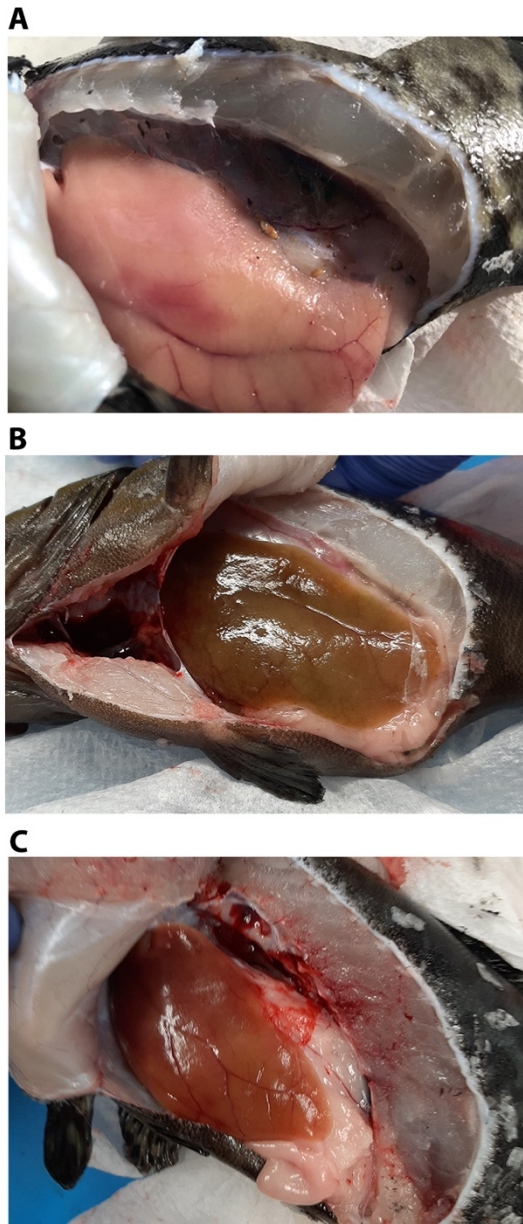
Figure 9. *Epinephelus lanceolatus* liver gross liver pathology on different diets.
A. High carbohydrate diet, January 2019, batch 1 (vaccinated). Fish weight ~300g. **B**. Alternate days high carbohydrate diet and low carbohydrate plus 1% taurine, December 2020. Batch 7, fish weight 282 g. **C.** Low carbohydrate diet plus 1% taurine, December 2020, Batch 8 fish weight 250 g. Fish batches are explained in the Appendix Table B2.

Cobia were also being grown in cages next to the grouper during both outbreaks (Fig 6). Cobia did not display any external signs of VER. During January 2019 (grouper batch 1 outbreak) the cobia stocked nearby weighed 1.8 kg on average and there were 28 deaths recorded among 4497 fish. When cobia, co-habiting with grouper, which had been confirmed to be infected with NNV, were tested for the presence of NNV by BSL all cobia were found to be negative. A VER outbreak in juvenile farmed cobia has previously been reported in Taiwan (Chi et al. 2003).

5.3.   On-farm serum specific IgM against the NNV capsid protein

We determined the specific anti-NNV IgM levels in vaccinated and unvaccinated fish on farm in batch 1 and batch 3 by ELISA. Batch 1, 2018-19 was the first group of vaccinated fish on the farm and, due to the larger size of the grouper (100-170 g), they were held in cages situated in the two lakes for grow-out immediately on arrival, lake 1 (unvaccinated) and lake 2 (vaccinated). Batch 3, 2019, is representative of the typical stocking scenario on the farm; with juvenile fish being initially raised in RAS and then transferred to lake 2 when larger (>120 g), for grow-out in the warmer months. No signs of VER were reported in batch 3 either in the RAS or lake grow-out and no NNV was detected in any of the samples tested by qPCR using the TaqMan qPCR method recommended by the OIE (Hick and Whittington 2010, OIE 2022). Metadata for batch 1 and batch 3 are provided in Appendix B Table B2 and B3 respectively. No significant infections by other pathogens were reported in these batches of grouper.

In batch 1, on entering the lakes in December 2018, both unvaccinated (lake 1) and vaccinated (lake 2) fish had antibodies which recognized the NNV capsid protein, but only vaccinated fish had a higher mean level and with less variation (Fig 10A). During the VER outbreak in lake 2, antibody levels dropped (or antibodies were consumed by binding virus), especially in moribund fish. Post outbreak, levels of anti-NNV IgM gradually recovered in vaccinated fish and increased in unvaccinated control fish, until levels were similar in April 2019 (Fig 10A).

In batch 3, vaccinated fish had higher levels of anti-NNV IgM than unvaccinated controls in the RAS. In fact, initially, most control fish had levels equivalent to naïve sera (Fig 10B). This difference became more pronounced after entering the lake. Subsequently, levels in both vaccinated and control fish increased as fish grew, and once they were 350-400 g in size both groups had similar levels of anti-NNV IgM (Jan 2020, Fig 10B). Clinical disease was not observed, despite temperatures reaching 30-30.5°C for 6 consecutive days at the end of January 2020.
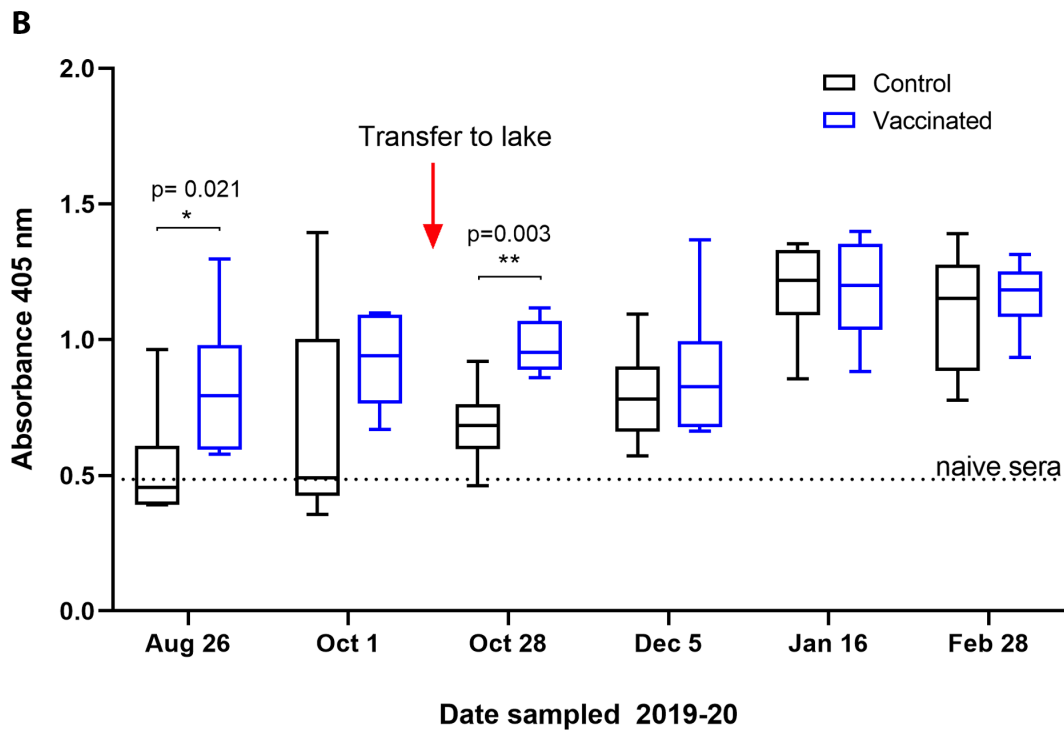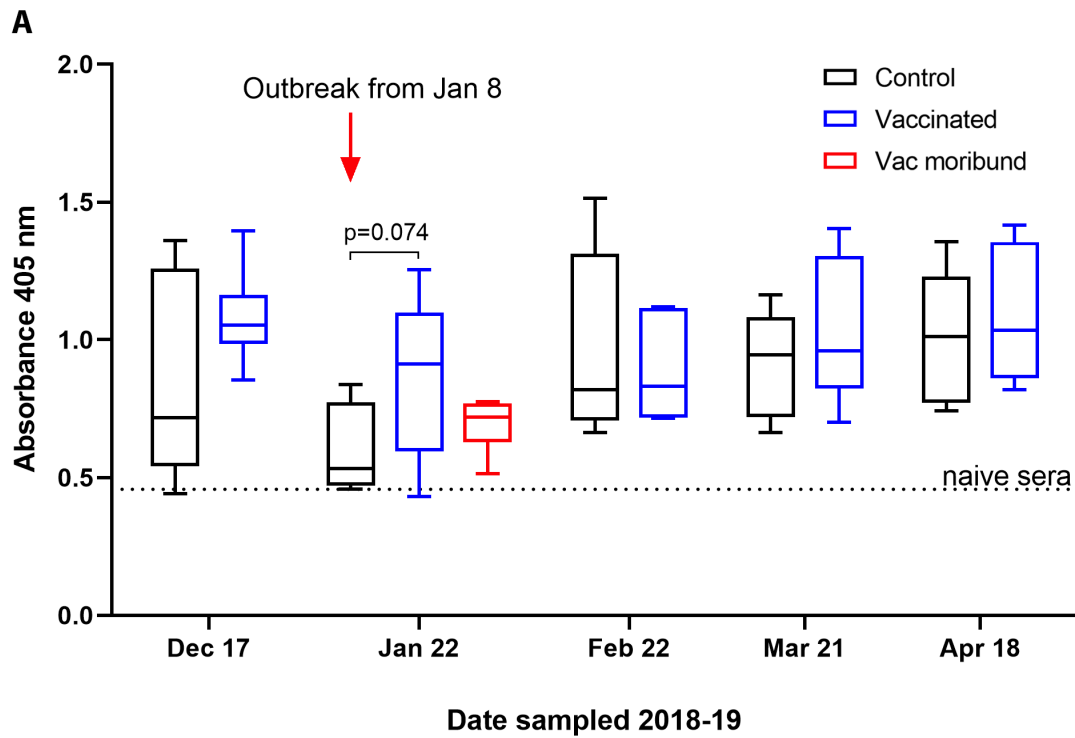
Figure 10: Anti-NNV IgM in vaccinated and unvaccinated (control) giant grouper during grow out (n=6).
A: Batch 1, lake cages: vaccinated fish in lake 2 (blue) including moribund fish with VER (red); controls in lake 1 (black). Box plot, 5-95th percentile with median. Statistical analysis: multiple t-tests comparing control and vaccinated fish at each timepoint. Significance level *p*<0.05. All data groups passed Shapiro-Wilk normality test, and false discovery rate was negative at Q= 1%. **B:** Batch 3 from RAS to lake 2: vaccinated (blue) and control (black) in adjacent cages. Box plot, 5-95th percentile with median. Statistical analysis: control and vaccinated fish compared at each time point by an unpaired two-sided t-test, when data were normally distributed (Shapiro-Wilk normality test). In the few cases when data were non-normal the Mann-Whitney non-parametric test was used. Significance level * *p*< 0.05 and ** *p*< 0.01

5.4.   Diagnostic TaqMan qPCR assay for NNV viral load

5.4.1. Method validation

Amplification curves for serial dilutions of plasmid standard according to copy number are seen in Figure 11.
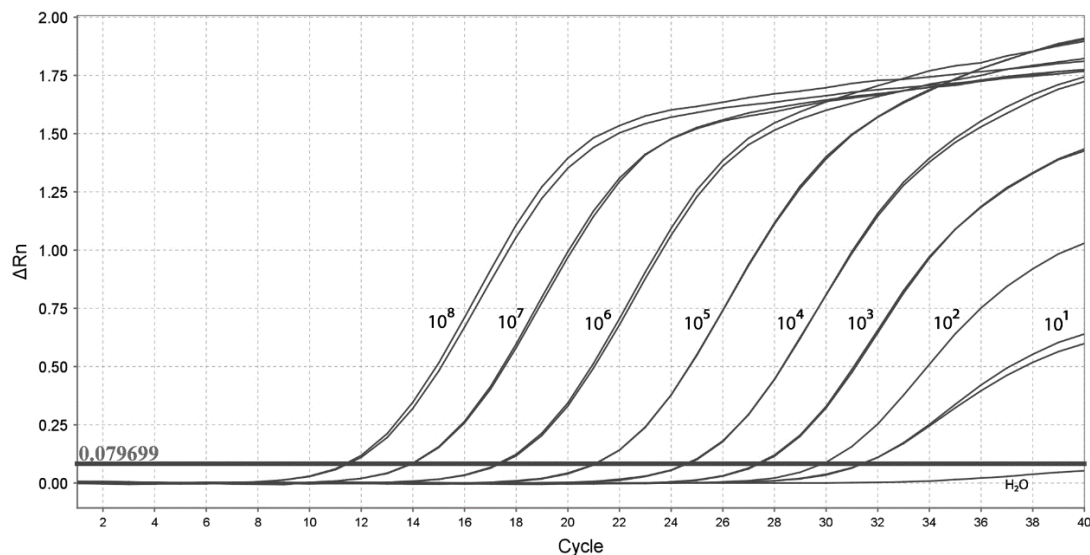
Figure 11: Amplification curves for plasmid standards.
Serial dilutions in duplicate from 10^8 to 10^1 copies of pcDNA3.1 containing an RGNNV RNA2 fragment were amplified with qR2T probes using TaqMan qPCR

A high efficiency (>95%) and an R^2 from 0.99-1.00 was attained in linear regression, plotting Ct values against $\log_{10}$ of copy number for the range of dilutions 10^7 to 10^2. Higher and lower dilutions did not conform as closely to linearity, see Figure 12a compared to 12b. When samples were interpolated, Cts yielding between 10^1 and 10^2 copies were considered borderline and when less than 10^1, negative. (10^1 copies of plasmid gave an average Ct of 34.049, with a high SD). Samples yielding copy numbers at the upper limit between 10^7 and 10^8 were reported numerically. The highest sample load detected was $10^{7.3}$. Inter-assay variation was determined by the coefficient of variation (CV= standard deviation/mean), for Cts of each standard (dilutions 10^7 to 10^2). CV ranged from 0.021 to 0.077; data from 5 runs.
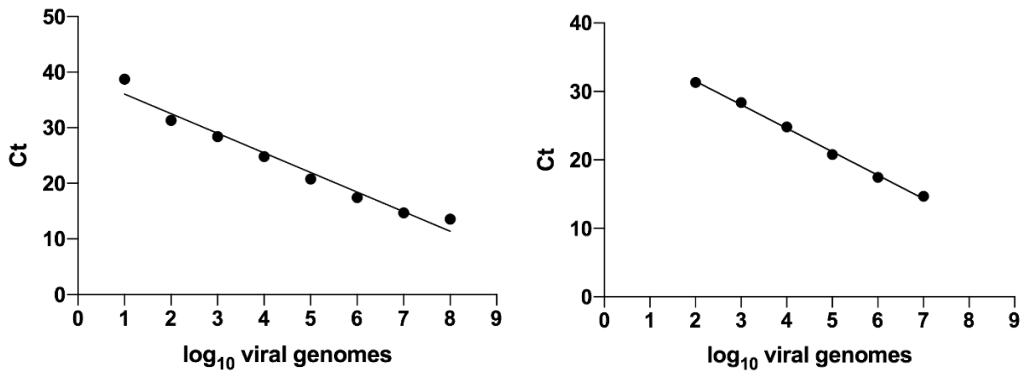
Figure 12: Linear regression of plasmid standards shown in Figure 11.
Standards were run in duplicate. Graph a (left) includes all dilutions tested. Graph b (right) shows linear range. Efficiency of b) 95.4%, $R^2 = 0.997$

To test for analytical sensitivity, serial 1:10 dilutions of positive control RNA isolated from NNV infected grouper brain were amplified in duplicate from 50 ng/ reaction to 0.005 pg (in a total volume of 10 µl). Amplification curves are shown in Figure 13. The mean Ct, SD and CV for two independent runs in duplicate of these serial dilutions are given in Table C3 Appendix C.
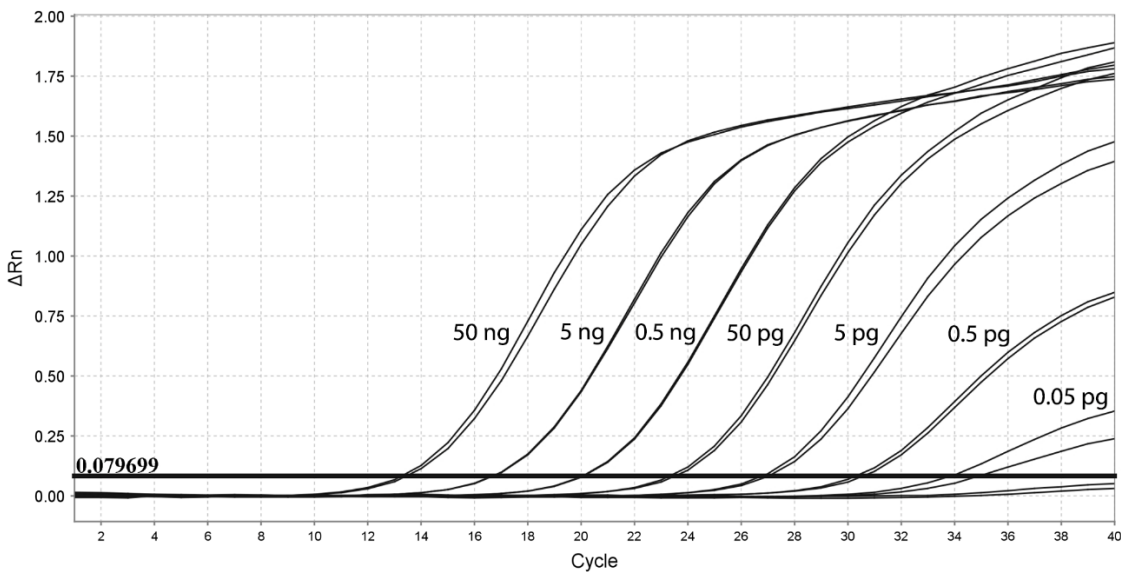
Figure 13: Amplification curves for serial dilutions of total RNA from NNV infected grouper brain.
Serial dilutions in duplicate from 50 ng to 0.05 pg were amplified with qR2T probes using TaqMan qPCR

We were able to detect virus in as little as 0.5 pg of RNA from clinically infected tissue. Note the average Ct value for 0.5 pg of RNA from infected brain (30.876) was similar but beyond the mean Ct value (30.236) for 10^2 copies of plasmid (see Figure 11), above which we considered detection was borderline. Therefore 1 pg of RNA, rather than 0.5 pg, should be considered our lowest limit for detection of NNV. The manufacturer's recommendation for the TaqMan®Fast Virus 1-Step Master Mix is to use from 1 pg to 100 ng of sample nucleic acid, which also is in line with these results.

With regard to controls, both infected fish from the January 2019 outbreak used as positive controls had similar levels of viral load. 50 ng of total RNA/reaction contained an estimated $10^{7.2}$ and $10^{7.3}$ copies of RGNNV RNA2 respectively, using the average Ct across plates for these samples. Negative controls, RNA from brains of small unvaccinated fish, gave a negative result for RGNNV RNA2 in all of the 5 runs, as expected.

5.4.2. Viral load in grouper brains batch 1 and 3

The results shown above indicated our method was sufficiently robust to proceed with detection of RGNNV RNA2 genomes in the grouper brains sampled in field trials. This was performed on batch 1 and batch 3 samples using 50 ng of total RNA per reaction in duplicate. Results for batch 1 are shown in Figure 14 and details are given in Appendix C, Table 4C.

Figure 14: NNV RNA2 viral genomes detected in grouper brains during Batch 1 grow-out.
Viral load detected by TaqMan qPCR following Hick and Whittington 2010; each triangle is a data point representing an individual fish

Clinical signs were only observed in fish sampled during the January 2019 outbreak in lake 2, which occurred after a prolonged period of low DO. All these moribund fish had viral loads > 10^6 RNA2 genome copies / 50 ng of total RNA from brain. Cumulative mortality reached 20%, but the majority of vaccinated fish were able to recover. A minor adverse event also occurred in late

March when some fire extinguisher foam seeped into lake 2. This environmental stress likely triggered VER which was observed in some fish, but cumulative mortality was <1%. This is reflected in several fish in lake 2 having medium-low viral load in mid-April. The presence of virus in both zones of the lake during this period is evidenced by several fish (within samples taken of n=6 fish) having subclinical NNV infection. Outbreaks occurred under the stressful conditions of adverse events in lake 2. Since the control group had been stocked distant from the vaccinated fish, we are unable to comment on vaccine performance in relation to these results.

In batch 3 none of the brains sampled had any detectable NNV RNA2. Controls and standards worked consistently compared to previous runs, thus validating the detection method. There were no clinical signs of infection at any stage in this cohort, nor any adverse events. Interestingly, levels of anti-NNV-IgM increased after transfer to the lake (Fig. 10B). Results indicate that NNV infection was not established in this cohort.

5.5. Sequencing of NNV genome from the Rocky Point isolate

The sequences obtained for NNV RNA1 and RNA2 genome segments were consistent with sequences for other isolates of RGNNV reported in the literature and were uploaded to NCBI GenBank. Sequence lengths were 3015 bp for RNA1 (MZ054261) and 1316 bp for RNA2 (MW590702).

5.6. Polyclonal anti-giant grouper IgM antibody raised in rabbit

Grouper IgM purified by protein A affinity chromatography was lyophilized and sent to Thermofisher, Scientific, Rockford USA to produce a polyclonal anti-giant grouper IgM antibody in rabbit, for routine ELISA use. We received 1.1 mL samples of serum from 2 rabbits immunized and boosted with grouper IgM and 1.5 mL of purified Rabbit anti-grouper IgM IgG from Rabbit 3152 (0.15 mg/mL). The performance of the antibody was tested in western blot and ELISA (Figure 15). The antibody specifically binds immunoglobulin bands in the sera and titres were > 512000 and 256000 for rabbits AB3152 and AB 3153 respectively. Further boost and terminal bleed sera were subsequently obtained; the final volume of sera received was 50 ml.
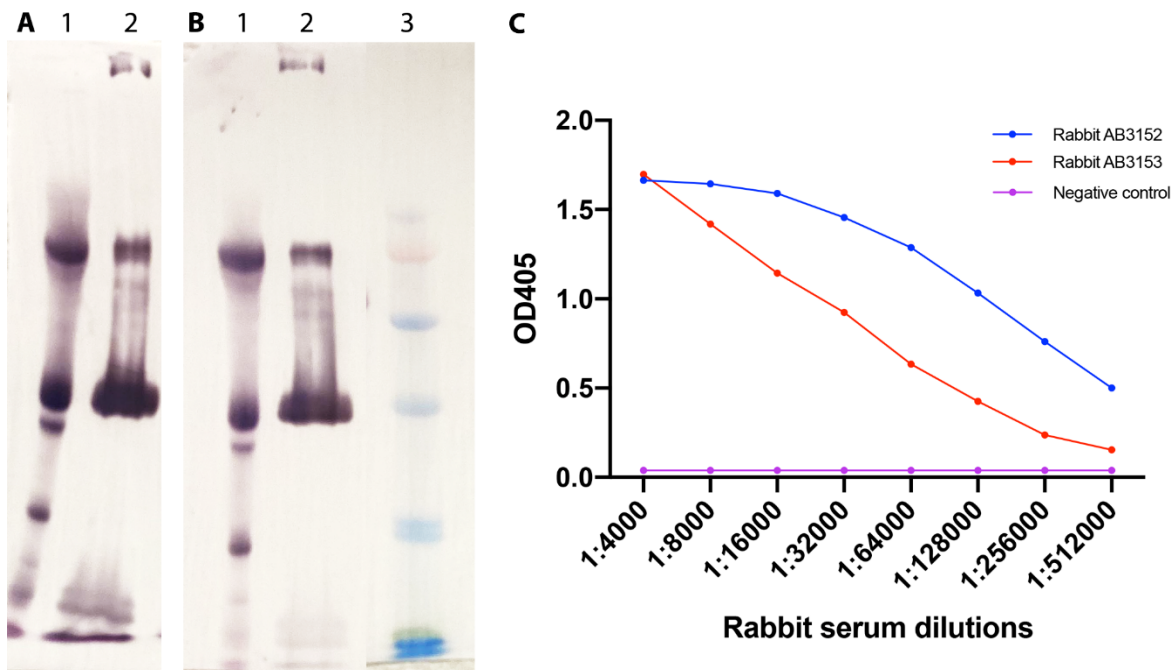
Figure 15: Western blot and ELISA testing the specificity and titre of anti-grouper IgM polyclonal antibodies harvested from 2 rabbits.
A and **B** (rabbit AB3152 and AB1353 respectively) Lane 1: *E. lanceolatus* crude serum; lane 2: *E. lanceolatus* purified IgM; lane 3: protein MW ladder. Detection: rabbit anti-grouper IgM (1:8000); sheep anti rabbit IgG 1:15000) **C. Antibody titre:** ELISA plate coated with *E. lanceolatus* crude serum (1: 512, approx. 4.5 μg protein per well). Detection: rabbit anti grouper IgM in serial dilution (1:4000 – 1:512000); sheep anti rabbit IgG (1:15000).

5.7. Aquarium RGNNV challenge trials at JCU

Pre-challenge dose-titration trials indicated that mortalities occurred more quickly than occurred in previous work (Condon, 2020), with the higher doses reaching 50% morbidity within 7 days, while the lowest doses, 8×10^3 viral copies per mL (about 800 copies per fish IM) resulted in 50% morbidity after 10 days (Fig. 16). All doses resulted in >90% morbidity over the duration of the challenge trial so the lowest dose was chosen for the vaccination challenge experiment (Fig. 16).

Fish vaccinated with Nodavax and HK-vaccine variants were challenged 972 °days post-vaccination by intramuscular injection of 8×10^2 viral copies per fish. Morbidity began to occur at 13 days post challenge and was most evident during days 15-17 post challenge (Fig. 16B). Morbidity of adjuvant-only control groups reached 92 and 93% and there was no significant difference between the adjuvant controls (Mantel-Cox Log Rank Test) (Fig. 16B). In contrast, morbidity in all vaccinated groups (recombinant protein and heat inactivated vaccines) ranged between 3 and 6%, regardless of adjuvant combination (Fig. 16B).
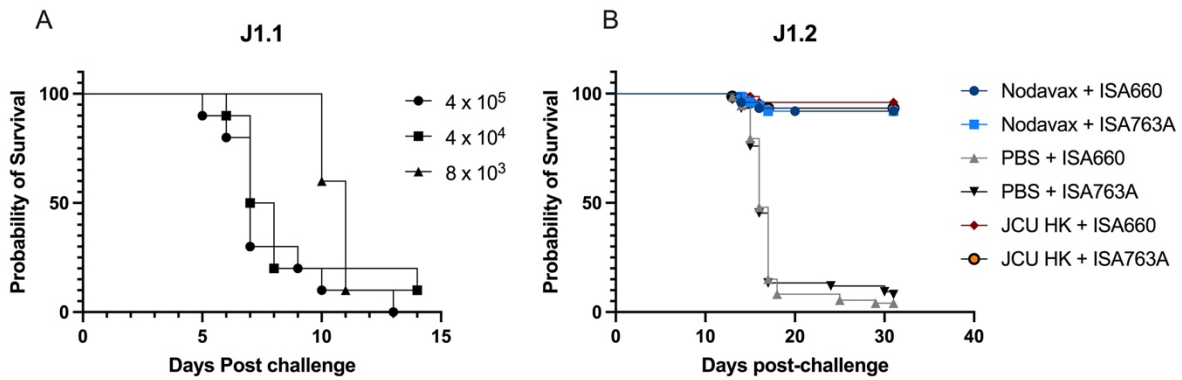
Figure 16: Survival curves from pilot challenges (A) and vaccination challenge (B).
A Pilot challenges with three different viral doses were conducted sequentially by intramuscular injection of 10 fish per dose. Doses are shown in approximate viral copies per mL, but 100 µL was injected so the dose per fish is 10-fold lower. **B.** Vaccination trial challenge morbidity.

All fish that were euthanised, displaying signs of VER in the first 32 days post RGNNV challenge, were positive for RGNNV by RT-qPCR. Ct values ranged from 12.38 to 28.97 across all groups. Similarly, virus was isolated in cell-culture from all tissue samples from fish showing signs of VER (Table 5). All surviving fish in Tank 1 were euthanised at day 31 to determine if NNV was detectable in tissue. Fish in Tanks 2 and 3, which were replicates of the groups held in Tank 1 were retained to observe if signs of VER became evident over the longer term.

Table 5: Viral loads determined by RT-qPCR and virus isolation from fish euthanised in Tank 1 (31 days post-challenge). Number tested are those fish exhibiting signs of VER
Summary of RT-qPCR and Virus Isolation Results of Fish that were Euthanised in the first 31 days displaying signs of VER

Experimental Group	RT-qPCR Ct value: Eye				RT-qPCR Ct value: Brain				No. RT-qPCR +ve		No. Virus Isolation +ve		No. Tested
	Min Ct	Max Ct	Average	Std.dev	Min Ct	Max Ct	Average	Std.dev	Eye	Brain	Eye	Brain	
Nodavax + Adjuvant 6	14.32	28.29	20.56	5.43	24.61	27.80	26.08	1.22	6	6	6	6	6
Nodavax + Adjuvant 7	18.44	27.64	22.05	3.14	21.45	25.98	24.80	1.53	6	6	6	6	6
Adjuvant 6	15.18	28.95	21.93	3.68	19.95	28.97	24.82	1.93	70	70	70	70	70
Adjuvant 7	12.38	28.96	21.24	3.67	19.76	28.71	23.72	2.14	69	69	69	69	69
JCU + Adjuvant 6	18.68	28.68	23.38	5.03	25.19	27.57	26.65	1.28	3	3	3	3	3
JCU + Adjuvant 7	17.60	25.66	22.19	4.15	20.71	24.78	22.70	2.04	5	5	5	5	5
All groups	12.38	28.96	21.60	3.74	19.76	28.97	24.39	2.10					

For the period between 31 days and 14 weeks post-challenge, approximately 67% of fish were positive, by RT-qPCR for the detection of RGNNV in the eye with Ct values ranging from 23.70 to 35.42 regardless of experimental treatment group (Table 5). However only 31% of samples were positive if brain was tested, with Ct values ranging from ~26.7 to 39.09. The five fish from Tank 2 that were euthanised prior to the 14-week termination had RT-qPCR Ct values less than 30 (range 26-29) and produced CPE in virus isolation assays. Although all vaccine groups displayed similar absence of VER signs (>90% appeared healthy), fish from both of the Nodavax (Tréidlia Biovet) recombinant vaccinated groups returned a lower proportion of RGNNV detected in eye tissue by

both RT-qPCR and virus Isolation (Table 6). In addition, the Ct value of detections from the Nodavax-injected groups tended to be higher, suggestive of lower viral copy number in fish vaccinated with this vaccine. Positive virus isolations were detected from a small number of surviving fish in all experimental groups. It should be noted, there were only a small number of fish remaining in the cages from the adjuvant-only injected groups. Similar to the RT-qPCR results, there fewer fish positive for the detection of RGNVV with Nodavax in virus isolation assays. During the 31 day to 14-week samples, virus isolation results displayed a correlation with Ct value, whereby, samples with positive RT-qPCR results with a Ct value less than 30 in both eye and brain tissue, were positive by CPE in virus isolation assays.

Table 6: Viral loads determined by RT-qPCR and virus isolation from fish euthanised in Tank 2 (14 weeks post-challenge).

Experimental Group	RT-qPCR Ct value: Eye				RT-qPCR Ct value: Brain				No. RT-qPCR +ve		No. Virus Isolation +ve		No. Tested
	Min Ct	Max Ct	Average	Std.dev	Min Ct	Max Ct	Average	Std.dev	Eye	Brain	Eye	Brain	
Nodavax + Adjuvant 6	26.44	37.82	35.42	3.58	27.06	39.09	35.63	4.50	9	6	2	1	24
Nodavax + Adjuvant 7	26.58	39.56	35.76	3.05	27.18	38.16	34.82	3.49	16	8	1	1	25
Adjuvant 6	26.03	26.03	26.03	na	35.23	35.23	35.23	na	1	1	1	0	1
Adjuvant 7	24.57	26.27	25.47	0.85	31.67	34.00	32.84	1.64	3	2	2	0	3
JCU + Adjuvant 6	31.26	39.34	35.76	2.47	32.10	34.96	33.69	1.11	22	6	6	1	24
JCU + Adjuvant 7	23.70	38.01	33.79	5.05	26.69	36.90	34.16	3.45	16	8	5	1	23
All groups	23.70	39.56	32.77	5.12	26.69	39.70	34.58	3.11	67	31	17	4	100

RT-qPCR results are presented as Cycle threshold (Ct) values, including maximum (Max), minimum (Min), average and standard deviation (Std.dev). Number (No.) of positive (+ve) results for RT-qPCR, virus isolation and tested are also indicated for each experimental treatment group.

There were no morbidities in Tank 3 during week 14 to 17 but 51/99 (~51%) fish that were tested, were positive for the detection of RGNNV by RT-qPCR (Table 6). Eye tissue was the most frequently positive tissue with only 20/99 (20%) brain tissues resulting in a positive detection of RGNNV by RT-qPCR. Average Ct value was similar and very low for either tissue type, 34.97 for eye and 34.02 for brain. Nodavax with adjuvant 7 (ISA763A) had the lowest proportion of positive detections of RGNNV, of which only 1 sample was also positive in virus Isolation (Table 7). Although the frequency of detection of RGNNV by RT-qPCR was quite high (51%) only approximately 12% of fish were positive for the detection of RGNNV by virus isolation (VI) at week 17. All of the tissues that were positive in VI samples originated from the eye of vaccinated fish. None of the four adjuvant only fish were positive for virus isolation, though this may reflect the very low number of control animals sampled at 17 weeks. CPE was only evident from one of the tissue culture wells from the twelve samples that were VI positive. The remaining samples were positive in VI based on RT-qPCR Ct value being approximately the same as that of the tissue homogenate rather than due to presentation of CPE (Table 7).

Table 7: Viral loads determined by RT-QPCR and virus isolation from fish euthanised in Tank 2 (14 weeks post-challenge).

Experimental Group	RT-qPCR Ct value: Eye				RT-qPCR Ct value: Brain				No. RT-qPCR +ve		No. Virus Isolation +ve		No. Tested
	Min Ct	Max Ct	Average	Std.dev	Min Ct	Max Ct	Average	Std.dev	Eye	Brain	Eye	Brain	
Nodavax + Adjuvant 6	31.17	36.87	33.69	1.69	35.46	39.28	37.58	1.94	12	3	5	0	23
Nodavax + Adjuvant 7	37.51	39.45	38.14	0.78	38.69	38.69	38.69	n/a	5	1	1	0	23
Adjuvant 6	39.44	39.44	39.44	n/a	n/a	n/a	n/a	n/a	1	0	0	0	2
Adjuvant 7	36.79	38.69	37.74	1.34	n/a	n/a	n/a	n/a	2	0	0	0	2
JCU + Adjuvant 6	32.67	38.37	34.70	1.59	34.14	35.55	34.71	0.52	16	6	1	0	25
JCU + Adjuvant 7	33.82	35.65	34.55	0.59	31.18	32.46	32.07	0.43	15	10	5	2	24
All groups	31.17	39.45	34.97	1.93	31.18	39.28	34.02	2.42	51	20	12	2	99

N/A. Not applicable (no survivors to sample at 14 weeks post-challenge).

5.8. Vaccine dose response and onset of protection trial at UQ

A pre-challenge dose-titration trial using filtrate prepared from infected brain and eye tissue homogenate diluted from 0-100,000 fold indicated that the dose began to titrate in terms of morbidity between a 100 and 1000 fold dilution of the filtrate (Figure 18, Q1.0). Intramuscular injection with 50 µL of a filtrate dilution of 500 x was therefore chosen for each challenge.

In the challenge conducted one- and two-weeks post-vaccination, there was no significant effect of vaccination compared to controls with high morbidity across all treatment groups (Mantel Cox log-rank, $P>0.05$, Figure 18, Q1.1 and Q1.2). By week three post-vaccination at 27°C there was significant protection against challenge ($P<0.0001$) in the 6x and 8x antigen doses with ISA763A adjuvant and the 2x and 8x dose with the ISA660 adjuvant (Figure 18, Q1.3). At week 4, protection was only achieved by the vaccine adjuvanted with ISA660 and this was only marginally significant ($p=0.0489$), although morbidity in fish vaccinated with 8x antigen in emulsified in ISA660 occurred late in the experiment, more than 30 days post challenge and after 58 days in the RAS (Figure 18, Q1.4). By week 6 there was no significant protection afforded by any of the treatment groups (Figure 18, Q1.5). However, due to dislodgement of a tank outlet pipe by the fish in one tank in the holding system the day after vaccination, sixteen fish from the group vaccinated with 8x antigen adjuvanted with ISA660 (group 9) escaped into the sump and were euthanised due to injury from the pipework or asphyxiation in the filter bags. Consequently, there were no animals remaining in this treatment group for the week 6 challenge.
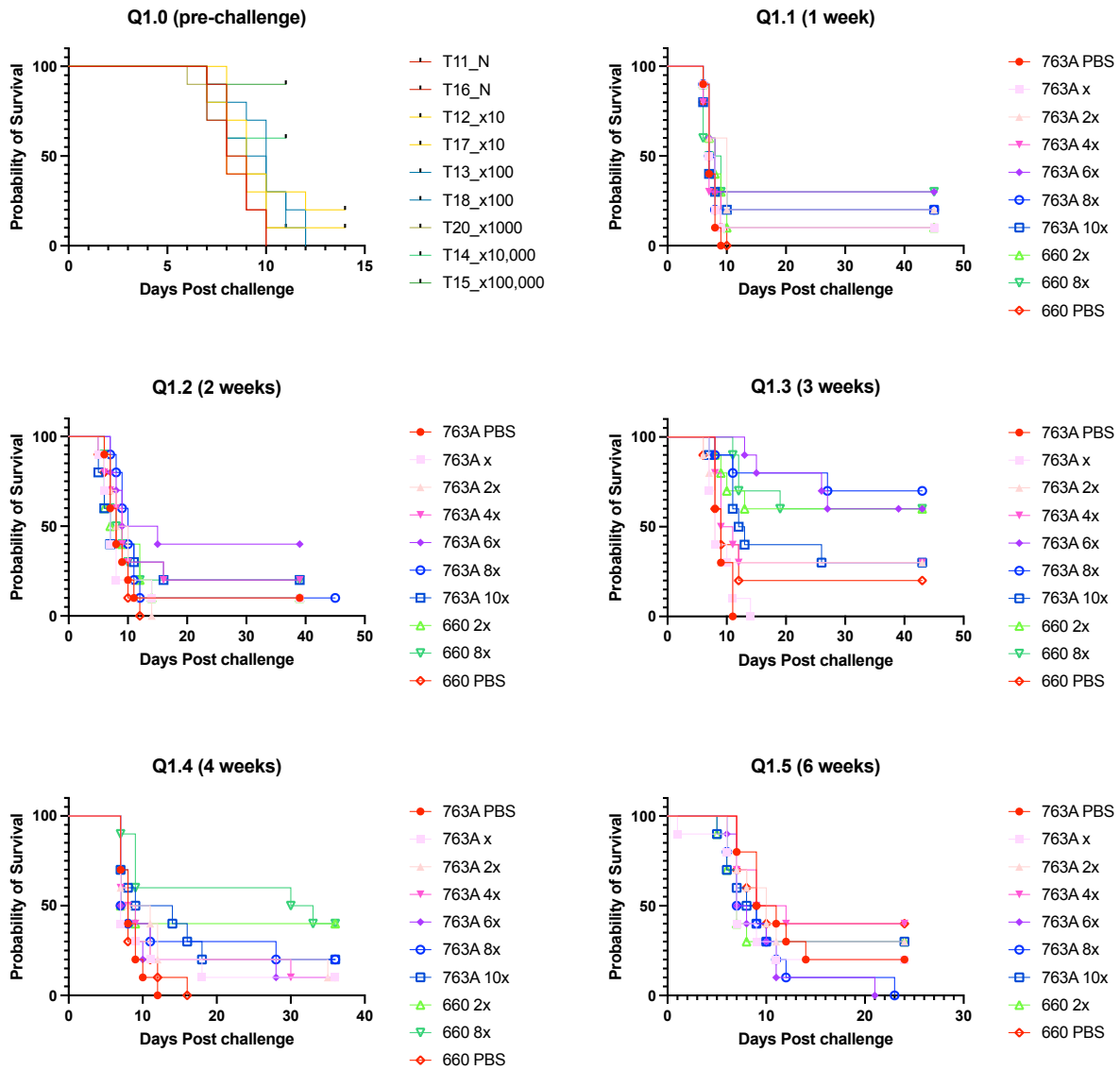
Figure 18. RGNNV challenge models in Giant Grouper.
Q1.0 Pre-challenge of unvaccinated fish to calibrate dose. Q1.1-1.5 morbidity post-challenge of vaccinated and respective control fish at 1-, 2-, 3-, 4- and 6-weeks post-vaccination by IM injection with 50 µL of a 500 x dilution of infectious tissue filtrate (see methods). Vaccine types: 763A= Montanide ISA763A VG adjuvant; 660= Montanide ISA660 VG adjuvant; *n*x: multiple of antigen concentration; antigen: capsid recombinant protein used in Nodavax

6. Discussion

6.1.   A safe subunit NNV vaccine was rapidly produced which stimulates anti-NNV antibody production in farmed giant grouper

A severe outbreak of VER caused by RGNNV in large farmed giant grouper at a farm in SE Queensland in the summer of 2018 precipitated the need for rapid intervention and vaccination was proposed as a preventative strategy. Australia has very strict biosecurity legislation so any imported animal derived products used in vaccine preparation for subsequent use in animals or discharge into the environment must be demonstrated to be free of bacteria, viruses and TSE, and then approved for use by the Australian Pesticides and Veterinary Medicines Authority. No permissive cell-lines for RGNNV are approved by the Commonwealth biosecurity regulator, the Department of Agriculture, Fisheries and Forestry, at the time, ruling out the timely production of an inactivated whole virus vaccine. As a consequence, a decision was taken to produce a recombinant capsid vaccine using an expression vector, bacterial expression system and fermentation media already approved by DAFF and APVMA, and in use locally in a livestock vaccine. Moreover, a very similar nodavirus vaccine had been developed overseas during the late 1990s/early 2000s as part of a commercial comparison of recombinant, DNA and inactivated nodavirus vaccines, in which inactivated lysates of *E. coli*, expressing recombinant constructs, were moderately effective (40-70% RPS) in controlled challenges in European sea bass (Dos Santos NMS, Ireland J, Simard N, Barnes AC, Horne MT, Aqua Health Ltd, unpublished); and (Dos Santos et al. 2004). Adopting this previously tested approach proved time efficient from the initial concept in May 2018, followed by vector construction and optimisation in July and August that same year, manufacture and safety testing in August and September and final deployment of the first commercially produced vaccine, Nodavax, on the farm in November under a restricted use permit from the APVMA in October 2018, a period of less than 6 months. Subsequently, in May 2019, a similar vaccine produced in Spain was reported to have a protective effect in tank trials (Gonzalez-Silvera et al. 2019).

The vaccine was determined to be safe for fish using 2x the normal dose adverse reaction test. As a proxy for efficacy, the ability to prime immune memory for the virus was investigated under controlled conditions. The vaccine antigen rapidly stimulated the production of specific anti-NNV IgM in juvenile fish, and secondary exposure to antigen three and a half months post-vaccination further stimulated specific antibody quantity and avidity. Anti- NNV neutralizing antibodies in *E.*

coioides larvae have been reported just one week post vaccination, with VLPs peaking at 7 weeks (Lai et al. 2014). Specific antibodies raised against a virus are considered to be a good indicator of protection in fish (Munang'andu and Evensen 2018) and have been associated with viral neutralization in NNV vaccine research (Costa and Thompson 2016).

The longitudinal specific serum antibody data from field trial vaccinated fish batches 1 and 3 showed vaccination enriched the pool of anti-NNV IgM antibodies in farmed grouper, especially in younger fish. In batch 3, vaccinated fish, weighing on average 85 g in the farm production RAS in late August 2019, had significantly higher levels of anti-NNV IgM three months post vaccination, than their unvaccinated counterparts. Most unvaccinated control fish had negligible levels of specific anti-NNV IgM. Antibodies detected in control fish are likely natural IgM, which are a normal non-specific defence in fish that increases with age (Magnadottir et al. 2009). After transfer from the production RAS to the lake in October, the difference in anti-NNV IgM between the two groups became more marked, likely due to the presence of virus in the waterbody. Wild fish in the lakes include mullet (*Mugil cephalus*) which are known to carry sub-clinical endemic *Betanodavirus* infections in the wild. This implies vaccinated fish may be better prepared to combat virus in the lake and have more anti-NNV antibody reserve if environmental circumstances increased vulnerability. Indeed, in December, there was a drop in anti-NNV IgM detected which may indicate some antibody consumption arising from increased viral loads that had occurred. Antibody may have been used to keep the virus at bay during the summer months, preventing outbreaks among all the fish, which were stocked adjacently, vaccinated fish comprising 75% of the total in the group. The farm has continued with the vaccination programme, and has had no VER outbreaks linked to a temperature and hypoxia since adverse events that occurred in batch 1 in 2019.

6.2.   Since deployment of the vaccine on farm, the only subsequent VER outbreak was greatly reduced in severity

Comparing the outbreak in vaccinated fish (January 2019) with the previous outbreak (March 2018), in which no fish were vaccinated, the contrast in outbreak dynamics is striking. In March 2018, the temperature was mild (24.5 to 26.7 °C) and the oxygen saturation was not critical, but NNV infection took off rapidly with severe effect in the as-yet unvaccinated stock. Emergency harvesting was performed to mitigate losses, with cumulative mortalities 86- 100 %. In January 2019, the average percentage oxygen saturation was < 50% in the week prior to outbreak in vaccinated stock (Batch 1), reaching a minimum 38.4% saturation in the morning. The DO was

consistently below 4 mg L^{-1}, and 3 mg L^{-1} the morning before rapid levels of mortality began. Temperatures were ~29°C, but the infection was quickly brought under control by introducing a current mover to aerate the water. Fish recovered without extended mass mortality. Cumulative mortality was 14.1%. and emergency harvesting was not required. Considering an infection outbreak as a multi-factorial, complex event, the main differences in the March 2018 outbreak scenario compared to January 2019 were: the stocking density was much higher, estimated at 40-60 kg m^{-3} compared to 4.5-7.5 kg m^{-3}, but water quality parameters were adequate; the fish were substantially larger, averaging ~840 g compared to ~350 g, and the fish were not vaccinated.

It is concerning that outbreaks occurred in larger juveniles and that the mortality was so severe in 2018. Titres of natural antibodies increase with age and fish with more mature immune systems are considered less susceptible than young fish. Severe outbreaks of VER are typically associated with larvae and juvenile fish, before full immunocompetency is acquired (Costa and Thompson 2016). The vaccine, nevertheless, appears to have substantially increased fish resilience to adverse events, which of themselves increase vulnerability to infection.

6.3.   Multiple factors contribute to outbreaks and survival

Continuing with the vaccination program, the farm did not experience further NNV outbreas after the VER outbreak in summer 2019 until summer 2022, when a persistent low-level infection occurred. Since NNV is endemic, the possibility of infection is always present during cage grow-out, particularly in summer months when the temperature is optimal for viral replication and a stressor for fish. Viral multiplication rate correlates with mortality in fish (Nishizawa et al. 2012). Thus, if viral load exceeds a threshold beyond which the infection can be contained by protective immunity, outbreaks will occur. This threshold can be lowered by environmental stresses which compromise the immune system (Tort 2011). As described above, in January 2019, the average percentage oxygen saturation was moderately hypoxic over an extended period. Moreover, the temperature was high and at elevated temperatures oxygen consumption rate increases in hybrid groupers (*E. fuscoguttatus* ♀ x *E. lanceolatus*)(TGxGG) (Das et al. 2021). But, if we compare a year later in January 2020, there were also several consecutive days of low % oxygen saturation, yet there was no outbreak in vaccinated fish comprising batch 3. In 2020, the temperature was 1-1.5 °C lower than in 2019 at the time of low % oxygen saturation, but the minimum DO was even lower than the previous year, reaching 2.7 mg L^{-1} one morning, when fish would be under oxygen stress. Fish were also larger in 2020, on average ~400 g compared to ~300 g in 2019, which should increase vulnerability to hypoxia. However, fish were not infected by the virus (negative by

TaqMan RT-qPCR). It is likely that there was less virus reservoir present in the lake in 2020 since successive cohorts of farmed fish had been vaccinated and likely resulting in a reduced viral load in the environment. Vaccination timing may also be important; fish batch 1 were immunized approximately two months prior to outbreak in January 2019, while in January 2020, fish of batch 3 had been vaccinated 7 months earlier and had been exposed to virus in the lake since October 2019. This was accompanied by an increase in specific anti-NNV antibodies.

Diet likely had impacts on fish health. In general fish had very large pale livers regardless of vaccination or disease status. Shortly prior to the January 2019 outbreak, histopathological examination of livers revealed inflammation, thick bile ducts, lipidosis and glycogen retention. Excessive hepatic glycogen deposition can directly injure hepatocytes and affect liver function. In carnivorous fish this is mainly attributed to high dietary carbohydrate intake (Goodwin et al. 2002, Deng et al. 2018). The feed in use was estimated to exceed 10-12% carbohydrate (likely ~16%) and liver pathology was evident in all fish sampled. Some fish also showed intestinal oedema. In hybrid grouper (TGxGG) dietary carbohydrate above 11.49% depresses serum lysozyme and reduces serum protein content, including IgM, as well as impairing the antioxidant capacity (Li et al. 2020). We consider that the formulation of the diets that were fed to the first three batches of vaccinated fish trialled was suboptimal, especially for batch 1. Gross clinical and histopathological evidence indicated diet caused considerable pathology in the liver and intestine of grouper sampled over the period. As a result, diets were reformulated to contain less carbohydrate (requested 8%, achieved 10 %) and were supplemented with 1% taurine, with a concomitant reduction in gross liver pathology. However, non-alcoholic fatty liver disease is also described as a consequence of exposure to xenobiotic compounds such as pesticides, which can be present as residue on raw materials. Changes in raw material inclusion rates in diets may alter the residue exposures.

6.4. The southeast Queensland NNV isolate is very similar to RGNNV isolates from southeast Asia

A high level of similarity in RNA coding sequence and in translated protein (99%) has been reported for RGNNV RNA2 in grouper across the Indo-Asian region (Knibb et al. 2017). This could be due to evolutionary constraints or homogenization via transportation of hosts within the region. However, the virulent reassortant strain RGNNV/SJNNV has recently been reported for the first time in Southeast Asia, with RGNNV/SJNNV detected in hybrid grouper (TGxGG), in the water and in feed in a Malaysian hatchery (Ariff et al. 2019). Translated RNA1 and RNA2 sequences from

the present grouper isolate were 99% identical to several RGNNV isolates from southeast Asia in a number of species. For instance, RNA dependent RNA polymerase (RNA1) had 99.49% identity with a cultured tiger grouper (*E. fuscoguttatus*) NNV isolate from Malaysia, (AEK48161.1) and capsid protein (RNA2) had 99.7% identity with an isolate from hybrid grouper in Taiwan (ATA63006.1). In the latter, the single amino acid change in the capsid was conservative (isoleucine to valine), and not in the protrusion (P) region of the protein in the C terminus which mediates entry into the host and is a major immunoreactivity determinant (Ito et al. 2008, Chen et al. 2015, Panzarin et al. 2016). This high degree of similarity implies the current subunit vaccine is likely relevant for use further afield.

6.5.   Commercial uptake and continued supply of Nodavax

The two farms which have rapidly deployed the subunit vaccine in response to clinical outbreaks of disease have continued to use it across repeated intake of cohorts. The use of experimental vaccine in combination with changes to husbandry and water quality management have continued to align with reduced impacts from clinical NNV outbreaks in the two different culture system environments of an intensive RAS and cages in outdoor saline lakes.

Extended experimental permits have allowed examination of the vaccine's effectiveness across seasons and different systems. Outbreaks of clinical disease in vaccinated fish have been of reduced severity, such that commercial production was able to be economically sustained and expanded to service a growing market demand for the species.The data from this project will be used to apply for an approval for the use of the subunit vaccine from APVMA under a Minor Use Permit.

6.6.   The vaccine was shown to be highly effective in retrospective controlled challenge trials in juvenile grouper.

Vaccination with the recombinant subunit vaccine resulted in protection greater than 90% in a challenge that resulted in 92-93% mortality in adjuvant-only injected controls. Subunit and killed vaccines are not expected to be sterilising vaccines, and virus nucleic acid was detected in some vaccinated survivors 17 weeks post-challenge by RT qPCR but at very high Ct values (low virus concentration). Viral retention within vaccinated fish may provide a source of re-infection and provide opportunity for viral recombination. Indeed, recombinant RGNNV/SJNNV has been detected in infected gilt-head sea bream (*Sparus aurata*) one year post-infection (Toffan et al. 2017).

One disadvantage of recombinant vaccines expressed in *E. coli* is that if the protein expressed is a glycoprotein, the glycosylation may be incorrect or even absent when expressed in *E. coli*, so rendering the antigen ineffective or less effective when improperly configured. Carbohydrates are important immunogens in fish. Consequently, proteins in killed virus vaccines, in which the virus is grown in fish cell lines, will presumably be glycosylated similarly to that which occurs when replicating in the fish. This means that a killed vaccine should be more effective than a bacterially expressed subunit. In the trials at JCU, there was no significant difference in protection between the heat killed virus or the bacterially expressed protein. Killed viral vaccines carry additional risk compared to subunits expressed in *E. coli* and therefore require additional regulatory evidence of safety. This includes not only demonstrating that the virus itself has been completely inactivated, but also that any known or unknown viruses or other infectious agents that may be in the cell lines used for culture are also inactivated. This can be challenging, particularly if the cell lines used for culture are exotic to Australia, such as the striped snakehead cell-line SSN-1 used for culturing *Betanodavirus*, as this constitutes a biosecurity risk. SSN-1 contains a retrovirus that makes it permissive for *Betanodavirus* (Iwamoto et al. 2000, Lee et al. 2002, Nishizawa et al. 2008). It is unknown whether this retrovirus can be transmitted to Australian fish species, or whether it is an infectious risk.

In the single kinetics trial, the immunity to nodavirus provided by the recombinant vaccine was constrained to the period when there are high levels of circulating serum antibody, from about 500 degree days to 750 degree days in the kinetics study. This short duration of protection is not because immune memory has failed to establish as we were able to induce a secondary response with purified antigen in vaccinated fish brought in from the farm; this response would not have been achieved if the primary immune response had not been first achieved. There are two plausible explanations for failure to protect for longer. The first is that the immune memory-based secondary response occurs too slowly after infection with virus to prevent disease occurring because, for example, the virus internalises in host cells before the secondary antibody concentration has elevated sufficiently. The second is that there may be insufficient antigen present during a natural virus infection (as opposed to injection with purified antigen) to elicit the secondary response. That is, no secondary response occurs at all under viral infection. Regardless, this suggests that the mode of protection of the vaccine is primarily by circulating antibody "mopping-up" virus and preventing severe infection from developing and allowing fish to recover. It is interesting that the vast majority of vaccinated fish that were protected in challenges three and four weeks post-vaccination continued to survive until the trial was terminated at 10 weeks in

spite of introduction of newly challenged fish, presumably shedding virus, into the same tanks in subsequent challenges. While the failure to protect at week six post vaccination is only a single datapoint and should be repeated, the observation in the experiment is consistent with continued intervention on the farm to manage persistent low-level outbreaks in vaccinated fish. Some further work to modify the formulation or delivery regimen to improve later protection is strongly recommended. This might include the evaluation of several different adjuvants, including ISA660, over longer challenge models, and also the use of a prime-boost strategy for vaccination. Further, increasing the concentration of antigen relative to *E. coli* lysate via purification to varying degree may improve the immune response.

7. Conclusion

The impetus for this project was to rapidly develop and administer a safe vaccine against *Betanodavirus* on a farm where the need for disease control was urgent. The efficacy of the vaccine in controlled challenge models exceeded 90% with viral load reduced close to the limits of detection in vaccinated fish 17 weeks post-infection. The usefulness of this simple recombinant subunit NNV vaccine is supported by the longitudinal farm data, in spite of the uncontrolled environmental variables. The speed of vector design and build, manufacture, test and deployment of the final vaccine is supportive of both the adoption of the existing platform technology and the flexibility of the Australian restricted use permit system for biological products. This is a regulatory model that could be replicated overseas to accelerate vaccine deployment to small and medium enterprise farmers throughout the region, where there is increasing focus on disease prevention in aquaculture. Most importantly, this longitudinal study illustrates the multifactorial nature of fish health, including stocking density, water quality and, most likely, the impact of incorrectly formulated diets. The farm trial results indicate the current non-purified subunit vaccine against NNV coupled with improved husbandry practice and continued veterinary support has enabled control VER on the farm.

The kinetics laboratory trial suggests that protection is useful after initial transfer from hatchery to farm and on farm data suggests that vaccination is beneficial. However, in a controlled trial at UQ, fish were not protected from direct challenge at 6 weeks, although effectively protected at 3 and 4 weeks. This timing correlates almost perfectly with the primary serum antibody response, implicating a necessity for circulating serum antibody for protection: Induction of a secondary antibody response based on immune memory, which the vaccine does establish in grouper, did not apparently offer good protection against disease. There are several adjuvants that are in late-stage development and have approval for use in animals that have shown promise in barramundi and grouper by extending substantially the primary antibody response (see Li et al manuscript in Appendix). As the required antigen dose has been determined by the trials in this project, a full investigation of alternative adjuvants and also alternative delivery regimes (the delivery and timing of a booster vaccination, for example) is warranted.

8. Implications

The availability of an efficacious NNV vaccine underpins the viability of the Australian grouper industry. Prior to the creation of the subunit vaccine, several growout operations failed due to NNV mortalities rendering them unviable.

The deployment of the subunit vaccine and improved management of the disease has created the opportunity for industry to gain confidence to invest in expanded grouper production to meet the developing market demand for this premium quality fish. Through the course of the project the volumes of production have continued to increase to the present.

9. Recommendations

- A minor use permit for the present formulation (dose and adjuvant) should be prioritised. The vaccine is useful in practice

- An investigation of alternative dosing regimes (prime-boost) to extend protection for the full farm cycle

- An investigation of available or close-to-market alternative adjuvants that extend the primary antibody response from vaccination and may improve protection late in the cycle.

- Establishment of an intellectual property framework to manage and secure the continued manufacture of the vaccine on behalf of the nascent grouper aquaculture industry.

10. Extension and Adoption

The vaccine is in commercial use, under veterinary prescription. It is used at TCO for fish supplied to Rocky Point and Noosa Ecomarine. It is also used by TCO in fingerlings for export. Vaccine pricing is proprietary, but the farms make a business decision based on the potential savings of reduced mortality. There was substantial veterinary input to the farm and hatchery during this project from Matt Landos and James Fensham of Future Fisheries Veterinary Services. Input extended well beyond the prescription and direction of vaccination with Nodavax and included substantial input into animal management to mitigate impact of diseases and parasites. Further, veterinary investigation of the feed formulations coupled with lobbying of the manufacturers to improve consistency and quality had substantial impact on the farm. Notably, changed practices have been adopted on the farm that has likely had substantial impact in the survival of fish through growout.

11. Glossary

Term	Explanation
Adjuvant	An additive to a vaccine that changes the properties of the vaccine so that the immune system reacts in a different way
Amplicon	The specific DNA product of a PCR (see PCR)
Antibody	A complex molecule with a variable region that can bind to molecular shapes or structures very specifically. The molecule also comprises a constant region that is recognised by cells in the immune system enabling recognition of whatever the antibody is bound to for further processing such as, for example, destruction.
Antigen	One component of a vaccine to which the immune system responds specifically, for example by production of antibodies that bind to the antigen. In terms of a vaccine it is the component that stimulates the specific immune protective effect.
Buffer	A solution containing chemicals that impede changes in pH. They are useful where pH needs to be constant. Choosing the correct buffer is complex. There are entire textbooks on buffers.
Chromatography	A means of separating substances in solution. In its simplest form (from which the technique derives its name) a dye is spotted onto a filter paper and a solvent applied to one end. The components of the dye separate as the solvent migrates revealing the complexity of the mixture. In the present study we used fast protein liquid chromatography in which the solvent is water or an aqueous buffer. In this case, the chromatography paper is replaced by a column packed with porous beads. The solvent (buffer) is pumped through the column over the beads at a fixed flow rate and the mixture of proteins applied to the top of the column. The proteins in the mixture separate based on their physicochemical properties, such as size (gel filtration), electrical charge (ion exchange), or how tightly they bind to the beads in the column (affinity). By collecting small fractions of the buffer as it flows through, proteins can be very effectively purified. Detection of protein peaks is achieved via a UV lamp and detector after they pass through the end of the column.
Degree days (°days)	The temperature in degrees centigrade multiplied by the time in days. This is a measurement of physiological time that is particularly relevant in cold-blooded animals, such as fish, because processes happen more slowly at low temperature than at high temperature. In a fish, a lot more happens in a week at 30°C than in a week at 10°C. The former would represent 210 °days while the latter only 70°days – a much shorter physiological time. By measuring in degree days we can make quite robust comparisons between different systems with variable temperatures.

DO%	The dissolved oxygen content of a liquid (e.g. farm water) measured as a percentage of saturation at the temperature and pressure. This is a useful way of reporting oxygen content because it is independent of temperature and pressure and it reflects how much might be available for the animals in the water to use, enabling direct comparisons between different systems that is useful in management.
Histology	The study of tissues at the cellular level. This is done via microscopy following staining of very thin sections of preserved tissue. Histopathology is the description of abnormality within histology sections and is a useful primary determinant of the health status of animal.
Immunoglobulin	Immunoglobulin (Ig) is a family of complex protein molecules that includes those that form antibody. There are several types of immunoglobulin that occur in different parts of an animal and they are named based on the largest component, the heavy chain, that comprises the complex immunoglobulin protein. In fish, the major circulating antibody found in the blood is Immunoglobulin M (IgM). In mammals, IgM is the major circulating antibody in infants, but in juveniles and adults, refinement of the immune system during formation of the antibody response means that IgM production ceases in favour of production of IgG. In this project, we used IgG raised in rabbits to detect the IgM from grouper blood so that we could measure the grouper response to the vaccine.
Inclusion bodies	Crystals of protein that form in bacteria, generally when they are asked to produce a protein that they don't usually produce in large amounts. It is a safety mechanism for the bacteria, but very useful if we want relatively pure protein crystals. It is problematic if the protein is needed in soluble form. In this case the bacteria might be grown at very low temperature so that the protein forms slowly and is less likely to form inclusion bodies.
PCR	Polymerase chain reaction. A very versatile method for amplifying very small quantities of DNA very specifically. It can be used for detection of genetic material for pathogens as a diagnostic tool or, as in the present study, to produce specific pieces of DNA in sufficient quantity that can be recombined to make something that you want, such as a subunit vaccine vector. We amplified the DNA for the nodavirus coat protein using PCR, and we amplified the bits of pET28 that we wanted, also by PCR.
Plasmid	Circular DNA, often found in bacteria, but also in the environment that is very stable. Naturally occurring plasmids allow exchange of genetic information between bacteria, such as antibiotic resistance, or the ability to use unusual nutrients. Because of their stability and the ease of transfer into bacteria they are useful tools in molecular biology
Primer	A short piece of DNA, often about 15-25 nucleotides in length, that precisely matches the end of a piece of DNA that one wishes to

	amplify by PCR. For most purposes, two primers, one matching opposite sides of each end of the target, are used.
Recombinant	DNA that has been modified by inserting or adding a piece of DNA from somewhere else. This can be somewhere else on the same chromosome, or it can be from a completely different taxon. The vaccine in this study is a recombinant vaccine because the piece of DNA that encodes the Betanodavirus coat protein has been "recombined" with plasmid pET28 by genetic engineering and then placed back into the bacterium E. coli so that the coat protein is expressed by the bacteria, usually as crystalline protein particles called inclusions or inclusion bodies (see Inclusion bodies)
Reverse transcription	It is not simple to amplify RNA directly. Nodavirus genetic material is RNA, it is an RNA virus. If we want to amplify nodavirus genetic material we first have to convert it to complementary DNA. This process is called reverse transcription and uses an enzyme called, unsurprisingly, reverse transcriptase.
RT-qPCR	Reverse transcriptase quantitative real-time PCR. A variation of polymerase chain reaction in which reverse transcription is performed either separately or in a single step with the PCR. The quantitative part of the PCR comes from the employment a fluorescent dye in the reaction that is incorporated into the amplicon in real time and is measured providing a kinetics curve. This makes it possible to use mathematical models to estimate how many copies of the target were in the original sample, which is quite useful. Because it produces a characteristic curve. Analysis of the curves can also tell you whether the amplification was specific, making qPCR a very rigorous diagnostic assay when performed and interpreted by competent scientists.
SDS-PAGE	Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis is a way of separating proteins in a mixture based primarily on their mass. Proteins are denatured in the detergent SDS which strips them of their native charge and applies a uniform positive charge to the proteins. A small volume of the protein mixture is then applied into a well in the top of a gel made from a mixture of acrylamide and methylene bisacrylamide and an electric current is applied across the gel. The polymerised acrylamide acts like a molecular sieve, with the pore size determined in part by the percentage acrylamide and also by the ration of acrylamide to bisacrylamide. The proteins are drawn towards the negative electrode but travel at different speeds through the acrylamide, based on their size. A ladder like pattern of proteins is formed in the gel with the larger proteins migrating the shortest distance while the smaller proteins migrate further through the gel. The gel can be stained with a variety of stains to visualise the ladder.
TaqMan	A very specific chemistry for qPCR that uses a proprietary probe, dye and quencher technology from Thermo Fisher Scientific, For TaqMan based qPCR, three oligonucleotide components are used,

	two amplification primers that match the ends of the target, and a third "probe" primer that matches somewhere within the target amplicon and has a fluorescent dye tag and quenching molecule attached. On incorporation into the extending target, the quenching molecule is released from the probe, permitting detection of the fluorescent tag.
Western blot	A process of identifying proteins using antibodies. Proteins are separated by SDS-PAGE, but instead of staining ALL the proteins directly in the gel, they are transferred by "blotting" onto a charged membrane such as nitrocellulose or polyvinylidene fluoride (PVDF) where they can by incubated with specific antibodies that are then detected via enzymes and chromogens to produce (a) coloured band(s). This enables very specific identification of proteins in complex mixtures. Blotting can be used for many purposes, but "Western" specifies that this blot is for immunostaining with antibody.

12. Project materials developed

Table 7: List of tangible outputs

#	Output	Details	Access
1	Recombinant vaccine	A recombinant nodavirus vaccines comprising lysed inactivated E.coli T7 expressing the RGNNV nucleocapsid protein. Marketed as Nodavax	Available by veterinary prescription via Tréidlia BioVet
2	Recombinant protein plasmid for RGNNV nucleocapsid	Complete nucleocapsid protein with 6 x Histidine tag to facilitate purification by chromatography has been cloned in plasmid pET28A+ and is stored in E.coli at The University of Queensland (UQ)	By request from UQ at cost
3	Heat inactivated vaccine	Prepared by James Cook University from cultured virus in SSN-1 E-11 cell lines. For laboratory use only.	By request from JCU subject to DAFF permit for use in live animals
4	Rabbit anti-Grouper IgM antiserum	A high affinity rabbit IgG raised against purified IgM from giant grouper. Aliquots are stored at The University of Queensland and they may be used for various immunological assays, including ELISA and Western blot	By request from UQ and at cost
5	Various immunology assays	Biolayer interferometry for detection of antibodies in various species of fish and other animals, including humans	By request from UQ and at cost
6	RGNNV sequences	Sequences of the complete genome of isolates from Rocky Point Aquaculture	NCBI accession numbers: MZ054261 and MW590702
7	Diagnostic test for NNV	In house diagnostic for NNV set up based on Hick and Whittington 2010, recommended by OIE. Method has independently achieved the same results as Queensland Biosecurity Science laboratory	Protocol in UQ lab available free on request
8	Recombinant bacterial isolates	QMA0553-7. Bacterial isolates containing pET28+ expressing RGNNV nucleocapsid sequences.	Held in UQ strain collection with copies at Tréidlia BioVet. Available to FRDC stakeholders from UQ by agreement and at cost.

		QMA0644 Bacterial isolate containing diagnostic reference plasmid for calibration of RT-qPCR.	
9	Scientific paper	Li, A., R. Thwaite, S. Kellie, and A. C. Barnes. (2021). Serum IgM heavy chain sub-isotypes and, light chain variants revealed in giant grouper (*Epinephelus lanceolatus*) via protein A affinity purification, mass spectrometry and genome sequencing. Fish Shellfish Immunol **113**:42-50.	DOI: 10.1016/j.fsi.2021.03.014
10	Scientific paper	Li A., Harris, RJ, Fry, BG., Barnes AC. (2021) A one-step dip-and-read Biolayer interferometry (BLI) assay to generate IgM real-time quantity and quality data directly from fish serum. Fish Shellfish Immunol. 119:231-237	DOI: 10.1016/j.fsi.2021.10.003
11	Scientific paper	Thwaite R, Li A, Kawasaki M, Lin C-H, Stephens F, Cherrie B, Knuckey R, Lanods M, Barnes AC (2021). A longitudinal field survey during deployment of an emergency autogenous vaccine against Betanodavirus in farmed giant grouper *Epinephelus lanceolatus*: Multiple factors contribute to outbreaks and survival. Aquaculture 548; 737599	DOI: 10.1016/j.aquaculture.2021.737599
12	Scientific paper	Oon CYJ, Rudenko O, Barnes AC and Thwaite R (in revision). Changes in innate antiviral immune gene expression in the brain of giant *grouper Epinephelus lanceolatus* during *Betanodavirus* outbreaks at a marine cage farm	Status: Short communication. In revision; Aquaculture
13	Scientific paper	Li A. Zoccola-Macfarlane E., Thwaite R., Barnes AC. (submitted Sept 22) Effects of oil-emulsion and gel adjuvant vaccines on antibody kinetics and avidity in barramundi (*Lates calcarifer*) and giant grouper (*Epinephelus lanceolatus*)	Status: Under review; Fish Shellfish Immunol.

13. Appendices

13.1. Appendix A: Recombinant protein vaccine design

Table A1: Primers used for NNV RNA2 sequencing and cloning of capsid protein coding region

Primer name	Sequence (5'–3')
pET28a+RC_F	CACCACCACCACCACCACTG
pET28NNVEco_F	gccatcatcatcatcatcacATGGTTCGTAAAGGTGAAAAAAAC
pET28Eco_Rnew	GTGATGATGATGATGATGGCTGCTGCTGCCC
pET28aT7_F	TTAATACGACTCACTATAGGG
pET28aT7_R	GCTAGTTATTGCTCAGCGG
NNVEcoStop_R	cagtggtggtggtggtggtgTTATTCGCTATCAACACGGGTAC
NNVEco_282F	TGCAGCACGTATTTTTCAGC
NNVEco_749R	ATATCCAGCGGTGTGCTACC
NNVRNA2_F1*	TCAMAATGGTACGCAARGG
NNVRNA2_R1*	TCACTGCGCGGAGCTAACGGTAAC

*Rasangan & Manin (2012)

Table A2: Complete sequence of vaccine plasmid pET28a+NNVEcoSTOP

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LOCUS         pET28a+NNVEcoSTOP         6271 bp

DEFINITION    pET28a+NNVEcoSTOP, Circular DNA 6271 bases.

ORIGIN

      1   TGGCGAATGG GACGCGCCCT GTAGCGGCGC ATTAAGCGCG GCGGGTGTGG

     51   TGGTTACGCG CAGCGTGACC GCTACACTTG CCAGCGCCCT AGCGCCCGCT

    101   CCTTTCGCTT TCTTCCCTTC CTTTCTCGCC ACGTTCGCCG GCTTTCCCCG

    151   TCAAGCTCTA AATCGGGGGC TCCCTTTAGG GTTCCGATTT AGTGCTTTAC

    201   GGCACCTCGA CCCCAAAAAA CTTGATTAGG GTGATGGTTC ACGTAGTGGG

    251   CCATCGCCCT GATAGACGGT TTTTCGCCCT TTGACGTTGG AGTCCACGTT

    301   CTTTAATAGT GGACTCTTGT TCCAAACTGG AACAACACTC AACCCTATCT

    351   CGGTCTATTC TTTTGATTTA TAAGGGATTT TGCCGATTTC GGCCTATTGG

    401   TTAAAAAATG AGCTGATTTA ACAAAAATTT AACGCGAATT TTAACAAAAT

    451   ATTAACGTTT ACAATTTCAG GTGGCACTTT TCGGGGAAAT GTGCGCGGAA
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 501    CCCCTATTTG TTTATTTTTC TAAATACATT CAAATATGTA TCCGCTCATG

 551    AATTAATTCT TAGAAAAACT CATCGAGCAT CAAATGAAAC TGCAATTTAT

 601    TCATATCAGG ATTATCAATA CCATATTTTT GAAAAGCCG TTTCTGTAAT

 651    GAAGGAGAAA ACTCACCGAG GCAGTTCCAT AGGATGGCAA GATCCTGGTA

 701    TCGGTCTGCG ATTCCGACTC GTCCAACATC AATACAACCT ATTAATTTCC

 751    CCTCGTCAAA AATAAGGTTA TCAAGTGAGA AATCACCATG AGTGACGACT

 801    GAATCCGGTG AGAATGGCAA AAGTTTATGC ATTTCTTTCC AGACTTGTTC

 851    AACAGGCCAG CCATTACGCT CGTCATCAAA ATCACTCGCA TCAACCAAAC

 901    CGTTATTCAT TCGTGATTGC GCCTGAGCGA GACGAAATAC GCGATCGCTG

 951    TTAAAAGGAC AATTACAAAC AGGAATCGAA TGCAACCGGC GCAGGAACAC

1001    TGCCAGCGCA TCAACAATAT TTTCACCTGA ATCAGGATAT TCTTCTAATA

1051    CCTGGAATGC TGTTTTCCCG GGGATCGCAG TGGTGAGTAA CCATGCATCA

1101    TCAGGAGTAC GGATAAAATG CTTGATGGTC GGAAGAGGCA TAAATTCCGT

1151    CAGCCAGTTT AGTCTGACCA TCTCATCTGT AACATCATTG GCAACGCTAC

1201    CTTTGCCATG TTTCAGAAAC AACTCTGGCG CATCGGGCTT CCCATACAAT

1251    CGATAGATTG TCGCACCTGA TTGCCCGACA TTATCGCGAG CCCATTTATA

1301    CCCATATAAA TCAGCATCCA TGTTGGAATT TAATCGCGGC CTAGAGCAAG

1351    ACGTTTCCCG TTGAATATGG CTCATAACAC CCCTTGTATT ACTGTTTATG

1401    TAAGCAGACA GTTTTATTGT TCATGACCAA AATCCCTTAA CGTGAGTTTT

1451    CGTTCCACTG AGCGTCAGAC CCCGTAGAAA AGATCAAAGG ATCTTCTTGA

1501    GATCCTTTTT TTCTGCGCGT AATCTGCTGC TTGCAAACAA AAAAACCACC

1551    GCTACCAGCG GTGGTTTGTT TGCCGGATCA AGAGCTACCA ACTCTTTTTC

1601    CGAAGGTAAC TGGCTTCAGC AGAGCGCAGA TACCAAATAC TGTCCTTCTA

1651    GTGTAGCCGT AGTTAGGCCA CCACTTCAAG AACTCTGTAG CACCGCCTAC

1701    ATACCTCGCT CTGCTAATCC TGTTACCAGT GGCTGCTGCC AGTGGCGATA

1751    AGTCGTGTCT TACCGGGTTG GACTCAAGAC GATAGTTACC GGATAAGGCG

1801    CAGCGGTCGG GCTGAACGGG GGGTTCGTGC ACACAGCCCA GCTTGGAGCG

1851    AACGACCTAC ACCGAACTGA GATACCTACA GCGTGAGCTA TGAGAAAGCG

1901    CCACGCTTCC CGAAGGGAGA AAGGCGGACA GGTATCCGGT AAGCGGCAGG

1951    GTCGGAACAG GAGAGCGCAC GAGGGAGCTT CCAGGGGGAA ACGCCTGGTA
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2001   TCTTTATAGT CCTGTCGGGT TTCGCCACCT CTGACTTGAG CGTCGATTTT

2051   TGTGATGCTC GTCAGGGGGG CGGAGCCTAT GGAAAAACGC CAGCAACGCG

2101   GCCTTTTTAC GGTTCCTGGC CTTTTGCTGG CCTTTTGCTC ACATGTTCTT

2151   TCCTGCGTTA TCCCCTGATT CTGTGGATAA CCGTATTACC GCCTTTGAGT

2201   GAGCTGATAC CGCTCGCCGC AGCCGAACGA CCGAGCGCAG CGAGTCAGTG

2251   AGCGAGGAAG CGGAAGAGCG CCTGATGCGG TATTTTCTCC TTACGCATCT

2301   GTGCGGTATT TCACACCGCA TATATGGTGC ACTCTCAGTA CAATCTGCTC

2351   TGATGCCGCA TAGTTAAGCC AGTATACACT CCGCTATCGC TACGTGACTG

2401   GGTCATGGCT GCGCCCCGAC ACCCGCCAAC ACCCGCTGAC GCGCCCTGAC

2451   GGGCTTGTCT GCTCCCGGCA TCCGCTTACA GACAAGCTGT GACCGTCTCC

2501   GGGAGCTGCA TGTGTCAGAG GTTTTCACCG TCATCACCGA AACGCGCGAG

2551   GCAGCTGCGG TAAAGCTCAT CAGCGTGGTC GTGAAGCGAT TCACAGATGT

2601   CTGCCTGTTC ATCCGCGTCC AGCTCGTTGA GTTTCTCCAG AAGCGTTAAT

2651   GTCTGGCTTC TGATAAAGCG GGCCATGTTA AGGGCGGTTT TTTCCTGTTT

2701   GGTCACTGAT GCCTCCGTGT AAGGGGGATT CTGTTCATG GGGGTAATGA

2751   TACCGATGAA ACGAGAGAGG ATGCTCACGA TACGGGTTAC TGATGATGAA

2801   CATGCCCGGT TACTGGAACG TTGTGAGGGT AAACAACTGG CGGTATGGAT

2851   GCGGCGGGAC CAGAGAAAAA TCACTCAGGG TCAATGCCAG CGCTTCGTTA

2901   ATACAGATGT AGGTGTTCCA CAGGGTAGCC AGCAGCATCC TGCGATGCAG

2951   ATCCGGAACA TAATGGTGCA GGGCGCTGAC TTCCGCGTTT CCAGACTTTA

3001   CGAAACACGG AAACCGAAGA CCATTCATGT TGTTGCTCAG GTCGCAGACG

3051   TTTTGCAGCA GCAGTCGCTT CACGTTCGCT CGCGTATCGG TGATTCATTC

3101   TGCTAACCAG TAAGGCAACC CCGCCAGCCT AGCCGGGTCC TCAACGACAG

3151   GAGCACGATC ATGCGCACCC GTGGGGCCGC CATGCCGGCG ATAATGGCCT

3201   GCTTCTCGCC GAAACGTTTG GTGGCGGGAC CAGTGACGAA GGCTTGAGCG

3251   AGGGCGTGCA AGATTCCGAA TACCGCAAGC GACAGGCCGA TCATCGTCGC

3301   GCTCCAGCGA AAGCGGTCCT CGCCGAAAAT GACCCAGAGC GCTGCCGGCA

3351   CCTGTCCTAC GAGTTGCATG ATAAAGAAGA CAGTCATAAG TGCGGCGACG

3401   ATAGTCATGC CCCGCGCCCA CCGGAAGGAG CTGACTGGGT TGAAGGCTCT

3451   CAAGGGCATC GGTCGAGATC CCGGTGCCTA ATGAGTGAGC TAACTTACAT
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3501   TAATTGCGTT GCGCTCACTG CCCGCTTTCC AGTCGGGAAA CCTGTCGTGC

3551   CAGCTGCATT AATGAATCGG CCAACGCGCG GGGAGAGGCG GTTTGCGTAT

3601   TGGGCGCCAG GGTGGTTTTT CTTTTCACCA GTGAGACGGG CAACAGCTGA

3651   TTGCCCTTCA CCGCCTGGCC CTGAGAGAGT TGCAGCAAGC GGTCCACGCT

3701   GGTTTGCCCC AGCAGGCGAA AATCCTGTTT GATGGTGGTT AACGGCGGGA

3751   TATAACATGA GCTGTCTTCG GTATCGTCGT ATCCCACTAC CGAGATATCC

3801   GCACCAACGC GCAGCCCGGA CTCGGTAATG GCGCGCATTG CGCCCAGCGC

3851   CATCTGATCG TTGGCAACCA GCATCGCAGT GGGAACGATG CCCTCATTCA

3901   GCATTTGCAT GGTTTGTTGA AAACCGGACA TGGCACTCCA GTCGCCTTCC

3951   CGTTCCGCTA TCGGCTGAAT TTGATTGCGA GTGAGATATT TATGCCAGCC

4001   AGCCAGACGC AGACGCGCCG AGACAGAACT TAATGGGCCC GCTAACAGCG

4051   CGATTTGCTG GTGACCCAAT GCGACCAGAT GCTCCACGCC CAGTCGCGTA

4101   CCGTCTTCAT GGGAGAAAAT AATACTGTTG ATGGGTGTCT GGTCAGAGAC

4151   ATCAAGAAAT AACGCCGGAA CATTAGTGCA GGCAGCTTCC ACAGCAATGG

4201   CATCCTGGTC ATCCAGCGGA TAGTTAATGA TCAGCCCACT GACGCGTTGC

4251   GCGAGAAGAT TGTGCACCGC CGCTTTACAG GCTTCGACGC CGCTTCGTTC

4301   TACCATCGAC ACCACCACGC TGGCACCCAG TTGATCGGCG CGAGATTTAA

4351   TCGCCGCGAC AATTTGCGAC GGCGCGTGCA GGGCCAGACT GGAGGTGGCA

4401   ACGCCAATCA GCAACGACTG TTTGCCCGCC AGTTGTTGTG CCACGCGGTT

4451   GGGAATGTAA TTCAGCTCCG CCATCGCCGC TTCCACTTTT TCCCGCGTTT

4501   TCGCAGAAAC GTGGCTGGCC TGGTTCACCA CGCGGGAAAC GGTCTGATAA

4551   GAGACACCGG CATACTCTGC GACATCGTAT AACGTTACTG GTTTCACATT

4601   CACCACCCTG AATTGACTCT CTTCCGGGCG CTATCATGCC ATACCGCGAA

4651   AGGTTTTGCG CCATTCGATG GTGTCCGGGA TCTCGACGCT CTCCCTTATG

4701   CGACTCCTGC ATTAGGAAGC AGCCCAGTAG TAGGTTGAGG CCGTTGAGCA

4751   CCGCCGCCGC AAGGAATGGT GCATGCAAGG AGATGGCGCC CAACAGTCCC

4801   CCGGCCACGG GGCCTGCCAC CATACCCACG CCGAAACAAG CGCTCATGAG

4851   CCCGAAGTGG CGAGCCCGAT CTTCCCCATC GGTGATGTCG GCGATATAGG

4901   CGCCAGCAAC CGCACCTGTG GCGCCGGTGA TGCCGGCCAC GATGCGTCCG

4951   GCGTAGAGGA TCGAGATCTC GATCCCGCGA AATTAATACG ACTCACTATA
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5001    GGGGAATTGT GAGCGGATAA CAATTCCCCT CTAGAAATAA TTTTGTTTAA

5051    CTTTAAGAAG GAGATATACC ATGGGCAGCA GCCATCATCA TCATCATCAC

5101    ATGGTTCGTA AAGGTGAAAA AAAACTGGCG AAACACGCGA CCACCAAAGC

5151    GGCGAACCCG CAGCCGCGTC GTCGTGCGAA CAACCGTCGT CGTTCTAACC

5201    GTACCGACGC GCCGGTTTCT AAAGCGTCTA CCGTTACCGG TTTCGGTCGT

5251    GGTACCAACG ACGTTCACCT GTCTGGTATG TCTCGTATCT CTCAGGCGGT

5301    TCTGCCGGCG GGTACCGGTA CCGACGGTTA CGTTGTTGTT GACGCGACCA

5351    TCGTTCCGGA CCTGCTGCCG CGTCTGGGTC ACGCGGCGCG TATCTTCCAG

5401    CGTTACGCGG TTGAAACCCT GGAATTCGAA ATCCAGCCGA TGTGCCCGGC

5451    GAACACCGGT GGTGGTTACG TTGCGGGTTT CCTGCCGGAC CCGACCGACA

5501    ACGACCACAC CTTCGACGCG CTGCAGGCGA CCCGTGGTGC GGTTGTTGCG

5551    AAATGGTGGG AATCTCGTAC CGTTCGTCCG CAGTACACCC GTACCCTGCT

5601    GTGGACCTCT TCTGGTAAAG AACAGCGTCT GACCTCTCCG GGTCGTCTGA

5651    TCCTGCTGTG CGTTGGTAAC AACACCGACG TTGTTAACGT TTCTGTTCTG

5701    TGCCGTTGGT CTGTTCGTCT GTCTGTTCCG TCTCTGGAAA CCCCGGAAGA

5751    AACCACCGCG CCGATCATGA CCCAGGGTTC TCTGTACAAC GACTCTCTGT

5801    CTACCAACGA CTTCAAATCT ATCCTGCTGG GTTCTACCCC GCTGGACATC

5851    GCGCCGGACG GTGCGGTTTT CCAGCTGGAC CGTCCGCTGT CTATCGACTA

5901    CTCTCTGGGT ACCGGTGACG TTGACCGTGC GGTTTACTGG CACCTGAAAA

5951    AATTCGCGGG TAACGCGGGT ACCCCGGCGG GTTGGTTCCG TTGGGGTATC

6001    TGGGACAACT TCAACAAAAC CTTCACCGAC GGTGTTGCGT ACTACTCTGA

6051    CGAACAGCCG CGTCAGATCC TGCTGCCGGT TGGTACCGTT TGCACCCGTG

6101    TTGACTCTGA ATAACACCAC CACCACCACC ACTGAGATCC GGCTGCTAAC

6151    AAAGCCCGAA AGGAAGCTGA GTTGGCTGCT GCCACCGCTG AGCAATAACT

6201    AGCATAACCC CTTGGGGCCT CTAAACGGGT CTTGAGGGGT TTTTTGCTGA

6251    AAGGAGGAAC TATATCCGGA T
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Shaded region is the expressed protein including N-terminal His. Green text in order is T7 promoter, Lac operator, ribosome binding site and start codon. Red text is stop codon and T7 terminator.

13.2. Appendix B: Cohort data of fish stocks used in vaccine field trials

Table B1: Table Stocking of vaccinated and control grouper at Rocky Point Aquaculture 2018-2021

Batch # & code	Date vaccinated	Arrived farm	Enclosure	Cohort and location sampled	# fish & (stocking weight in g)	Stocking density (kgm⁻³)
1 EL04 -18 & ELO5-18	- 12.11.18	Dec '18	ponds	control: pond 6	5575	0.64
				vaccinated: pond 9	4715 (100)	0.29
			lake 1	control: cage 1	274	0.91
			lake 2	vaccinated: cage 7	2600 (100)	4.42
				vaccinated: cage 8	2510 (100)	2.43
				vaccinated: cage 9	2544 (170)	6.93
2 EL01-19	-		RAS	*Unvaccinated Tank 3	743 (48)	1.78
				*Vaccinated Tanks 4,5,6,7	8874 (41)	4.55
3 EL02-19	 27.5.19	Aug '19	RAS then lake 2	vaccinated: tank 14 -> cage 6	3845 (30.5)	5.75
				vaccinated: tank 14 -> cage 6	4703 (32)	7.5
4 EL03-19	- 15.9.19	Oct '19	ponds	control: pond 9	10015 (75)	0.15
				vaccinated: pond 6	10499 (79)	0.16
5 EL09-19	- 25.11.19	Dec '19	RAS	control: tank 3	1934 (35)	3.35
				vaccinated: tank 5	2728 (30.8)	4.1
6 EL01-20	- 15.2.20	May '20	RAS	control: tank 11	1825 (61.7)	5.6
				vaccinated: tank 12	5642 (41.8)	11.55
7 EL02-20	- 20.5.20	June '20	RAS	control: tank 17	4593 (71)	16.3
				vaccinated: tank 18	3250 (48)	7.8
8 EL03-20	- 22.6.20	Aug '20	RAS then lake A	control: tank 13-> cage A10	5335 (59)	15.7
				vaccinated: tank 21-> cage A9	6420 (58)	18.9
9	-			control: tank 6-> cage A4		

| EL06-20 | 1.12.20 | Jan '21 | RAS then lake A | vaccinated: tank 21-> cage A5 | | |

*Truck driver mixed up vaccinated and unvaccinated transport tanks

Table B2: Batch 1 samples used for ELISA
S= sampling, C= control, V= vaccinated on 12.11.18

Timepoint	Sampling date	Code	Cage in lake	Av. Weight (g)	Comments & weight range (g)
1	17.12.18	S1_C1-C6	1	374.6	299-431
		S1_V1-V6	6,7,9	250.2	151-364
NNV outbreak first suspected 8.1.19, confirmed 14.1.19 by Biosecurity Sciences lab Qld					
2	22.1.19	S3_C7-C12	1	505	381-680
		S3_V7-V12	6,7,9	445.2	moribund vaccinated 351-541
		VH7-VH12	9	blood only	healthy vaccinated
3	22.2.19	S4_C7-C12	1	851.7	771-947
		S4_V7-V12	6,7,9	793.2	652-886
4	21.3.19	S5_C7-C12	1	930	810-980
		S5_V7-12	9	991.7	890-1110
5	18.4.19	S6_C7-12	1	1246.8	1172-1348
		S6_V7-12	7,9	696.3	Larger fish already had been harvested 548-867

Table B3: Batch 3 samples used for ELISA

S= sampling, C= control, V= vaccinated on 27.5.19

Timepoint	Date	Code	Site	Av. Weight (g)	Comments & weight range
9	26.8.19	S10_C1-C6	Tank 12	94.7	Fish in RAS 70-121
		S10_V1-V6	Tank 14	80.25	35-140
10	1.10.19	S11_C1-C6	Tank 12	103.2	72-137
		S11_V1-V6	Tank 14	111.8	60-153
11	28.10.19	S12_C1-C6	Cage 7	132.2	Fish in lake cages. Some controls escaped and were replaced 116-145
		S12_ V1-V6	Cage 6	206.7	155-268
12	5.12.19	S13_C1-C6	Cage 7	186.9	155-224
		S13_V1-V6	Cage 6	257.8	180-320
13	16.1.20	S14_C1-C6	Cage 7	347.6	256-383
		S14_V1-V6	Cage 6	460.1	291-578
14	28.2.20	S15_C1-C6	Cage 7	571.9	462-717
		S15_V1-V6	Cage 6	481.4	194*-640 *1 fish very small

13.3.   Appendix C: Molecular analyses

Table C1: Primers for cloning NNVRNA2 694 bp fragment, TaqMan RT-qPCR and genome sequencing

Primer name	Sequence 5' to 3'	Purpose
NNVRNA2_F2	TGATGCAACCATCGTCCCC	694 bp amplicon
NNVRNA2_R2	CAACGCCATCTGTGAACGTC	"
NNVRNA2_F2H	Ggcttatcgaaattaatacgactcactataggga TGATGCAACCATCGTCCCC	694 amplicon + overhangs
NNVRNA2_R2H	Agatggctggcaactagaaggcacagtcgaggc CAACGCCATCTGTGAACGTC	"

pcDNA3.1 F	GCCTCGACTGTGCCTTC	Vector with overhangs
pcDNA3.1 R	TCCCTATAGTGAGTCGTATTAATTTCGATAA	"
qR2T_F*	CTTCCTGCCTGATCCAACTG	TaqMan assay NNV viral load
qR2T_R*	GTTCTGCTTTCCCACCATTTG	TaqMan assay NNV viral load and genome sequencing
R2probe2*	FAM–CAACGACTGCACCACGAGTTG–QSY	TaqMan hydrolysis probe
NNVRNA2_R1**	TCACTGCGCGGAGCTAACGGTAAC	genome sequencing
NNVRNA2_F4	TAATCCATCACCGCTTTGCAATC	"

*Hick and Whittington (2010) **(Ransangan and Manin 2012)

Table C2: Primers used for NNV RNA1 genome sequencing

Primer name	Sequence (5'–3')	Position on RNA1
Forward primers		
NNV1_F7	ACATCACCTTCTTGCTCTGTTG	3–24
NNV1_F4	TTGCTGGCGGGAAGGATG	650–667
NNV1_F6*	TAGTGCCTACGACACTGATC	1110–1129
NNV1_F9*	GCACTCGCACGCATGTCTGGA	2145–2165
NNV1_F10	GTCTGTCGCATTAGACGGGG	2673–2692
Reverse Primers		
NNV1_R8	ACACAATGTTACGATGCTCACCCA	865– 842
NNVRNA1_R**	TTGTGGCAAGCTCGTTGGAA	1390–1370
NNV1_R3	GTCAGTGTAGTCTGCATACT	2331–2312
NNV1_R1	GAAGCGTAGGACAGCATAAAGC	3079–3058

*(Agnihotri et al. 2016) **(Kim et al. 2019)
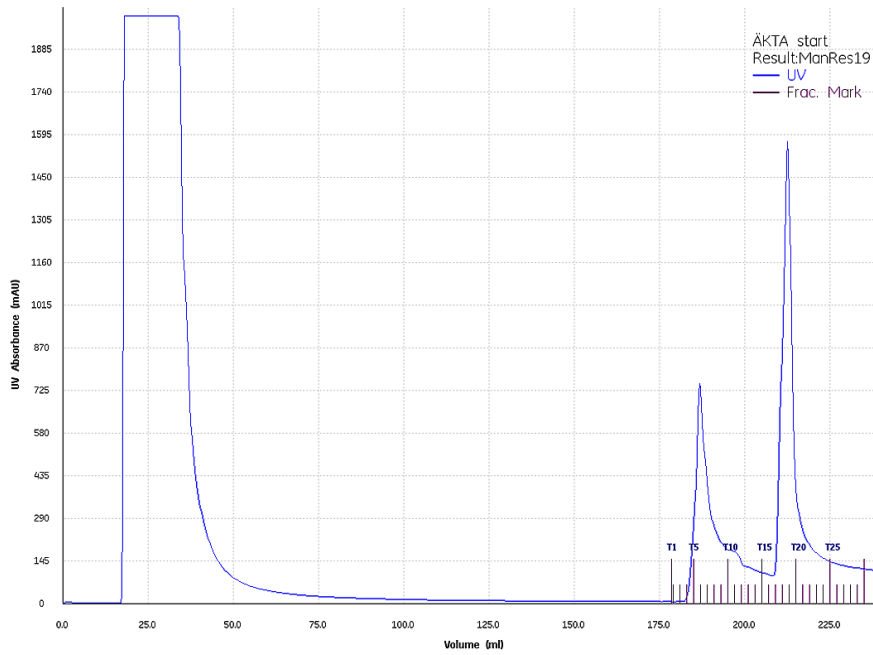
Fig C.1 Chromatogram NNV capsid protein purification by FPLC.
Recombinant 6xHis-NNV-C protein purified on IMAC column loaded with 0.1M NiSO$_4$ on ÄKTA start. Large peak (left) is flow through. Stepwise elution (2 peaks) with 30% and 100 % elution buffer.
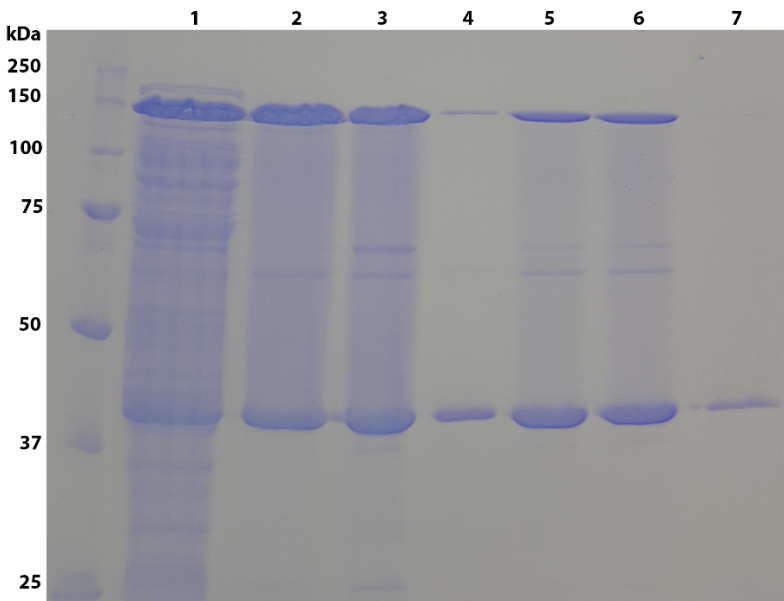
Fig C2 SDS-PAGE of fractions retrieved from FPLC of recombinant 6xHis-NNV-C protein and subsequent dialysis.
Lanes 1: Total protein soluble fraction (1:10) loaded onto IMAC column 2: Pooled fraction T17-21 prior to dialysis 3: Dialyzed pooled fractions 4: Dilution 1:10 of sample lane 3. 5: Dilution 1:2 of dialyzed pooled fractions for aliquots, pre-freeze thaw 6: Sample lane 5, following 24 h freeze at -80 °C and thaw 7: 1:10 dilution of freeze-thawed sample

Table C3: Cts for serial dilutions of total RNA from NNV infected grouper brain, 2 independent runs

RNA from NNV infected brain	Mean Ct	SD	CV
50 ng	13.434	0.156	0.012
5 ng	16.719	0.069	0.004
0.5 ng	20.317	0.386	0.019
50 pg	23.942	0.637	0.027
5 pg	27.634	0.903	0.033
0.5 pg	30.876	0.589	0.019
0.05 pg	35.492	2.129	0.060
0.005 pg	undetected	-	-

Table C4: Batch 1 NNV_RNA2 viral load in brain from December 2018 to April 2019 (n=6)

Date sampled	Code	Weight (g)	Ct Mean	log10 NNV_RNA2	viral load	health status	Anti-NNV-IgM (Ab 405 nm)
17.12.18	S1C1	375	undetermined		-		0.573
	S1C2	367	24.137	4.32	medium		1.224
	S1C3	398	30.589	2.81	low		0.601
	S1C4	431	undetermined		-		0.442
	S1C5	299.5	undetermined		-		1.361
	S1C6	377	36.009	0.09	negative		0.835
	S1V1	152	undetermined		-		1.048
	S1V2	153	21.626	4.99	medium		1.027
	S1V3	250	undetermined		-		1.085
	S1V4	364	undetermined		-		1.059
	S1V5	362	undetermined		-		0.853
	S1V6	218	undetermined		-		1.396
Outbreak	began	8.1.19	lake 2				
22.1.19	S3C7	414	undetermined		-		0.458

	S3C8	418	undetermined		-		0.837
	S3C9	547	undetermined		-		0.564
	S3C10	590	undetermined		-		0.503
	S3C11	381	undetermined		-		0.751
	S3C12	680	undetermined		-		0.476
	S3V7	393	13.273	7.33	high	moribund	0.767
	S3V8	541	14.5075	7.01	high	moribund	0.666
	S3V9	351	13.643	7.21	high	moribund	0.776
	S3V10	424	13.0885	7.36	high	moribund	0.678
	S3V11	440	13.269	7.32	high	moribund	0.759
	S3V12	522	16.54	6.42	high	moribund	0.513
22.2.19	S4C7	892	undetermined		-		0.821
	S4C8	771	30.725	2.22	low		0.817
	S4C9	974	undetermined		-		0.72
	S4C10	866	undetermined		-		1.514
	S4C11	806	undetermined		-		0.664
	S4C12	801	undetermined		-		1.244
	S4V7	736	undetermined		-		0.718
	S4V8	881	undetermined		-		0.872
	S4V9	652	undetermined		-		1.119
	S4V10	725	undetermined		-		0.716
	S4V11	879	undetermined		-		0.789
	S4V12	886	undetermined		-		1.114
21.3.19	S5C7	970	undetermined		-		0.663
	S5C8	890	undetermined		-		1.009

	S5C9	980	undetermined		-		1.162
	S5C10	960	undetermined		-		1.054
	S5C11	970	undetermined		-		0.738
	S5C12	810	undetermined		-		0.882
	S5V7	1110	undetermined		-		1.405
	S5V8	1000	undetermined		-		0.701
	S5V9	890	undetermined		-		0.976
	S5V10	950	undetermined		-		0.864
	S5V11	1070	34.372	1.16	borderline		1.269
	S5V12	930	undetermined		-		0.943
outbreak	28.3.19	lake 2	very minor <1%	mortality			
18.4.19	S6C7	1348	25.087	4.01	medium		1.357
	S6C8	1216	undetermined		-		0.982
	S6C9	1250	34.082	1.24	low		0.781
	S6C10	1317	undetermined		-		1.039
	S6C11	1178	undetermined		-		1.185
	S6C12	1172	38.490	-	negative		0.743
	S6V7	705	23.863	4.37	medium		1.334
	S6V8	867	34.538	2.25	low		1.179
	S6V9	686	29.396	2.61	low		0.818
	S6V10	783	undetermined		-		1.416
	S6V11	549	33.123	1.52	borderline		0.875
	S6V12	589	undetermined		-		0.888

Note, in Table C4 control fish (grey shading, zone A) were located in a distant part of the lake to where vaccinated fish (white background, zone B) were stocked and where adverse events and outbreaks occurred. "Undetermined" indicates no amplification signal was detected above the minimum threshold, namely no virus was detected (-). In Table 3 the viral load was classified as follows: high >10^6, medium > 10^4-10^6, low > 10^2- 10^4 RNA2 genome copies/ 50ng

total RNA from brain and borderline between 10^1 and 10^2 copies. ELISA results for specific anti-NNV-IgM are also included.

13.4. Appendix D: Project personnel

Table D1: List of project personnel

Role	Contact details	Company name
Principal investigator	Prof. Andrew C. Barnes Research Group Leader Email: a.barnes@uq.edu.au Business Ph: 07 33469416	University of Queensland (UQ)
Co-investigator	Dr Richard Knuckey General Manager Email: rknuckey@thecompanyone.com Business Ph: 07 31234863	The Company One
Co-investigator	Mr Mark White Director Email: markw3@iprimus.com.au Business Ph:	Tréidlia BioVet
Co-investigator	Mr Brad Cherrie Hatchery Manager Email: bradleyc@rockypointprawns.com Business Ph:	Rocky Point Aquaculture Company Pty Ltd
Co-investigator	Mrs Serena Zipf Director Email: serenaz@rockypointprawns.com Business Ph: 07 55461588	Rocky Point Aquaculture Company Pty Ltd
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13.5. Appendix E: Permits and approvals

Table E: Permits and approvals granted for this project

Applicant	Purpose	Permit identifier	Approving Authority
TCO	Ethics: Vaccination of grouper for safety and efficacy trials	SA2018/10/659	Queensland Government Dept. Ag & Fisheries
Tréidlia BioVet	Research Permit for vaccine field trials in fish		APVMA
Tréidlia BioVet	Biosafety: Approved media batches for cultivation of organisms for in vivo use		DAWE
UQ	Import: Antisera and genetic material for in vitro and in vivo use	0002699905 0003845304	DAWE
UQ	Biosafety: Physical containment 2 certification, rooms 60/327-330	Cert-2099	OGTR
UQ	Biosafety: Exempt dealings. Cloning into standard vectors and laboratory organisms	IBC/923/SBS/2015	UQ Institutional Biosafety Committee
UQ	Translocation: Shipment of fish from TCO to UQ	UQ22-02	Queensland Government Dept. Ag & Fisheries
UQ	Ethics: Field trial sample collection. Secondary antibody response in vaccinated and unvaccinated farm fish (as amendment 1)	SBS/506/18	UQ NEWMA AEC
UQ	Ethics: Challenge model and vaccination trial in grouper	2022/SBS/000386	UQ NEWMA AEC
JCU	Ethics: Challenge model and vaccination trial in grouper	A2709	JCU AEC

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