The effect of barramundi nodavirus on important freshwater fishes

I.G. Anderson and N. Moody

Queensland Government
Department of Primary Industries and Fisheries

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Table of Contents

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OBJECTIVES:

1. To establish a standard infection model for barramundi nodavirus in barramundi larvae and fry.
2. To more accurately define the range of fish species that can actually be infected by barramundi nodavirus, the effect of the virus on these fishes and the infectious dose.
3. To determine the sensitivity of the barramundi nodavirus to a range of environmental conditions and to disinfectants.
4. To evaluate the cell culture isolation system as a method of detecting nodavirus in asymptomatic carrier fish.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED

This project has confirmed the knowledge that management of barramundi translocations outside their natural range requires fisheries authorities take into account the risk that barramundi nodavirus may lethally infect native freshwater fishes. The project has shown barramundi nodavirus can multiply and spread throughout the body of freshwater fishes, and that the spread of infection from fish to fish is a possibility in freshwater.

The project has created knowledge on the Australian application of a sensitive molecular detection test for nodavirus in healthy fishes. This knowledge has lead to industry and government support for further research on diagnostic test development for nodaviruses which will lead to a national Standard Diagnostic Procedure. Further, this knowledge has led the barramundi hatchery sector to support a research project applying the molecular detection test to screen captive barramundi breeders for nodavirus with the aim to produce nodavirus-free barramundi fry.

Need

The culture of barramundi outside its natural range has been raised as a risk to native freshwater fishes because of the possible spread of disease from translocated barramundi fry. The barramundi disease considered of most significance is Viral Nervous Necrosis, or Viral Encephalopathy and Retinopathy, which is caused by a virus in the nodavirus family. Viral Nervous Necrosis is a disease of the brain, spinal cord and eyes seen in, at least 32 different, marine finfish larvae and fry. Outbreaks of Viral Nervous Necrosis have occurred in Australian barramundi hatcheries. Changes to hatchery husbandry and management minimised the impact of this disease and the disease did not impede the development of the barramundi culture industry through the 1990s. Despite this, as recently as this year, significant losses of young barramundi were still observed in some hatcheries. Some research done in the mid-1990s found that the barramundi nodavirus (or barramundi nervous necrosis

virus) was apparently able to infect some species of freshwater fishes in experimental infection studies. In this research the nodavirus was purified from the tissues of diseased barramundi fry. While some aspects of these studies were considered incomplete, state fisheries authorities implemented strict controls on the movement to, and culture of barramundi in areas where they do not normally live.

One of the constraints when studying any of the fish nodaviruses is their inability to grow in the laboratory in artificially cultured fish cells. The ability to isolate and grow viruses in the laboratory on cells is important for the diagnosis of infections but also to produce enough virus for detailed analysis of their molecular makeup and biology. In 1998 Dr Nick Moody at the Oonoonba Veterinary Laboratory (Biosecurity, Queensland Department of Primary Industries and Fisheries, Townsville) successfully developed a barramundi brain cell line that supported the growth of barramundi nodavirus in the laboratory. This provided the opportunity to produce large numbers of live virus in the laboratory. Consequently it became possible to repeat infection studies to determine if freshwater fishes could really become infected by the barramundi nodavirus and what, if any, effect the infection had on them. The laboratory grown virus, and the barramundi brain cell line, also meant that diagnostic tests to detect barramundi nodavirus in fish not showing signs of disease (carrier status) could be evaluated.

Barramundi nodavirus infections

Initially experiments to infect barramundi were done to be sure that subsequent barramundi nodavirus infections of freshwater fishes would be comparable. Young barramundi were infected by bath exposure (virus added to water with fish) and by direct injection of the virus into the barramundi fry's body. The bath challenge would be considered the route of infection closest to what we expect would happen in natural infections. The signs of Viral Nervous Necrosis, deaths and pathology typical of outbreaks of Viral Nervous Necrosis in barramundi in culture facilities were seen in both bath and direct injection infections. A bath infection of barramundi was achieved in freshwater. This finding suggested a barramundi nodavirus infection was theoretically possible outside a seawater environment. We also found that the older the barramundi larvae/fry, the less effect the virus had in regard to number of deaths and the number of fish with damage to the brain or eyes.

Bath and injection infections were attempted in six and twelve week old silver perch, golden perch, sleepy cod and Barcoo grunter fry. These four species of fish were selected as young fry were available from commercial native fish hatcheries and they are important recreational fishes. At no time were we able to infect these fishes by bath exposure to the virus. All the fishes, at both ages, were susceptible to barramundi nodavirus by the injection route of infection. In the experimental infections up to 46% of the silver perch fry, 15% of the golden perch fry, 98% of the sleepy cod fry and 7% of Barcoo grunter fry became sick and died in the 28 days following injection of barramundi nodavirus. Even in those fry that survived many had evidence of the virus persisting in the brain and, or eye tissues at the end of the experiment. We provide a detailed comparison of the microscopic tissue lesions seen in each of the freshwater fishes as an aid in the diagnosis of fish nervous tissue lesions for fish pathologists. It could be suggested that the failure to transmit the barramundi nodavirus infection by the bath infection route, the more natural infection route, indicates freshwater fishes would not be susceptible to nodavirus infections in natural environments. This may not be correct, as barramundi fry are more susceptible the younger they are, freshwater fishes less than six weeks of age may still be able to be bath infected. Further research is needed to rule out that possibility.

Barramundi brain cell line and detection tests

The development of the barramundi brain cell line allowed the production of barramundi nodavirus in the laboratory. This provided the virus for the infection experiments. Unfortunately the cell line transformed after dividing and regrowing about 35 times making the cells no longer able to support the growth of barramundi nodavirus in the laboratory. Prior to this transformation the culture methods for the barramundi brain cell line and the initial diagnostic sample processing methods were optimised. There was a 100% agreement between Viral Nervous Necrosis in a sample of fish and growth of the virus in the barramundi brain cell line. Once the barramundi brain cell line cells transformed some aspects of the project could not be completed. It was intended to calculate the actual dose or number of viruses used in the infection experiments. This would have made detailed comparisons between infections in the different species and ages of fish more precise. One objective was to determine the effect of disinfectants, salinity, temperature and so on, on the viability of barramundi nodavirus. For both these aspects use of a nodavirus-susceptible cell line was necessary. A range of techniques, including adding chemicals to enhance the susceptibility of the barramundi brain cell line cells and starting new cell lines from barramundi brain tissue, were attempted to produce a cell line we could use in the laboratory to grow the barramundi nodavirus. The importance of cell lines for the diagnosis and culture of nodaviruses was recognised nationally. Further research is now underway to develop nodavirus-susceptible cell lines under the Australian Government funded new budget initiative to develop diagnostic tests for important aquatic animal diseases (Fisheries Research and Development Corporation project 2001/626).

Through this project we also implemented and did some initial optimisation of a sensitive molecular detection test for the barramundi nodavirus. This technology utilises a precise molecular key to recognise the nodavirus genetic sequence (a primer) and the polymerase chain reaction (PCR) to amplify a molecular product so that a positive test result can be easily detected in the laboratory. The best methods to extract the nodavirus genetic material from fish samples were evaluated, as were the best reagent and chemical concentrations to use in the test reaction for a one step test and the more sensitive two step test. It was demonstrated the one step test (RT-PCR) did miss some infections, in comparison to the two step test (nested RT-PCR), when samples from the ovaries of adult barramundi were tested for nodavirus. The molecular detection tests were applied successfully to confirm the presence of barramundi nodavirus in the fishes used in the infection experiments. Further validation of this molecular detection test is being done in the national nodavirus diagnostic test development project (FRDC 2001/626).

Application of the two step nodavirus molecular detection test (the nested RT-PCR) to detect nodavirus in wild freshwater fishes in northern Queensland is underway (Fisheries Research and Development Corporation project 2002/043). This new project has also involved barramundi hatcheries to see if use of this test technology can identify breeding barramundi adults that carry the infection without disease. The possibility that removal of these carrier fish from the breeding program will prevent any further outbreaks of viral nervous necrosis in barramundi larvae and fry is being evaluated.

KEY WORDS: Barramundi, sleepy cod, silver perch, golden perch, Barcoo grunter, betanodavirus, nodavirus, barramundi nervous necrosis virus, viral nervous necrosis, viral encephalopathy and retinopathy, pathology, histology, experimental infections, cell culture, RT-PCR, virus testing.

2. Background

The culture of barramundi outside its natural range has been raise as a possible risk to endemic freshwater fishes because of the transmission of a barramundi disease. The disease Viral Nervous Necrosis (VNN) in barramundi larvae (9 to 24 days old) is caused by a nodavirus, originally named Barramundi Picorna-like Virus (Glazebrook *et al* 1990; Munday *et al* 1992). Viral Nervous Necrosis is a common disease of marine finfish larvae reared in intensive conditions. At least 32 different marine fish species have been recorded as having VNN caused by a nodavirus (see Munday *et al* 2002) and the list continues to be added to regularly with VNN reported more recently in white seabass and Atlantic cod in north America (Curtis *et al* 2001; Johnson *et al* 2002) and red drum in South Korea (Oh *et al* 2002). VNN was reported in yellow-wax pompano and cobia in Taiwan (Chi *et al* 2003). Further, in 1999 we isolated a nodavirus from the freshwater fish, sleepy cod showing a granulomatous encephalitis (unpublished data) and it was shown that tilapia were susceptible to experimental nodavirus infections (Skliris and Richards, 1999). More recently, an outbreak of VNN was reported in European seabass (50-80 grams in size) cultured in freshwater in Greece (Athanassopoulou *et al* 2003). As this species is initially hatched and reared in a marine hatchery environment it was not known if the nodavirus was present in asymptomatic carrier fish on transfer or originated in the freshwater culture system. A marine grouper nodavirus was isolated from diseased, farmed freshwater guppies in Singapore (Hedge *et al* 2003). Infection trials using the guppy isolate did not cause disease in experimental guppies but the nodavirus was reisolated at 10 days post-infection. Again, the origin of the guppy isolate was not clear but Hedge *et al* (2003) suggest the nodavirus spread from marine fish to freshwater fish. Chi *et al* (2003) have reported mass mortalities of the freshwater Chinese catfish caused by a nodavirus.

Glazebrook (1995) apparently demonstrated that two to three month old Macquarie perch, silver perch and Murray cod could be lethally infected by barramundi nodavirus. The accuracy of these results has been questioned by fish health specialists because of the methods used although there are now a few more reports implicating nodavirus effects in freshwater fishes. Cross species transmission studies by Arimoto *et al* (1993) had previously shown striped jack nodavirus did not cause disease in red sea bream, goldstriped amberjack and yellow tail larvae. Another study, using sevenband grouper nodavirus, showed that bath challenge did result in disease in Japanese flounder and tiger puffer fish fry but not in kelp grouper or red sea bream (Tanaka *et al* 2003). The four clusters or genotypes of fish nodaviruses (SJNNV, RGNNV, TPNNV and BFNNV) originally grouped by Nishizawa *et al* (1997), based on the nucleotide sequence of the virus coat protein gene, may also define the phenotypic property of host specificity. These genotypes are in part consistent with 3 serotypes identified when 20 nodavirus isolates were examined by virus neutralisation tests using rabbit antisera raised against nodavirus strains representative of each genotype (Mori *et al* 2003). Serotype A includes SJNNV strains, serotype B the TPNNV strain and serotype C includes RGNNV and BFNNV strains. A cross species pathogenicity study found that striped jack nodavirus (SJNNV group) did not replicate in Atlantic halibut and, that, Atlantic halibut nodavirus (BFNNV group) did not replicate in striped jack larvae (Totland *et al* 1999). It has also now been shown that the transmission of a nodavirus infection does not always lead to disease. Castric *et al* (2001) found that healthy sea bream were the source of the nodavirus which caused an epizootic in sea bass on the same cage farm. Even when the isolated nodavirus was injected into virus-free sea bream fingerlings there was no disease. Increasingly there is evidence that confirms there are specific strains of fish nodavirus which can only infect specific host species. The presence of a nodavirus in one species does not automatically imply other fish in contact with that species are threatened by viral nervous necrosis outbreaks.

Because of Glazebrook's findings, Victoria had banned the culture of barramundi and New South Wales and South Australia permitted limited barramundi culture under strict license conditions. Similar concerns about the risk to endemic freshwater fishes have been raised in respect to proposed barramundi farms west of the dividing ranges in southern Queensland. There has now been more than ten years of successful barramundi culture in southern Australia with no effect on endemic freshwater fishes. State fisheries managers and recreation fishers still have concerns about the risk barramundi nodavirus poses to fishes in the Murray-Darling catchment.

VNN epizootics had a significant effect on the one commercial barramundi hatchery in the late 1980's, but was rare in government hatcheries. Once the viral cause of VNN was recognised in Australia, changes in husbandry, hygiene, improved water quality, improved nutrition and reduced stocking density in larval rearing tanks in hatcheries (Glazebrock and Heasman 1992; Anderson *et al* 1993), resulted in prevention of any further VNN epizootics. Through the mid-1990s the majority of barramundi larval production changed to extensive pond and "green-water" tank rearing systems. VNN epizootics were never seen and it was assumed nodavirus infections were probably endemic but rarely caused disease. In the late 1990s the nature of these infections appeared to change. Infrequently, mild brain lesions began to be detected on histological examination of clinically normal pond-reared barramundi fry. Further, larval mortalities due to VNN occurred in several "green-water" larval rearing runs, always associated with high stocking densities (40-50 larvae per litre). Several epizootics occurred in barramundi of 40-45 days of age, just after transfer to the nursery. Mortalities were low, less than 10% and more usually less than 1%. In 1999 one commercial barramundi hatchery experienced mass mortalities of barramundi around 15 days of age in larval rearing ponds. This was the first time any significant VNN was seen outside of intensive larval rearing systems. Late in 1999, a lethal disease of sleepy cod fingerlings in a recirculation tank system was diagnosed as a severe, granulomatous encephalitis. Electron microscopy and nodavirus RT-PCR on brain tissue demonstrated this was a VNN syndrome. Preliminary sequence analysis of amplicons suggested that the sleepy cod nodavirus was slightly different than barramundi nodavirus. An investigation failed to identify any contact between these hatchery-reared sleepy cod and barramundi. Mortalities due to VNN were observed in archer fish fingerlings in 2001. These fish had been cultured with barramundi in a larval rearing pond prior to transfer to a tank nursery where the mortalities occurred. Resources did not permit a full investigation in this case, but it was assumed the archer fish could have been infected with barramundi nodavirus during co-culture. The emergence of larval/fry VNN epizootics in rearing ponds, and the more common incidence of mortalities in nursery systems, resulted in the barramundi farming industry placing a high priority on developing protocols to produce nodavirus-free fingerlings and, or minimizing any impact of nodavirus infections or VNN in 2001/02.

While high level mortalities in intensive larval cultures are the common effect of fish nodaviruses in aquaculture, there are also records of disease in advanced juveniles. Le Breton *et al* (1997) reported a daily mortality rate in sea cages of up to 3.6% in 350-450 gram European sea bass due to VNN. This significant mortality was associated with elevated seawater temperatures. A syndrome of VNN where there was also over-inflation of the swim bladder was reported in 170-1,850 gram sevenband grouper, with one farm having a cumulative mortality of about 40% over six weeks (Fukuda *et al* 1996). These reports emphasize the possibility that fish nodaviruses could cause significant mortalities in any class of cultured marine finfish. In the absence of chemotherapy for viral diseases of fishes there is the potential for significant economic loss.

For state fisheries managers and recreational fishers the major problem of barramundi nodavirus remains the translocation issue. Though in more recent years, as indicated above, the barramundi aquaculture sector are again seeking to develop procedures or test protocols that will allow avoidance of any nodavirus disease impacts.

Detection tests for nodaviruses have undergone a continuous development in the past 5-10 years. The traditional tool of cell culture isolation systems (Chew-Lim *et al* 1994; Frerichs *et al* 1996) have not been readily available in Australia. Detection tests for nodaviruses, including immunodiagnostics (IFAT, immunohistochemistry and ELISA) and molecular tests based on the polymerase chain reaction (RT-PCR and nested RT-PCR), have been published and some have been applied in Australia.

Fluorescent antibody detection tests have been developed using anti-striped jack nodavirus serum (Arimoto *et al* 1992; Mushiake *et al* 1992). Munday *et al* (1994) demonstrated the fluorescent antibody test could also detect the barramundi nodavirus in tissue sections. Unfortunately, the fluorescent antibody test is not sensitive enough to detect sub-clinical infections. ELISA tests to detect antibodies to nodavirus in serum have been used (Mushiake *et al* 1992; Breuil and Romestand 1999; Breuil *et al* 2000; Watanabe *et al* 2000). Despite this success, Mushiake *et al* (1992) found the presence of nodavirus antigen in the ovary did not correlate with the presence of antibody in the serum. Watanabe *et al* (2000) found that only barfin flounder broodstock with ELISA antibody titers >40 also returned a positive RT-PCR. The reverse transcription-polymerase chain reaction (RT-PCR) detection test was developed by Japanese researchers (Nishizawa *et al* 1994). This test is more sensitive than immunodiagnostic tests and can successfully detect the nodavirus in asymptomatic broodstock and juvenile stripped jack. Although Nishizawa *et al* (1996) did report that striped jack broodstock females could test negative to RT-PCR on gonadal fluids and produce larvae which suffered from nervous necrosis. The primers used in this RT-PCR test recognise all sub-groups or genotypes of the nodavirus from different fish species (Nishizawa *et al* 1997; Munday *et al* 2002). We have used the Japanese RT-PCR detection test at the Oonoonba Veterinary Laboratory (OVL) and have confirmed it detects barramundi nodavirus (unpublished data). A two-step or nested RT-PCR, estimated at 100 times the sensitivity of the one-step RT-PCR, was developed by French scientists (Thiery *et al* 1999; Dalla Valle *et al* 2000). Dalla Valle *et al* (2000) emphasised the addition of the nested RT-PCR appeared an absolute requirement to ensure a sensitivity level adequate to screen for nodavirus-free broodstock. Their study using the nested RT-PCR also found that 72% of brain and 59% of blood samples were positive in the adult and juvenile European sea bass while only 35% of the ovarian biopsies were PCR positive. Similarly in gilt-head seabream, 39% of brain and 34% of blood samples were PCR positive.

One of the constraints using molecular detection tests is that the viability of the virus remains unknown and consequently without sequential testing, the significance of test results can be difficult to interpret. At the time the preliminary work for this project began there were only two cell lines reported from other countries which supported the growth of the nodaviruses (Frerichs *et al* 1996 – SSN-1; Chew-Lim *et al* 1994 – a seabass cell line, Singapore). Neither were readily available in Australia at that time and both cell lines had persistent infections with other viruses. No other laboratory in Australia has successfully developed a cell culture system to culture the barramundi nodavirus in the laboratory. In 1998, Dr Nick Moody at OVL successfully developed a barramundi cell line that supported the growth of barramundi nodavirus in the laboratory. At the time this project began the cell line was in its 20th passage. A confluent cell monolayer grew in just 4 days, at 25ºC and at a split ratio of 1:10. Isolation of the barramundi nodavirus was from clarified tissue homogenates of barramundi fry with clinical VNN. The virus causing cytopathic effect (CPE) was confirmed as a nodavirus using

the Japanese RT-PCR detection (unpublished data). The new cell line provided the opportunity to produce large amounts of the barramundi nodavirus without relying on an involved procedure to purify the virus from infected barramundi tissue. The virus produced in cell culture makes it possible to carry out challenge trials to determine minimum infectious dose and species susceptibility. Further, the use of the barramundi cell line means that, in the absence of clinical disease, fish tissue samples could be tested to determine whether viable nodavirus is present. There are now a range of continuous cell lines reported to support the growth of fish nodaviruses. These include GF-1 (grouper fry; Chi *et al* 1999); E-11 (a clone of SSN-1; Iwamoto *et al* 2000); GB, GF and GL (grouper brain, fin and liver; Lai *et al* 2000; Lai *et al* 2001; Lai *et al* 2003); SF (seabass fry; Chang *et al* 2001) and TF (turbot tail fin; Aranguren *et al* 2002b). These cell lines remain unavailable for use in Australia.

For all these detection technology areas, further refinement can be proposed, as can the need for validation across a range of clinical materials in Australia and a need to produce reagents locally. The importance of developing locally applicable immunodiagnostic, molecular detection and cell culture isolation systems was recognised as a national priority. Outputs from this project in relation to cell culture isolation systems and molecular detection tests led to a project, FRDC 2001/626 "Aquatic Animal Health Subprogram: development of diagnostic tests for the detection of nodavirus". The nodavirus diagnostic test development project is funded under the DAFF Commonwealth Government's New Budget Initiative, 'Building a National Approach to Animal and Plant Health', to improve diagnostic techniques for important aquatic animal diseases.

The susceptibility of freshwater fishes to the barramundi nodavirus was the critical issue for fisheries managers and other stakeholders. Using the barramundi cell line and the clinical isolates we have in storage at OVL we were able to confirm the susceptibility of important freshwater fishes to the virus. In the event a fish showed no signs of disease attempts to confirm virus in their tissues will determine if a true infection (without disease) had occured. Unfortunately, after the production of nodavirus in the barramundi cell line for challenge trials, at around passage 35-45, the cells transformed and were no longer susceptible to infection by the nodavirus. It was planned that suspensions of the barramundi nodavirus be exposed to different physical conditions and disinfectant resumes. Failure to reisolate viable virus would have provided more accurate information for recommendations on methods that minimise the risk of virus transfer between farms, hatcheries and catchments. With the transformation of the cells we were not able to complete this component of the project. The importance of cell culture isolation systems that are susceptible to nodavirus was recognised as a national priority and on going attempts to develop new cell lines was funded under FRDC project 2001/626 "Aquatic Animal Health Subprogram: development of diagnostic tests for the detection of nodavirus".

3. Need

Susceptibility of freshwater fishes to barramundi nodavirus.

- There is a need to address concerns about the risk of possible lethal transmission of barramundi nodavirus to freshwater fishes already under threat in their natural habitat.
- Important freshwater fishes will be exposed to the nodavirus from cell cultures to determine their susceptibility
- By using the OVL isolation facility in Townsville, well outside the Murray-Darling Region, there is minimal risk to the natural fish populations.
- Confirmation that barramundi nodavirus can cause lethal infections in freshwater fishes will strengthen the application of strict licence conditions on barramundi farming in southern Australia.
- Confirmation that barramundi nodavirus does not affect freshwater fishes will possibly allow expansion of the barramundi farming into regions needing new sustainable economic development.

A barramundi infection model.

- To establish a realistic virus dose and route of infection for the challenge trials, an infection model using barramundi will be developed.
- OVL has ready access to barramundi larvae and fry of all ages.
- The model is also necessary to help quantify the effect of virus exposure to disinfectants and different environmental conditions.

What is the viability of barramundi nodavirus?

- Fish health management requires good information on how to effectively decontaminate facilities following outbreaks of VNN.
- Knowledge of the persistence of a barramundi nodavirus in the environment will allow fisheries managers to decide on effective conditions for barramundi farming licences that minimise the risk of transferring virus outside the culture facility.

Sensitivity of the cell culture isolation system.

- While sensitive detection tests are available, the barramundi cell line offers a more practical diagnostic method that can be used by any laboratory with cell culture/ virology capability.
- An evaluation of the cell culture isolation system's ability to detect virus in carrier (no disease) fish, and standardization of the cell culture presentation, is required before the method can be recommended.

4. Objectives

1. To establish a standard infection model for barramundi nodavirus in barramundi larvae and fry.

2. To more accurately define the range of fish species that can actually be infected by barramundi nodavirus, the effect of the virus on these fishes and the infectious dose.

3. To determine the sensitivity of the barramundi nodavirus to a range of environmental conditions and to disinfectants.

4. To evaluate the cell culture isolation system as a method of detecting nodvirus in asymptomatic carrier fish.

5. Infection model for barramundi nodavirus in barramundi larvae and fry

Under Objective One " establish a standard infection model for barramundi nodavirus in barramundi larvae and fry".

5.1. Methods

Virus prepared from infected fish

Whole larvae, or heads from fry, were homogenised in viral transport medium (M199 supplemented with 1000IU/ml benzylpenicillin, 1mg/ml streptomycin sulphate and 2µg/ml amphotericin B). The homogenate was transferred to screw-cap microcentrifuge tubes, clarified by centrifugation at 10,000×*g* for 10 minutes at 5°C and the supernatant stored at -80°C.

Cultured virus – tissue culture supernatant

Barramundi brain (LCB) cells were routinely cultured in Earle's modification of Eagle's medium (EMEM) supplemented with 20% FBS (fetal bovine serum) in 25cm^2 tissue culture flasks at 25°C. When cells were 60% confluent, the media was removed, 1ml inoculum was added and the flasks were incubated at 25°C for 60 minutes. The inoculum was removed and 10ml EMEM, supplemented with 10% FBS, was added. Flasks were incubated at 25°C and monitored daily for the presence of cytopathic effect (CPE). When typical barramundi nervous necrosis virus (BNNV) CPE had occurred, cells and media were harvested from the flasks and stored in 0.9ml aliquots at -80°C until required.

Histology

Selected fish samples for histology were preserved as whole fish or were divided into just head, half a fish (mid-line longitudinal cut) or half a head (mid-line longitudinal cut). The tissues were fixed in Bouin's solution (750 ml saturated picric acid (1-2%); 250 ml formaldehyde solution; 50 ml glacial acetic acid) for 24-48 hours, then transferred to 70% ethanol and held until processing. Standard processing procedures for histology were followed. After embedding in Paraplast©, 4µm sections were cut and stained with haematoxylin and eosin (H&E). Photomicroscopy was on an Olympus BX51 microscope with a DP12 digital camera system.

RT-PCR

RNA was extracted from 200µl aliquots of cell cultured virus and homogenised barramundi using a commercial kit (High Pure Viral RNA Extraction Kit, Roche), according to the manufacturer's instructions. Extracted RNA was either used immediately or stored at -80°C until required.

RT-PCR was performed using the R3-F2 primer set and method described by Nishizawa *et al* (1994), with some modifications. RNA was incubated at 90°C for 5 minutes then reverse transcription was conducted at 37°C for 60 minutes in 20µl Omniscript RT buffer (Qiagen) containing 9.5µl template, 4U Omniscript RT (Qiagen), 0.5mM each dNTP, 1U ribonuclease inhibitor (Promega) and 1.0µM reverse primer. PCR amplification was carried out in a 50µl reaction mix containing 5µl of the reverse transcription reaction, 0.2µM of each primer, 2.5mM $MgCl_2$ and 25µl HotStarTaq Master Mix (Qiagen). Thermal cycling was conducted

in an Eppendorf Mastercycler programmed as follows; 1 cycle at 95°C for 15 minutes, 25 cycles at 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 40 seconds and 1 cycle at 72°C for 10 minutes.

Nested RT-PCR

The nested RT-PCR was performed using the R'3-F'2 primer set and method described by of Thiery *et al* (1999), with some modifications. Amplification was carried out in a 50µl reaction mix containing 1µl of the RT-PCR reaction, 4.0µM of each nested primer, 2.0mM $MgCl_2$ and 25µl HotStarTaq Master Mix (Qiagen). Thermal cycling was conducted in an Eppendorf Mastercycler programmed as follows; 1 cycle at 95°C for 15 minutes, 25 cycles at 94°C for 40 seconds, 50°C for 40 seconds and 72°C for 40 seconds and 1 cycle at 72°C for 10 minutes.

RT-PCR products were separated by electrophoresis through 2% Agarose MS (Roche) using TAE buffer (40mM tris-acetate, 1mM EDTA) and DNA was visualised by UV illumination after staining with ethidium bromide.

Fish source, maintenance and larval rearing.

Barramundi larvae used in these trials originated from a commercial hatchery or the Northern Fisheries Centre (Cairns, QDPI) as day old hatchlings. The fry originated from the Northern Fisheries Centre, the larval rearing ponds at the Oonoonba Animal Health Station or larvae reared at the laboratory, were weaned onto a commercial crumble diet and maintained until used for transmission trials.

Larval rearing: A small green water larval rearing system in a 3,000L tank was used to rear the day old larvae until used in trials. A separate live food production area was used to culture algae, rotifers and enriched *Artemia* to supply live feed for the larval rearing tank and the experimental aquaria during transmission trials. Standard aquaculture techniques, although scaled down, were used for the live food and larval cultures (Palmer *et al* 1992). The rearing protocol (a summary) used for this project was:

a. Clean and sterilise (60mg/L chlorine for one hour) rearing tank.
b. Fill tank with 1,800L clean seawater (25-30ppt) and chlorinate to clean airstones.
c. Dechlorinate and leave for 15 minutes with air on.
d. Add 300L of algae culture so water has a light green/clear colour.
d. Add rotifers in the afternoon to make up 20-30 per ml in culture tank, 40-50 x 10^6, leave overnight.
e. Check rotifers have survived overnight and adjust culture water to light green (add algae).
f. Barramundi larvae are added to rearing tank on arrival as day old hatchlings, however they are not fed because they are still using yolk sac. Gently add rearing tank water to a bin containing larvae over half an hour (eg., 0.5L every 5 minutes) until both have the same pH, temperature and salinity. Then gently add larvae to the rearing tank either by bucket or siphon to a density of 40-160 larvae/L. A low aeration was maintained throughout tank so as to keep the larvae in the water column but not to rigorous to cause physical damage.
g. Check rotifer numbers and algae in rearing tank every morning and check water quality (pH, temperature, salinity and ammonia).
h. Add rotifers and clean seawater/algae as needed each day, approximately 200L per day.

i. Examine larvae microscopically after day 3 to day 10 to check for metamorphosis and swim bladder inflation. (The fishes swim bladder is formed by swallowing air at night therefore it is important for aeration to be low to allow fish to surface.)
j. Once swim bladders have formed, around day 8, the larvae start consuming more rotifers, thus increase amount to 40-60 per ml in rearing tank.
k. When the larvae grow to 8mm or greater, around day 14, *Artemia* nauplii can be added. Initially a small number of *Artemia* are added, then check to see nauplii in the gut of the barramundi larvae, if none wait before adding any more to the culture tank.
l. Keep adding rotifers and *Artemia* so that small larvae still have something to eat. After a maximum of a week of adding both zooplankton, start to feed only *Artemia*, but addition of algae continues.
m. Once the larval rearing tank is full of water do a 50% water exchange and continue this water exchange every day, adding 200L of algae and the rest clean seawater.
n. Do not always add newly hatched *Artemia*, keep some over night and feed the following day.
o. Once larvae are 10-15mm in size (total length), this is between 25-30 days depending on growth, they can be weaned and harvested.
p. Harvesting is done by syphoning water out of the larval rearing tank so larvae are in 10-15cm of water, removing the larvae by net into a bucket of clean water and transferring them to a smaller tank or floating cage for weaning.

All transmission trials were done in a secure isolation building. The experimental unit was a 120 litre glass aquarium (100 litres of water) with two canister filters providing aeration and removing particulate matter from the water. Water temperature was controlled to 26±2ºC by reverse cycle air conditioning. For fry, 20% of the water in the aquaria was changed twice a week following water quality checks for ammonia and nitrite levels. The filters were cleaned once a week. Any waste on the bottom of the aquaria was siphoned out each day. Waste water was discharged to storage tanks which, when full, were disinfected by chlorine then pumped into the sewage system.

The larvae, when stocked into the 120L aquarium (100L water) for transmission trials were feed with algae, rotifers and/or *Artemia* nauplii at the same level as that described in the larval rearing protocol for the age of the larvae in the trial. Water exchange and monitoring were also as that described for the green water larval rearing protocol. The fry, already weaned before use in transmission trials, were fed twice daily to satiation with a commercial crumble. The fry were usually acclimatised in the aquaria for 4-7 days prior to application of the selected infection treatment. A sub-sample of each batch of barramundi larvae or fry examined histologically to assess health immediately prior to the start of each trial. The fish were formally monitored twice a day and informally during routine maintenance activities on the aquaria.

Collection of survival data

Sick and moribund fish were removed and euthanased as soon as they were detected, normally at one of the twice daily formal monitoring events. Clinical signs and mortalties recorded twice a day. Euthansed fish were then preserved in Bouin's solution for subsequent histological examination or were frozen at -80°C in VTM (viral transport medium) for subsequent viral isolation or PCR analysis. At times the fish were divided in half along the long axis, with half for Bouin's fixation and the other half (or half head for the older fish) frozen at -80°C in VTM.

Experimental design

These trials were done individually over an extended period of time as larvae or fry of different ages are not available at the one time. The first three trials were principally to develop methodologies, for example a comparison of infected tissue culture supernatant with infected fish tissue homogenate as a source of infectious nodavirus or to confirm the period post-infection when infection could be detected histologically etc., and as such did not have a standard protocol for control treatments. Trials 4, 5 and 6 use control treatments of uninfected tissue culture supernatant as a mock treatment.

Trial 1: 4 day larvae (4.5mm TL average).
Two treatments (bath infection with infected tissue culture supernatant (ITCS) and bath infection with clarified, infected tissue homogenate from fry with viral nervous necrosis [40 gram tissue/100ml VTM]) and a control (tissue culture supernatant) were duplicated. Approximately 2,000 four day old larvae were stocked into each aquaria. The aquaria had water levels reduced by 50% and 10ml of the ITCS, infected homogenate or TCS were added. The aquaria were left for one hour then refilled with clean seawater. The trial ran for 14 days.

Trial 2: 19 day fry (16mm TL average).
Nine aquaria were stocked with approximately 2,000 fry when they were 14 days old. One aquarium was an untreated control. The 8 treatment aquaria had water levels reduced by 50% and 6ml of infected homogenate (40 gram tissue/ 100ml viral transport media) in 100ml of seawater was added when the larvae were 19 days of age. After one hour the aquaria were refilled with clean seawater. The trial ran for 14 days.

Trial 3: 10 week fry.
Ten aquaria were stocked with approximately 60 fry of 10 weeks age. Two aquaria were untreated controls. The eight treatment aquaria had water levels reduced by 50% and 10ml of infected tissue culture supernatant (ITCS) was added. After one hour the aquaria were refilled with clean seawater. Dead and moribund fish were removed as soon as they were detected. In addition, ten randomly selected fry were removed each day, five for histology to assess the development of any central nervous system tissue lesions over time and five for virological analysis if required. The trial ran for 28 days.

Trial 4: 20 day fry.
Post-metamorphosis barramundi fry at 16 days of age were harvested from an extensive larval rearing pond at the Oonoonba Animal Health Station and were held in a 1,000L tank for conversion to freshwater over four days. *Artemia* nauplii were feed for the first two days as the fry were weaned onto a commercial barramundi crumble. Three freshwater aquaria were stocked with 150 fry when they were 20 days age. One aquarium was a control and two were used as replicated treatments. The water levels were reduced by 50% and the two treatment aquaria had 15ml of infected tissue culture supernatant (ITCS) added and 15ml tissue culture supernatant (TCS) was added to the control aquarium. After one hour the aquaria were refilled with clean water. The trial was terminated on day 18 after all the fish in the infection treatment aquaria had died.

Trial 5: 6 week fry (a control in conjunction with the sleepy cod 6 week infection trial).
Two freshwater aquaria were stocked with 100 fry at 6 weeks of age. One aquarium was a control where the fish were intraperitoneally injected with 0.02ml of uninfected tissue culture supernatant (TCS) and, in the other, fish were infected by intraperitoneal injection of 0.02ml of ITCS. Following some initial losses during acclimatisation, the control aquarium

contained 93 fish and the injection treatment aquarium had 97 fish on day 1 of the trial. The trial ran for 28 days.

Trial 6: 12 week fry (a control in conjunction with the barcoo grunter 12 week infection trial).
Two freshwater aquaria were stocked with 40 fry at 12 weeks of age. One aquarium was a control where the fish were intraperitoneally injected with 0.02ml of uninfected tissue culture supernatant (TCS). The other was an injection infection treatment where fish were intraperitoneally injected with 0.02ml of ITCS. The trial ran for 28 days.

5.2 Results and discussion

Trial 1: 4 day larvae.

By day 6 post-infection both bath infection treatments were estimated to have had 50-70% mortalities, while there were significantly fewer larval mortalities in the control aquaria. There was no noticeable difference in the level of mortalities between the aquaria infected by ITCS and clarified tissue homogenate, or the replicates. Pre-trial histology revealed no abnormalities in the larvae. In those surviving larvae harvested at the end of the trial, some had a marked vacuolation and neuronal cell necrosis in the brain and retina. Tissue homogenates from larvae in both treatments were nodavirus positive by virus isolation on LCB cells and nodavirus RT-PCR. These results confirmed the barramundi nodavirus in infected tissue culture supernatant was as infectious as the virus from barramundi fry with viral nervous necrosis.

Trial 2: 19 day fry.

The effect of the bath infection with nodavirus infected tissue homogenate is shown in Fig. 1.

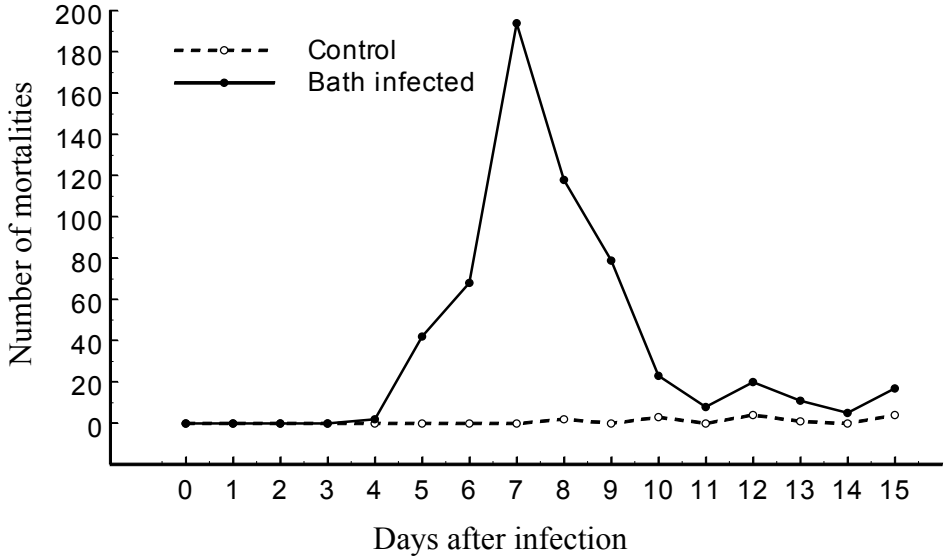

Figure 1: The number of daily mortalities of 19 day old barramundi fry bath exposure to nodavirus infected tissue homogenate (total from eight aquaria).

The pattern of mortalities in these bath infected 19 day old barramundi fry is similar to that seen in actual clinical outbreaks. The first mortalities occurred on day 4, peaked on day 7 and continued from day 10 at a low but continuous level until the trial ended. Tissue homogenates from selected moribund fry were nodavirus positive by the nested RT-PCR. This confirmed that a realistic experimental infection model was possible in larval barramundi of this age.

Trial 3: 10 week fry.

Mortality effect: Total mortalities in all eight treatment aquaria were 15 fish (or approximately 3.1%). No mortalities were recorded in the two untreated control aquaria. As ten fish were removed each day, the estimate of mortality rate must be considered very approximate as a fish removed on day 1 or 2 post-infection (PI) may have subsequently died on day 10 or 13 PI. In this trial the first mortality was observed on day 7 PI and the last on day 22 PI. The typical acute, rapidly increasing mortality pattern, as seen in Trial 2, was not seen in this trial. It still can be concluded that barramundi nodavirus is infectious to 10 week old barramundi by bath exposure, albeit not as a highly lethal infection.

Histopathology of sequentially sampled barramundi: At day 6 PI a single cell in the spinal cord of one fish was seen with scant but basophilic cytoplasm, suspicious of a nodavirus-caused lesion (Glazebrook et al 1990; Munday et al 1992). In fish sampled at day 8 PI single vacuolations, typical of viral nervous necrosis, were detected in the olfactory lobe, the granular cell layer of the optic tectum and the granular cell layer of the cerebellum (Fig. 2). A few cells with basophilic cytoplasms were also present in the outer edge of the olfactory lobe. In all subsequent days of the trial at least one of the five fish sampled for histology would have central nervous system tissue lesions. Single vacuoles were first present in the external and internal nuclear layers of the retina on day 13 PI. Degenerating mononuclear cells were present in the posterior compartment (vitreous humor) of the eye in these fish as well. By day 15-17 PI the severity and extent of vacuolation and neuronal degeneration in the brain and retina (Fig. 3) was at its greatest. In the brain, vacuolation, degeneration and cells with basophilic cytoplasms were present in the granular cell layers of the optic tectum and cerebellum, in the periventricular area of the hypothalamic region and in the outer edge of the olfactory lobe. By day 23 PI vacuolation was rare in the brain and retina. The lesion detected was typically a cell with a basophilic inclusion (Fig. 4), although some fish still had some degenerate mononuclear cells in the posterior compartment of the eye. No histopathology was present in control fish. No histopathology was seen in another tissue or organ.

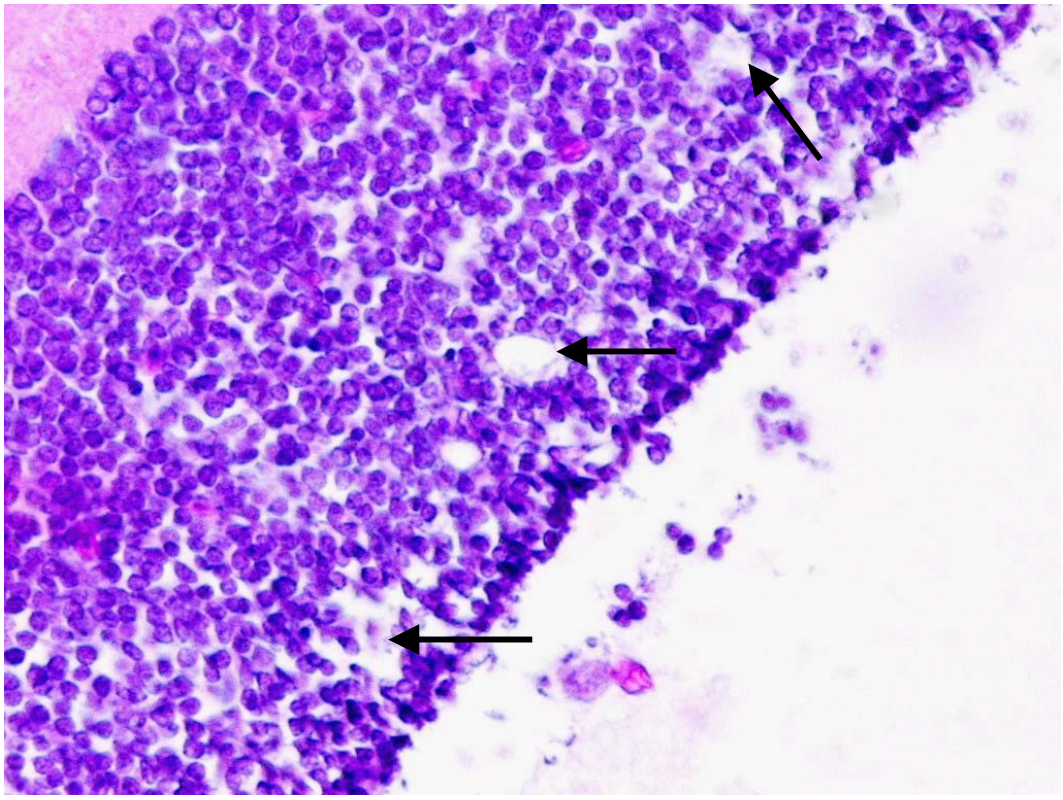

Figure 2: Barramundi fry 8 days after bath infection with nodavirus infected tissue culture supernatant. The arrows indicate single vacuoles in the granular cell layer of the optic tectum. x400, H&E.

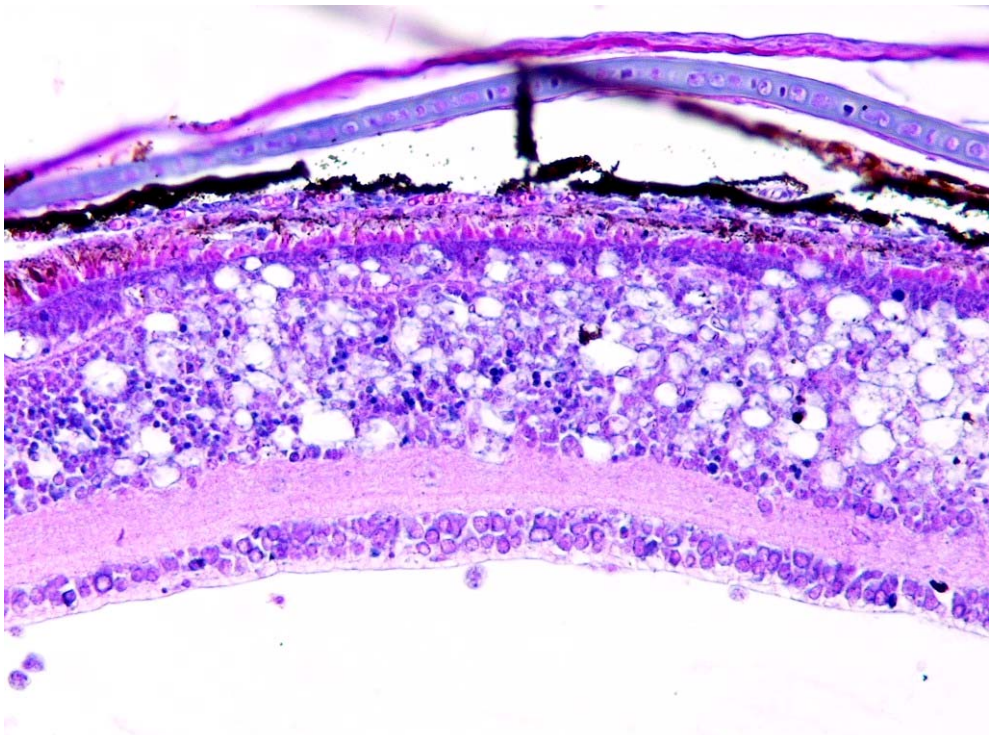

Figure 3: Barramundi fry 16 days after bath infection with nodavirus infected tissue culture supernatant. The vacuolation and degeneration in the internal nuclear layer of the retina is extensive and extends to the external plexiform and external nuclear layers of the retina. Note that basophilic inclusions are common and some of the cells in the ganglion cell layer have basophilic cytoplasms. x200, H&E.

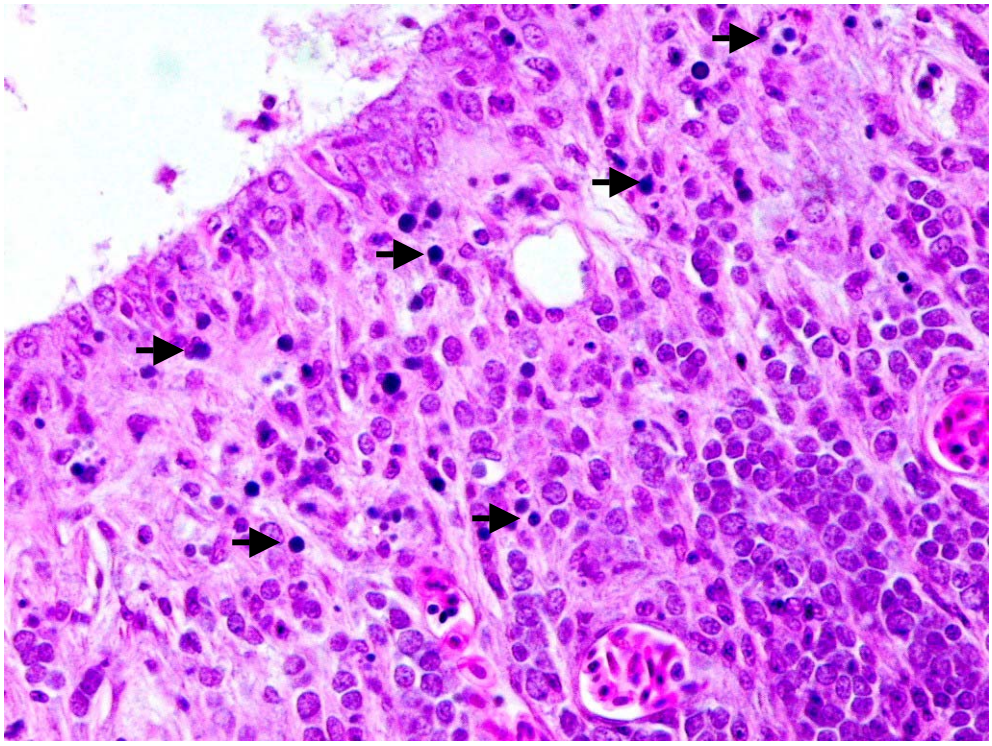

Figure 4: Barramundi fry 26 days after bath infection with nodavirus infected tissue culture supernatant. The arrows indicate some of the cells containing basophilic inclusions present in this section of the granular cell layer of the optic tectum. x400, H&E.

The sequential histopathology study clearly shows that, while there were only a few deaths caused by this bath challenge, transmission of the nodavirus infection occurred successfully to many of the experimental barramundi fry and typical viral nervous necrosis pathology was produced in central nervous system tissues. It also indicated that the infection did not cause permanent nervous tissue damage in the fry. By the end of the trial there was a distinct progress to resolution of the lesions, although even in the day 28 PI sample, one of the fish had a few cells at the edge of the olfactory lobe with intracytoplasmic basophilic inclusions.

Trial 4: 20 day fry.

The effect of the bath infection in freshwater with nodavirus infected tissue culture supernatant is shown in Fig. 5. Note the trial ended on day 18 PI when all the fish in the treatment aquaria were dead. This does not total 100% as a significant number of fish that were moribund or dead were identified as victims of cannibalism. Eighteen from the control aquarium and 61 from the two treatment aquaria were removed as victims of cannibalism and were not recorded as a mortality for purposes of illustrating the effect of nodavirus infections in this trial. The genetics of the barramundi used in these trials is essentially that of a wild animal and divergent growth rates between individual fish are observed. As a consequence when barramundi are reared at densities used in aquaculture, and when they are in a very rapid growth phase of their life cycle, a fish will try to cannibalise other fish that are smaller in size.

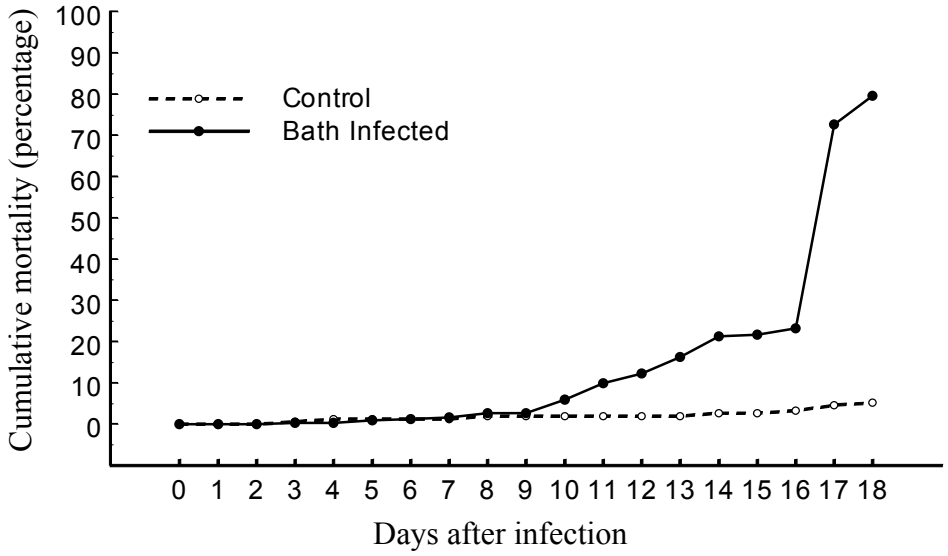

Figure 5: The mortality of 20 day old barramundi fry after bath exposure to nodavirus infected tissue culture supernatant.

A cumulative mortality of 79.7% (mean, n=2) was recorded, although this probably would have been lower as the day 17 and 18 PI fish examined histologically not only had central nervous system tissue lesions but also a white spot (*Ichthyophthirius multifiliis*) infection of the skin. The mortality pattern did not have the acute appearance of that seen in Trial 2 or in clinical epizootics. The first mortality were not observed until day 10 post-infection (PI) and steadily increased until day 16 PI when the white spot infection probably became the principal pathogen. Selected moribund fish removed during the trial were examined histologically and most had central nervous system tissue lesions of neuronal degeneration typical of viral nervous necrosis.

Despite the early end to the trial it can be concluded that transmission of a lethal BNNV infection by bath exposure in freshwater is possible in barramundi of this age. Although the onset of first mortalities was delayed as compared to that seen in Trial 2 and the nodavirus-alone mortalities were leveling off at around 23%. An effect of virus dose, ITCS verses tissue homogenate and freshwater on ITCS when it was added to the treatment aquaria, may also explain the differences between Trial 2 and this trial.

Trial 5: 6 week fry.

The effect of intraperitoneal injection of nodavirus infected tissue culture supernatant (ITCS) is shown in Fig. 6.

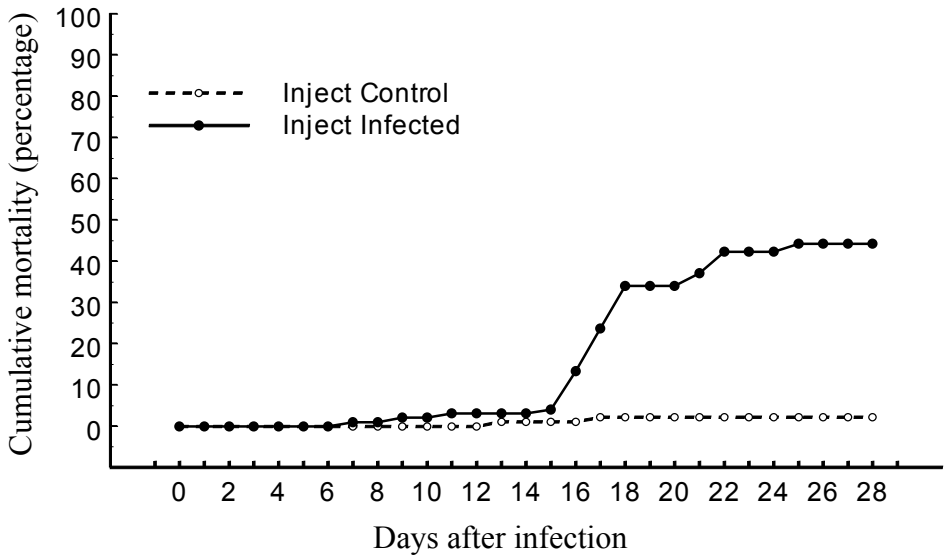

Figure 6: The mortality of 6 week barramundi fry after injection of nodavirus infected tissue culture supernatant.

This infection route and dose was directly equivalent to that used in the 6 week sleepy cod infection trial (see section 6.1.2.3). A cumulative mortality in the one treatment aquarium was 44.3% at 28 days PI was recorded. While the first mortality was seen on day 7 PI, these initial few mortalities were also seen in the control aquarium. The significant increase in fish deaths began on day 16 PI. Selected moribund fish sampled for histology through the trial had vacuolation and neuronal cell degeneration in the brain and retina. When surviving ITCS infected fish were examined, three of 54 had brain cells with basophilic inclusions. The results indicate that an experimental infection in barramundi at 6 weeks of age is possible by the direct infection route of intraperitoneal injection.

Trial 6: 12 week fry.

The effect of intraperitoneal injection of nodavirus infected tissue culture supernatant is shown in Fig. 7.

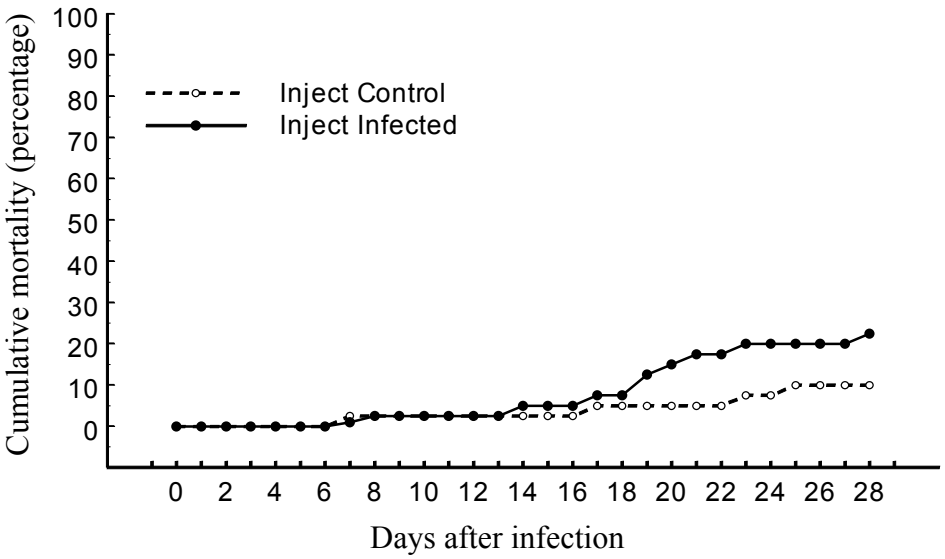

Figure 7: The mortality of 12 week barramundi fry after injection of nodavirus infected tissue culture supernatant.

Similar to Trial 5, the infection route and dose was equivalent to that used in the 12 week barcoo grunter infection trial (see section 6.1.2.4). A cumulative mortality in the one treatment aquarium was 22.5% at 28 days PI. Again the onset of increasing mortalities was not really seen until day 18 PI. The control aquarium had total of 10% mortalities at the end of the trial. This was attributed to bacterial infections of the skin, probably secondary to trauma from fish-to-fish interactions. Three of the moribund fish removed during the trial had scattered, single vacuoles in the granular cell layer of the cerebellum or the outer edge of the olfactory lobe. Some cells with basophilic, intracytoplasmic inclusions were also present in these areas of the brain. Seven of the 52 fish surviving ITCS infection had a few cells in the outer edge of the olfactory lobe or cerebellum with basophilic inclusions. The results indicate that an experimental infection in barramundi at 12 weeks of age is possible by the direct infection route of intraperitoneal injection. The level of mortality is higher than that achieved when ten week fry were bath infected (22.5% vs ≈3.1%).

Discussion

The objective to establish a standard infection model for barramundi nodavirus or barramundi nervous necrosis virus (BNNV) was met. It was confirmed the BNNV cultured in the barramundi brain cells (LCB) i.e., ITCS, was as infectious as the virus recovered from the tissues of barramundi fry with viral nervous necrosis. A bath transmission of BNNV was achieved in barramundi fry. The bath infection is expected to be closer to the natural route of infection. A bath transmission of BNNV in freshwater was achieved in 20 day old barramundi fry. This indicated that a bath challenge of freshwater fishes was a possibility. There was also confirmation that direct parental infection by injection of ITCS could cause VNN in 6 and 12 week old barramundi fry, with a more severe effect seen in the younger fish. This age effect, with older fish more resistant to the effects of BNNV, was also shown in the

bath infection of 20 day fry (Trial 4) and 10 week fry (Trial 3). The issue of age and nodavirus infection is more fully discussed in section 6.1.2.5.

It was intended to titrate the BNNV infectious dose so that a standard number of viruses could be used in transmission trials to more accurately compare the effect of BNNV in different fish species. With the transformation of the barramundi brain cell line (LCB) at around passage 35, a CPE was not produced when the LCB was infected with ITCS or homogenates from diseased barramundi fry. This prevented titration of the virus and thus standardizing an infectious dose. Detailed comparisons of the dose effects of BNNV on various fish species was not going to be possible but some level of comparison was possible by standardizing the infection methods used. The addition of 15ml of ITCS to a 120 litre aquarium filled with as little water as possible to sustain the experimental fish for one hour (there by maximising the fishes exposure to BNNV) and injection of 0.02ml into the abdominal cavity was the standard decided for use in the freshwater fish transmission trials.

6. The effect of barramundi nodavirus on freshwater fishes.

Under Objective Two "more accurately define the range of fish species that can actually be infected by barramundi nodavirus, the effect of the virus on these fishes and the infectious dose".

6.1. Freshwater fish infection by barramundi nodavirus – survival effects.

6.1.1. Methods

Cultured virus – tissue culture supernatant

Barramundi brain (LCB) cells were routinely cultured in Earle's modification of Eagle's medium (EMEM) supplemented with 20% FBS (fetal bovine serum) in 25cm^2 tissue culture flasks at 25°C. When cells were 60% confluent, the media was removed, 1ml inoculum was added and the flasks were incubated at 25°C for 60 minutes. The inoculum was removed and 10ml EMEM, supplemented with 10% FBS, was added. Flasks were incubated at 25°C and monitored daily for the presence of cytopathic effect (CPE). When typical BNNV CPE had occurred, cells and media were harvested from the flasks and stored in 0.9ml aliquots at -80°C until required.

Fish source and maintenance

Four species of freshwater fishes were used for these trials; silver perch (*Bidyanus bidyanus*), golden perch (*Macquaria ambigua*), sleepy cod (*Oxyeleotris lineolatus*) and barcoo grunter (*Scortum barcoo*). The selection of these species was mainly based on the ability to purchase or obtained specimens of a defined age from commercial fish hatcheries or from government breeding programs. Despite the commercial-scale production of these species, they are all valued freshwater recreational fishes as well as species, or candidate species, for aquaculture. Silver perch and golden perch are native to the Murray-Darling system, quite isolated from the natural distribution of barramundi which are found in the south from the Mary River, north across northern Australia. Barcoo grunter are native to the Lake Eyre and Bulloo-Bancannia catchments (including the Barcoo River from where the fish got its name), again isolated from the coastal distribution of barramundi. In recent years all three species have been bred and stocked or farmed east of the dividing ranges from northern New South Wales to northern Queensland. Sleepy cod are naturally found in freshwater streams and lagoons from the Dawson River northwards both coastal and inland to the Gulf of Carpentaria. Sleepy cod can overlap with the distribution of barramundi in these northern coastal rivers. The natural isolation of silver perch and golden perch from native barramundi was the principal basis for the concern on the risk of translocation of barramundi nervous necrosis virus and the possible lethal effects on these freshwater fishes.

Two ages of fish were used for the experimental infections, approximately 6 weeks of age and 12 weeks of age. The aim was to obtain fry as young as possible and then fish of the age when they are normally moved from the hatchery nursery to farms for stocking in grow-out ponds. As all these species are commercially produced through the use of extensive larval rearing ponds, we were not able to get the hatchery operators to collect fish from the ponds before they were harvested in the fourth week post stocking with free-swimming larvae. As the project was not funded to breed and rear the larvae of freshwater fishes we had to compromise and accept the 4-5 week old fry as the youngest age group. On arrival at the laboratory, the fish were acclimatised for five to seven days prior to application of the

selected treatment. As a consequence the age of the fish at the start of each trial was 5-6 weeks old or 11-12 weeks old. A sub-sample of each batch (age, species) was examined histologically to assess health status prior to commencing a trial.

All transmission trials were done in a secure isolation building. The experimental unit was a 120 litre glass aquarium (100 litres of water) with two canister filters providing aeration and removing particulate matter from the water. Water temperature was controlled to 26±2ºC by reverse cycle air conditioning. 20% of the water in the aquaria was changed twice a week following water quality checks for ammonia and nitrite levels. The filters were cleaned once a week. Any waste on the bottom of the aquaria was siphoned out each day. Waste water was discharged to storage tanks which, when full, were disinfected by chlorine then pumped into the city sewage system.

The fish were fed once or twice daily depending on species preference, and each time were fed until satiation. The 5+ week old fish were fed purchased frozen brine shrimp or zooplankton mix as they were weaned onto commercial aquarium fish flakes then crumble. The 11+ week old fishes were feed commercial aquarium fish flakes then crumble. The fish were formally monitored twice a day and informally during routine maintenance activities on the aquaria. Sick fish were removed and euthanased as soon as they were detected, normally at one of the twice daily formal monitoring events.

Experimental design

Each trial was set up with duplicate aquaria for the infection treatments and a single aquarium for the equivalent control treatment. The source of the nodavirus was infected tissue culture supernatant (ITCS). The method of infection consisting of injection of 0.02ml of ITCS intra-peritoneally or bath exposure in water. The bath infection was done by reducing the water level in the aquaria to a 5 cm depth (approximately 15 litres) and adding 15ml of ITCS in three lots of 5ml with 15 minutes gap, then leaving the fish in this reduced volume containing nodavirus for one hour before refilling the aquaria. The step wise addition of ITCS was needed as some species showed a toxicity to the tissue culture supernatent (infected or not). Control treatments followed the same method except that uninfected tissue culture supernatant was used. We aimed to stock about 40 fish per aquarium. The actual number of fish used did vary (see Table 1) depending on initial survivals on acclimatisation and the number of fish in the batch purchased.

The first trials were done with silver perch and it was found there was no successful infection following bath exposure. Subsequently infection trials only used the bath infection treatment in the six week old fish groups, the 12 week old fish only had ITCS injected. The infection trials were run for up to 28 days or when all fish had died.

Collection of survival data

Moribund fish were removed when detected. Clinical signs and mortalties recorded twice a day. Fish were euthansed and then preserved in Bouin's solution for subsequent histological examination. Frequently the sick fish were divided in half along the long axis, with half for Bouin's fixation and the other half (or half head for the 12+ week fishes) was frozen at -80°C for subsequent PCR analysis.

Table 1: The actual number of fish used in each of the infection trials.

Trial	Fish – age	Treament and replicate	Number of fish/ aquarium
1	Silver perch – 6 weeks	Bath infect 1 & 2	39, 41
		Bath control	36
		Inject infect 1 & 2	27, 38
		Inject control	33
2	Silver perch – 12 weeks	Bath infect 1 & 2	30, 30
		Bath control	30
		Inject infect 1 & 2	30, 30
		Inject control	30
3	Golden perch – 6 weeks	Bath infect 1 & 2	41, 40
		Bath control	40
		Inject infect 1 & 2	38, 40
		Inject control	41
4	Golden perch – 12 weeks	Inject infect 1 & 2	42, 41
		Inject control	41
5	Sleepy cod – 6 weeks	Bath infect 1 & 2	39, 40
		Bath control	42
		Inject infect 1 & 2	37, 41
		Inject control	40
6	Sleepy cod – 12 weeks	Inject infect 1 & 2	39, 38
		Inject control	41
7	Barcoo grunter – 6 weeks	Bath infect 1 & 2	40, 40
		Bath control	40
		Inject infect 1 & 2	43, 41
		Inject control	41
8	Barcoo grunter – 12 weeks	Inject infect 1 & 2	41, 41
		Inject control	40

6.1.2. Results and Discussion

Table 2: Summary of the results of the freshwater fish infection trials – mortality ([a]mean of duplicate aquaria) and presence of central nervous system ([b]CNS) pathology in sick and moribund fish removed through the trials.

Fish - age	Infection route	Percent dead at 28 days[a]	CNS pathology[b]
Silver perch – 6 weeks	Bath	3.8	No
	Injection	46.2	Yes
Silver perch – 12 weeks	Bath	8.3	No
	Injection	25.0	Yes
Golden perch – 6 weeks	Bath	2.5	No
	Injection	6.4	Yes
Golden perch – 12 weeks	Injection	14.5	Yes
Sleepy cod – 6 weeks	Bath	0	No
	Injection	98.7	Yes
Sleepy cod – 12 weeks	Injection	92.3	Yes
Barcoo grunter – 6 weeks	Bath	0	No
	Injection	4.8	Yes
Barcoo grunter – 12 weeks	Injection	7.3	Yes

6.1.2.1. Silver perch

At 6 weeks of age the silver perch had moderate mortality (mean = 46.2%) when ITCS was injected into the abdomen. Figure 8 shows the mortality curves (a simple average of the duplicated treatment tanks) following infection by nodavirus. Control mortalities were present (11.1% bath; 3.0% injection) and were similar to the 3.8% mortality seen in the bath infection treatment aquaria. Subsequent histological examination (see section 6.2.2.1) of dying fish sampled during the trial and fish that survived the 28 day trial, confirmed that only the silver perch that were injected with ITCS had neuronal degeneration of the central nervous system.

A moderate mortality was also seen in the 12 week old silver perch (mean = 25.0%) injected with ITCS. Bath infected fish had a mean cumulative mortality of 8.3% but, as in the 6 week silver perch trial, subsequent histological examination of dying fish and of survivors found no neuronal degeneration or necrosis. All control fish (3.3% mortality in bath and injection controls) had normal central nervous system tissue. The mortality curve is shown in Fig. 9.

No clinical signs were observed in bath infected silver perch. The clinical signs seen in the diseased silver perch injected with ITCS were similar in the 6 and 12 week trials. Initially fish would begin to loose coordination of their swimming, then loose buoyancy and sink to the bottom of the tank unless they were actively swimming. As the silver perch became more lethargic a darkened body colour would become obvious and the fish would lie on their side at the tank bottom. Spiral swimming was seen in one of the diseased silver perch.

6.1.2.2. Golden perch

In the infection trial for 6 week old golden perch there was a low level of cumulative mortality (mean = 6.4%) in the fish that were injected with ITCS. Even though this was low, bath infection, injection control and bath control mortalities were all lower, with 2.5%, 4.9% and 5% observed respectively. Further, histological lesions of neuronal degeneration of central nervous system tissues were only present in the dying fish from the injection infect treatment aquaria. This confirms nodavirus infection and disease did occur in the 6 week golden perch.

A higher cumulative mortality was seen in the 12 week old golden perch (mean = 14.5%) injected with ITCS. Control survival was 100%. No attempt was made to bath infect these older golden perch because of the failure to achieve an experimental infection in the 6 and 12 week old silver perch. The higher mortality seen in the 12 week old fish was not expected as we had seen from the barramundi infection trials that increasing age of fry was associated with lower nodavirus effects. Examination of dying fish on the ninth day of the trial did reveal an ectoparasite infection of the gills by *Ichthyobodo necator*, but all subsequent fish removed during the trial did have central nervous system tissue lesions of neuronal degeneration. As the trials for 6 week and 12 week old golden perch had to be done at different times (availability of fish of defined ages) we assume the ITCS used in each trial had a different infectious dose of the nodavirus. The transformation of the LCB cells meant we were not able to titrate the viable virus dose as planned at the start of this research. The mortality curves for the golden perch trials are shown in Fig. 10 and 11.

No clinical signs were observed in bath infected 6 week old golden perch. The 6 week old golden perch that were injected with ITCS, and became diseased, showed darkening of body colour, some were emaciated and would be found moribund on the tank bottom. At 12 weeks of age a loss of coordination, often swimming on their side and at the water surface or in mid-water, was seen as were some fish with emaciation (diseased fish were seen to attempt to feed but often missed food particles when striking) and some darkening of body colour. In the end the golden perch would be moribund on the bottom of the tank.

6.1.2.3. Sleepy cod

In both the 6 week and 12 week old sleepy cod there was a marked mortality in the fish injected with ITCS. A mortality curve typical of an acute, virulent infection was seen (see Figs. 12 and 13). Mortalities began on the sixth day after infection and rapidly increased until day 10 when they levelled off. The cumulative mortality in the six week old fish was 98.7% (mean, n=2) and in the 12 week old fish was 92.3% (mean, n=2). The control mortalities in the 6 week old sleepy cod were 14.3% in the bath aquarium and 7.5% in the injection aquarium, both higher than the bath infect fish where no mortalities were recorded. No mortalities were seen in the injection control fish in the 12 week trial. Histological examination of the bath infect 6 week fish at the end of the trial found no lesions. Similarly no tissue lesions were seen in the 6 week control fish examined at the end of the trial.

No clinical signs were observed in bath infected 6 week old sleepy cod. Both 6 and 12 week old sleepy cod injected with ITCS, and became diseased, showed a loss of buoyancy control with fish floating to the water surface. These fish would lie on their sides or back and, when disturbed, could not right themselves and would swim off with an uncoordinated spiral swimming. These fish would remain lethargic and anorexic until they became moribund. In

the trial at 12 weeks of age, diseased sleepy cod also had enlarged abdomens (dilated swim bladders).

6.1.2.4. Barcoo grunter

The barcoo grunter that were injected with ITCS at six weeks of age had a cumulative mortality of 4.8% (mean, n=2), higher than the bath infect treatment, where no mortalities were seen, and the controls (2.4% injection, 0% bath). The mortality curve is shown in Fig. 14. Histological examination of dying fish sampled during the trial, and fish that survived the 28 day trial confirmed that the barcoo grunter injected with ITCS had neuronal degeneration of central nervous system tissues. Bath infect and control fish examined at the end of the trial had normal central nervous system tissue.

A cumulative mortality of 7.3% (mean, n=2) was seen in the 12 week old barcoo grunter injected with ITCS (see Fig. 15). Histological examination found the injection infect fish had neuronal degeneration. While the control injection fish had a 2.5% mortality during the trial, none of the fish had central nervous system tissue lesions when examined at the end of the trial.

At the end of the trial of bath infected 6 week old barcoo grunter it was observed that about two-thirds of the fish were anorexic, lethargic and had a darkened body colour. The 6 week old barcoo grunter that were injected with ITCS, and became diseased, were anorexic, had a darkened body colour and swam erratically. Some fish were positively buoyant and would drift to the water surface if not swimming. The diseased older barcoo grunter (12 week) lost buoyancy, sinking to the tank bottom if not swimming. At the tank bottom the fish would lie on their sides and, if disturbed, swim off in an abnormal manner showing no balance. The fish were anorexic and had a dark body colour.

6.1.2.5. Discussion

The possible effect of barramundi nodavirus on native freshwater fishes has been a concern for fisheries managers from the time barramundi fingerlings began to be translocated outside their natural range for aquaculture. This concern about disease translocation was further highlighted by research done in 1994 (Glazebrook, 1995) which apparently demonstrated experimental, lethal transmission of barramundi nodavirus, as a clarified homogenate of diseased barramundi fry, to 2-3 month old Macquarie perch, Murray cod and silver perch by bath infection. These findings have been questioned as Glazebrook reported both indirect fluorescent antibody tests (IFAT) (using anti-striped jack nodavirus rabbit serum) and nodavirus RT-PCR tests failed to confirm nodavirus in the tissues of dying experimental fishes despite reported histological nervous lesions. The development of the barramundi brain cell (LCB) culture system at OVL created an opportunity to produce barramundi nodavirus *in vitro* to again look at the susceptibility of Australian freshwater fishes to barramundi nodavirus.

The results reported here show that silver perch, golden perch, sleepy cod and barcoo grunter are susceptible to barramundi nodavirus, the infected tissue culture supernatant can cause mortalities in all four species and that, even in surviving fish, the virus can persist in central nervous system tissues for at least 28 days (as assessed by histology and nested RT-PCR; see results in the following section 6.2.4). This susceptibility was only demonstrated on intra-peritoneal injection. At both ages, 6 and 12 weeks, we could not transmit the infection via the bath exposure method, the route of infection which would be considered more closely replicating a natural infection route. None of the fish examined from the control aquaria or

the bath infect treatments had histological lesions indicating nodaviral pathology in central nervous system tissues. This is in direct contrast to the Glazebrook (1995) report of 100% mortalities in 3 month old silver perch following bath infection.

The failure to transmit the infection via bath exposure may be attributed to the age of the experimental fish or the concentration of the nodavirus used in the bath challenge. A number of studies have shown a fish age effect in experimental nodavirus infections. As the only young (larvae or fry) freshwater fish available in numbers as experimental fish are from commercial hatcheries, we were unable to obtain a supply of fish younger than 4-5 weeks. The extensive larval rearing methods in fertilised ponds only allowed access to fish at the drain harvest. The infection model trials done in this project found that a bath transmission was possible when barramundi were 20 days of age and even in 10 week old fish but the effects on the 10 week old barramundi were much less severe. Grove *et al* (2003) attempted to infect 8 month old Atlantic halibut with Atlantic halibut nodavirus by bath exposure and intraperitoneal injection. No clinical disease was seen but CNS histopathology was present, and nodavirus was detected in tissues by RT-PCR, 13 days post intraperitoneal infection. No virus was detected in the bath exposed Atlantic halibut. Attempts to infect sea bream fry with seabass nodavirus demonstrated a lethal transmission with intramuscular injection of 2 and 4 gram fry but no disease was seen in immersion challenge (Aranguren *et al* 2002). Using a clarified homogenate of striped jack with viral nervous necrosis, Arimoto *et al* (1993) successfully transmitted an infection to 1 day old striped jack larvae via bath exposure but could not transmit an infection to 81 day old juvenile striped jack. It was planned that all our transmission trials would use a known titre of virus, so as to standardise the infection challenge. With the transformation of the barramundi brain cell line (LCB) at passage 35-45, a CPE was not produced when the LCB was infected with ITCS or homogenates from diseased barramundi fry. This prevented us from standardizing the infection dose and we may have been able to transmit the infection in a bath challenge if sufficient virus was present. Nodavirus bath infectious doses for various species of marine fish fry have been limited to reports of single dose rates, i.e., $10^{5.1}$ $TCID_{50}$/ml for 2 hours, 3.7 x 10^{7} $TCID_{50}$/ml for 1 hour and 6 x $10^{5.2}$ $TCID_{50}$/ml for 2 hours (Tanaka *et al* 2003, Húsgard *et al* 2001, Hegde *et al* 2002), but there is a report of the effect of virus dose on disease induction with intramuscular injection of an infected tissue homogenate. Tanaka *et al* (1998) reported the effects of injection of 0.04ml of a serial dilution of a homogenate prepared from sevenband grouper fry. At 10^{-1} and 10^{-2} all sevenband grouper juveniles were dead by 6 days post-infection but at 10^{-5} and 10^{-6} there were no mortalities and no virus detected in survivors. Infection doses of 10^{-3} and 10^{-4} resulted in intermediate mortalities.

We would conclude that, while we could not demonstrate a bath transmission of BNNV to the freshwater fishes, it still does not rule out the possibility younger fish may be susceptible to the barramundi nodavirus in water. It would still be useful to determine the minimum infectious dose for the immersion route of infection on fish younger than 5-6 weeks of age. This would allow a better measure of the susceptibility of the freshwater fishes to BNNV and a better understanding of the true risk posed by diseased barramundi to other fishes.

The occurrence of viral nervous necrosis in freshwater fishes was, until recently, unknown. The betanodavirus infections in finfish are considered a marine fish disease (see Munday, Kwang and Moody 2002). In 1999 Skliris and Richards report an experimental infection of tilapia intraperitoneally injected with sea bass nodavirus (with the tilapia held in seawater). While no mortalities occurred, there was some minor neuronal pathology and the virus was reisolated from brain tissue indicating viral replication. A nodavirus was isolated from listless, emaciated guppys in a farm in Singapore (Hegde *et al* 2003). Again, while no disease was seen in an experimental bath challenge using the isolated nodavirus, virus was detected in

experimental guppys on day 3 and 10 post-infection indicating viral replication. As the guppy nodavirus was the same as the grouper nodavirus endemic to marine fish in Singapore, Hegde *et al* (2003) suggested the nodavirus in the guppys could have been horizontally spread from marine finfish. A natural outbreak of viral nervous necrosis was reported in 8 month old sea bass in a freshwater recirculation system in Greece where 30% mortalities occurred over a month (Athanassopoulou *et al* 2003). The source of the nodavirus was not known but it seemed reasonable to expect the infection was carried by the fish from the hatchery (marine environment). The results from this project clearly demonstrate the four species of freshwater fishes are susceptible to experimental BNNV infection and show clinical signs as a result.

The variation in the way each of the four species responded to the inject infection is not surprising and would not be attributed just to variation in virus dose. While most nodaviruses appear relatively non-specific as far as fish hosts that they can infect (Munday *et al* 2002), there are examples where the effect of a nodavirus can vary depending on the species of fish infected. Early research in Japan found that striped jack nodavirus (as a homogenate) could be transmitted to striped jack larvae but not to red seabream, goldstripe amberjack nor yellow tail larvae (Arimoto *et al* 1993). When sevenband grouper nodavirus (as a homogenate) was intramuscularly injected into sevenband grouper juveniles 100% died by day 6 post-infection but only 20% of redspot grouper had died by day 19 post-infection (Tanaka *et al* 1998). Castric *et al* (2001) showed that a nodavirus isolated from asymptomatic sea bream was pathogenic to sea bass but did not cause disease in sea bream in experimental infections. Experimental infection of striped jack larvae held at 20°C with Atlantic halibut nodavirus did not cause disease. Similarly infection of Atlantic halibut larvae held at 6°C with striped jack nodavirus did not cause disease (Totland *et al* 1999). The failure of cross-species transmission in this study may have also have been related to temperature adaptation of the different nodaviruses. In a comprehensive study investigating cross-species transmission, Tanaka *et al* (2003) used sevenband grouper nodavirus to bath challenge seven species of marine finfish fry. After 15 days 4% of the sevenband grouper fry had died but 42% of Japanese flounder and 32% of tiger puffer fish fry had died. Rock fish, kelp grouper, kelp cross sevenband grouper hybrid and red seabream fry were not affected by the sevenband grouper nodavirus.

Strain differences in nodavirus isolates have been defined by variations in the nucleotide sequence of the coat protein gene (Nishizawa *et al* 1997). More recently, Mori et al (2003) have identified 3 nodavirus serotypes (A, B and C) which are mostly consistent with the four genotypes. It does also appear from cross-infection studies there are strain differences in host specificity. At this time no information is available on the presence of nodavirus in different marine finfish around Australia, nor on whether these nodaviruses are the same or different genotypes. This may be significant when management of disease risk on translocation is considered. A nodavirus strain may have a limited effect on one particular fish species but can be highly pathogenic to another species. This was shown in this project, although a direct comparison was not possible due to our inability to titrate the virus dose. Intraperitoneal injection of ITCS caused a cumulative mortality of approximately 23% at day 16 PI in 6 week old barramundi and 25.5% in 12 week old barramundi. This contrasts with the 98.7% and 92.3% mortalities recorded in sleepy cod fry. It is interesting to note the only time a natural outbreak of viral nervous necrosis in freshwater fish in Queensland was diagnosed was in fingerling sleepy cod (unpublished data).

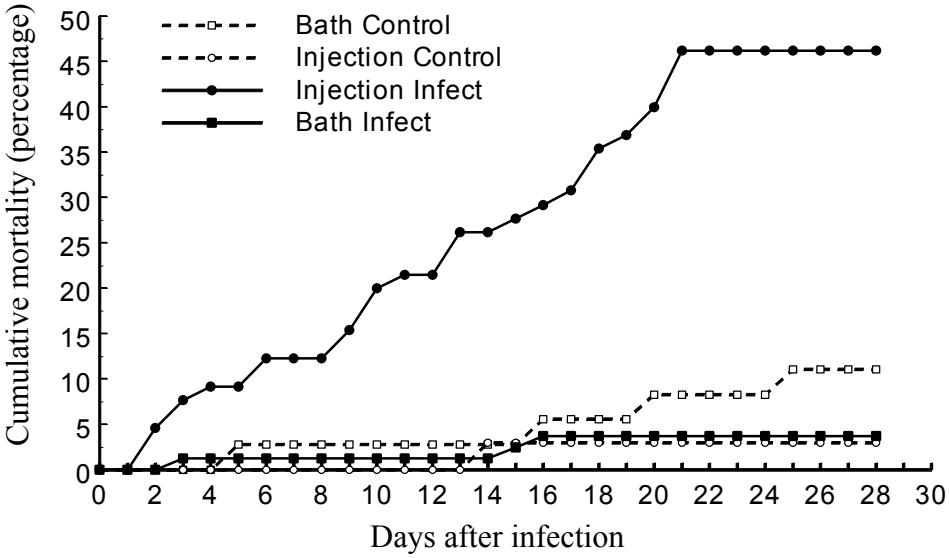

Figure 8: The mortality of 6 week old silver perch after injection and bath exposure to nodavirus infected tissue culture supernatant.

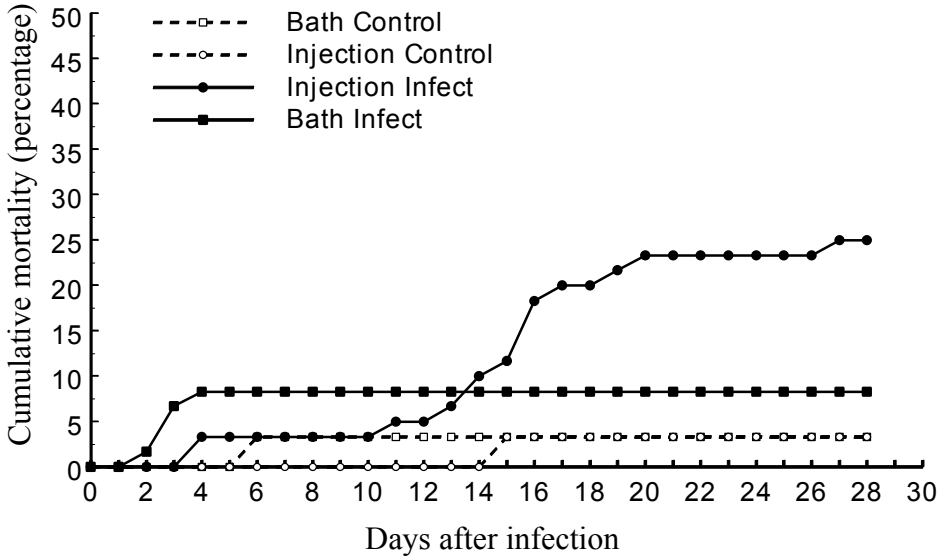

Figure 9: The mortality of 12 week old silver perch after injection and bath exposure to nodavirus infected tissue culture supernatant.

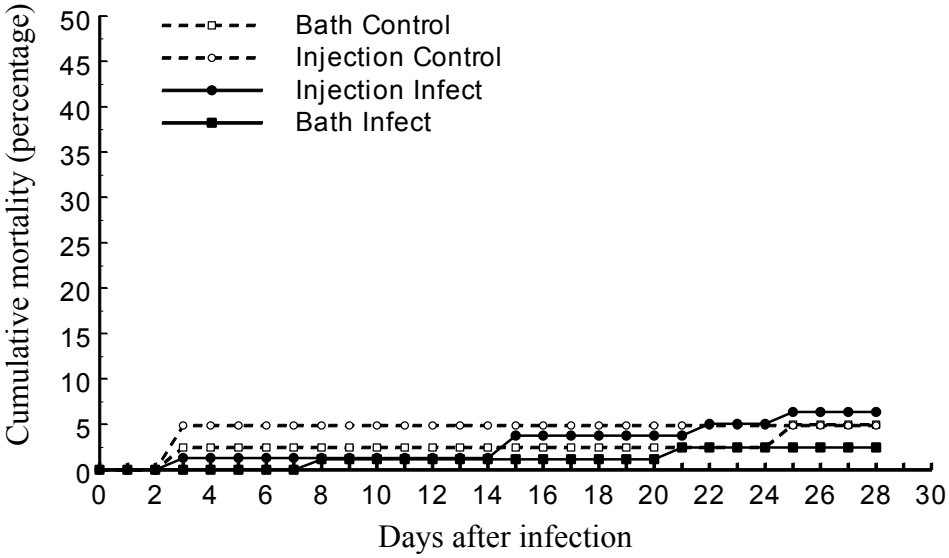

Figure 10: The mortality of 6 week old golden perch after injection and bath exposure to nodavirus infected tissue culture supernatant.

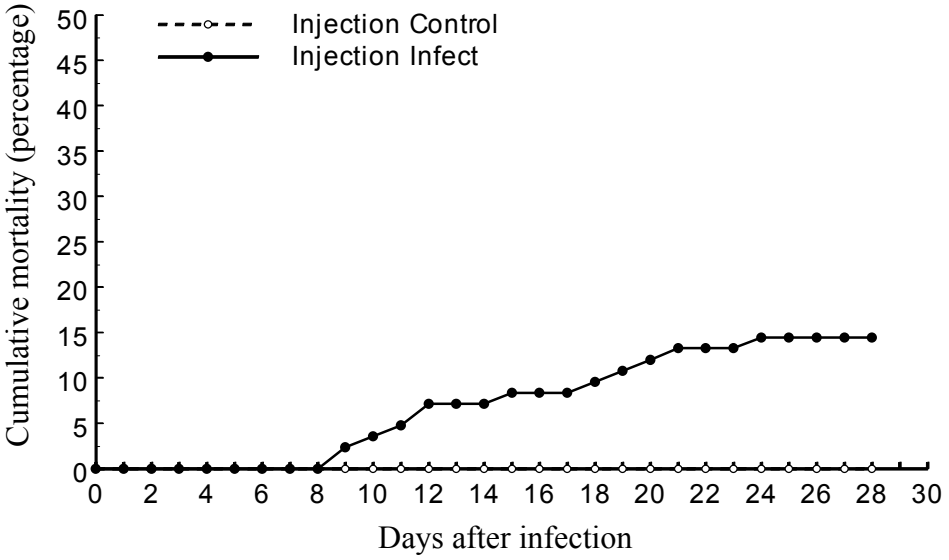

Figure 11: The mortality of 12 week old golden perch after injection of nodavirus infected tissue culture supernatant.

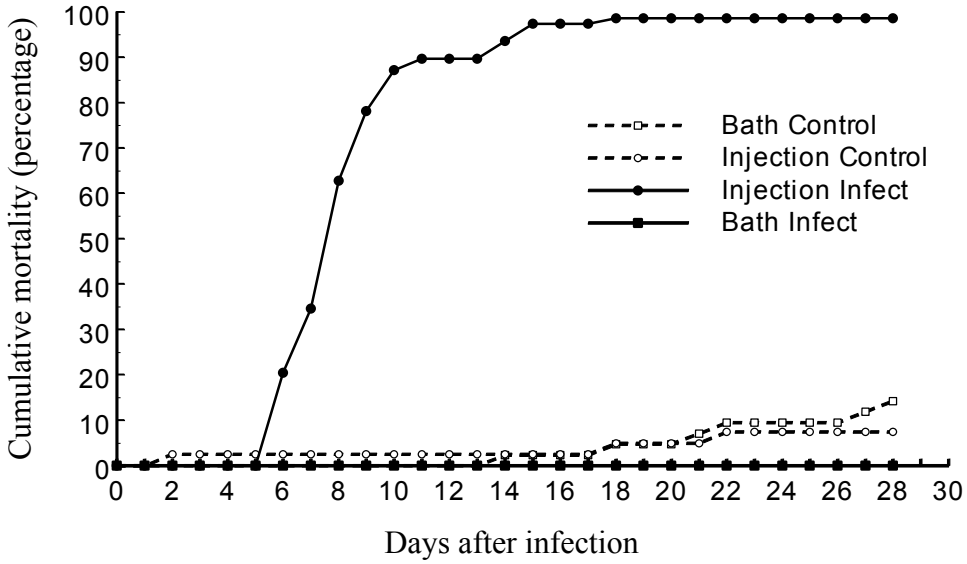

Figure 12: The mortality of 6 week old sleepy cod after injection and bath exposure to nodavirus infected cell culture supernatant.

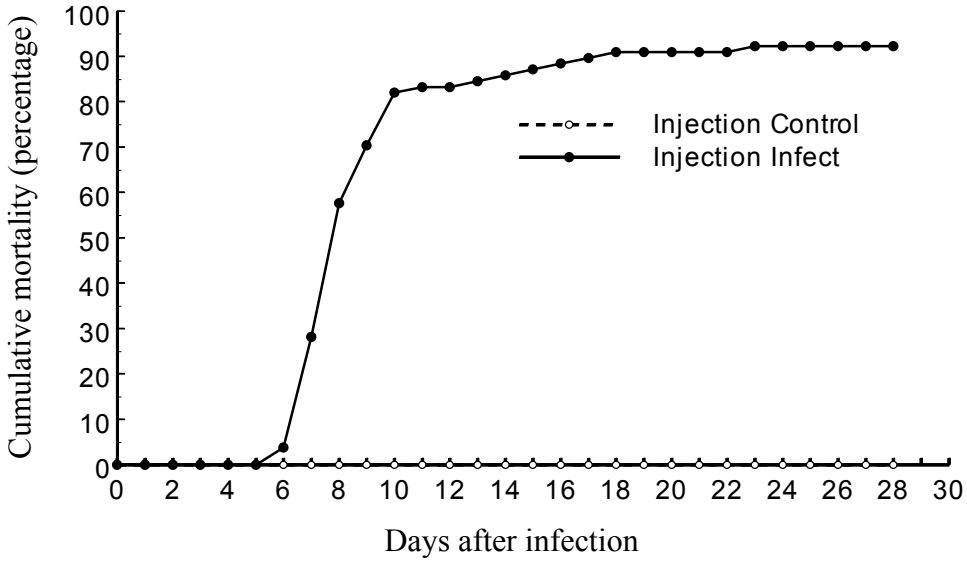

Figure 13: The mortality of 12 week old sleepy cod after injection of nodavirus infected cell culture supernatant.

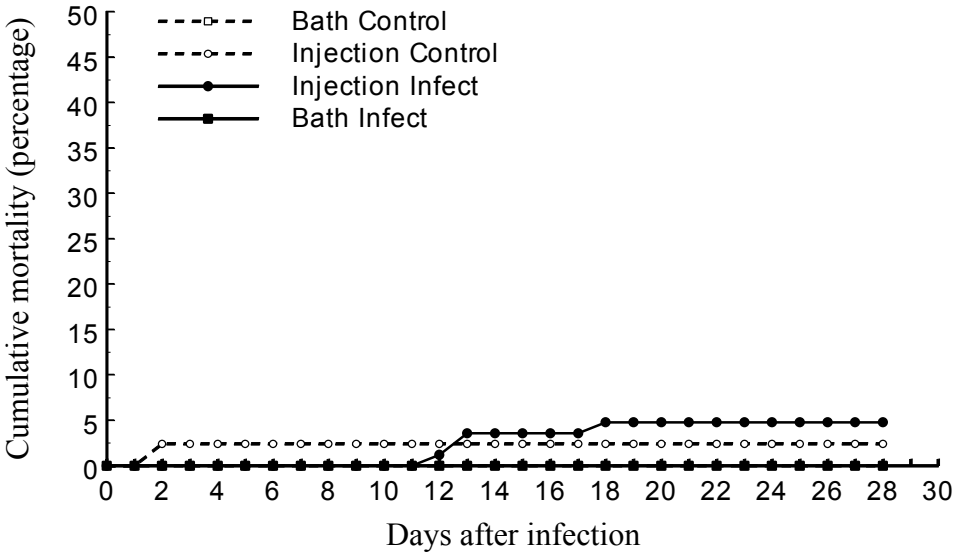

Figure 14: The mortality of 6 week old barcoo grunter after injection and bath exposure to nodavirus infected tissue culture supernatent.

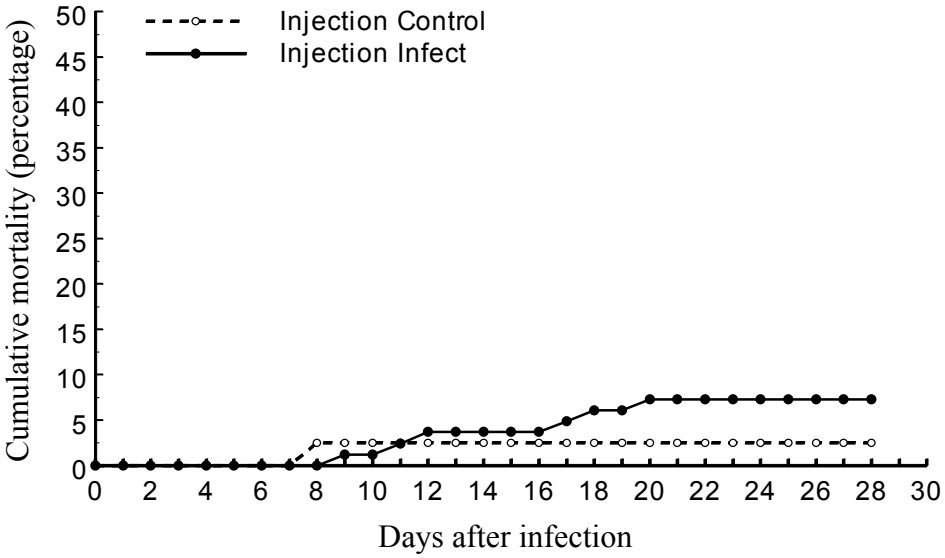

Figure 15: The mortality of 12 week old barcoo grunter after injection of nodavirus infected tissue culture supernatent.

6.2. Barramundi nodavirus histopathology in freshwater fishes and virus persistence

6.2.1. Methods

Histology (and photomicroscopy)

Selected fish samples for histology were preserved as whole fish or were divided into just head, half a fish (mid-line longitudinal cut) or half a head (mid-line longitudinal cut). The tissues were fixed in Bouin's solution (750 ml saturated picric acid (1-2%); 250 ml formaldehyde solution; 50 ml glacial acetic acid) for 24-48 hours, then transferred to 70% ethanol and held until processing. Standard processing procedures for histology were followed. After embedding in Paraplast©, 4µm sections were cut and stained with haematoxylin and eosin (H&E). Photomicroscopy was on an Olympus BX51 microscope with a DP12 digital camera system. The terminology used to describe the micro-anatomy of the fish followed that of Groman (1982).

Electron microscopy

Selected fish were euthanased by cervical dislocation, posterior to the cranium, and the brain removed into a petri dish. The brain and eye were removed, chopped into 1mm^3 pieces, with continual flooding with 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), and then the pieces were transferred to a 1.5ml microcentrifuge tube. The tissue was fixed in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 60 minutes at room temperature, washed twice in 0.1 M cacodylate buffer (pH 7.2), post-fixed in 0.1% (w/v) osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 60 minutes, washed as above, stained *en bloc* with saturated uranyl acetate for 30 minutes, washed twice with deionised water and dehydrated for through 50%, 70%, 80%, 85%, 90%, 95% and 100% (v/v) ethanol in deionised water for 5 minutes in each solution. The pellet was then incubated in two changes of absolute ethanol for 10 minutes before being infiltrated for 3 hours with 50% and 75% (v/v) Spurrs' resin in absolute ethanol at room temperature. The pellet was transferred to a mould, covered with 100% Spurrs' resin for 3 hours at room temperature and the resin was then cured by incubation at 60ºC for 16 hours. Semithin (1µm) sections were cut with a LKB ultratome and stained with toluidine blue for light microscopy. The principal lesions were located and the block trimed for ultrathin sections. Ultrathin (70nm) sections were cut and collected on naked gold grids, stained with lead citrate and examined in a Philips CM10 transmission electronmicroscope.

PCR testing preparation

Selected fish samples for PCR testing were collected as whole fish or were divided into just head, half a fish (mid-line longitudinal cut) or half a head (mid-line longitudinal cut), with the other half used for histology. The fish or part fish were frozen at -80°C until they were homogenized and stored as described in section 5.1. To reduce costs, some of the PCR testing of the larger groups of experimental fish was done in pools of 2 to 4 fish.

RT-PCR

RNA was extracted from 200µl aliquots of homogenised fishes using a commercial kit (High Pure Viral RNA Extraction Kit, Roche), according to the manufacturer's instructions. Extracted RNA was either used immediately or stored at -80°C until required.

RT-PCR was performed using the R3-F2 primer set and method described by Nishizawa *et al* (1994), with some modifications. RNA was incubated at 90°C for 5 minutes then reverse transcription was conducted at 37°C for 60 minutes in 20µl Omniscript RT buffer (Qiagen) containing 9.5µl template, 4U Omniscript RT (Qiagen), 0.5mM each dNTP, 1U ribonuclease inhibitor (Promega) and 1.0µM reverse primer. PCR amplification was carried out in a 50µl reaction mix containing 5µl of the reverse transcription reaction, 0.2µM of each primer, 2.5mM $MgCl_2$ and 25µl HotStarTaq Master Mix (Qiagen). Thermal cycling was conducted in an Eppendorf Mastercycler programmed as follows; 1 cycle at 95°C for 15 minutes, 25 cycles at 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 40 seconds and 1 cycle at 72°C for 10 minutes.

Nested RT-PCR

The nested RT-PCR was performed using the R'3-F'2 primer set and method described by Thiery *et al* (1999), with some modifications. Amplification was carried out in a 50µl reaction mix containing 1µl of the RT-PCR reaction, 4.0µM of each nested primer, 2.0mM $MgCl_2$ and 25µl HotStarTaq Master Mix (Qiagen). Thermal cycling was conducted in an Eppendorf Mastercycler programmed as follows; 1 cycle at 95°C for 15 minutes, 25 cycles at 94°C for 40 seconds, 50°C for 40 seconds and 72°C for 40 seconds and 1 cycle at 72°C for 10 minutes.

RT-PCR products were separated by electrophoresis through 2% Agarose MS (Roche) using TAE buffer (40mM Tris-acetate, 1mM EDTA) and DNA was visualised by UV illumination after staining with ethidium bromide.

6.2.2. Histopathology in diseased fish and survivors.

There were a range of lesions seen in the fish sampled through the trials but some trends were apparent that seemed to relate to the time since infection. All the tissue changes described here were in fish that had been injected with ITCS. No lesions were seen in any other tissue or organ other than those described here.

6.2.2.1. Silver perch

Silver perch 6 weeks

The initial moribund fish removed during the trial had scattered, single vacuoles in the internal and external nuclear cell layers of the retina, in the granular cell layers of the optic tectum, valvula cerebelli and cerebellum (corpus cerebelli). The vacuoles had no clear cellular structure but occasionally a pyknotic nucleus would be apparent at the vacuole edge, and there would be pale, eosinophilic, stringy cellular debris at the edge and extending across the vacuole. Some of the cells in the retinal layers affected by vacuolation would have a basophilic inclusion in the cytoplasm in these early sampled fish (Fig. 16). Latter sampled fish (>15 days after infection) would have numerous vacuoles in the retina and brain. The vacuolation coalescing into extensive areas of degeneration of the retinal nuclear layers to an extent where there was disruption of the external plexiform layer (Fig. 17). Odd single vacuoles could also be seen in the ganglion cell layer and the visual cell layer of the retina. In these latter diseased fish there were always degenerating mononuclear cells in the posterior compartment (vitreous humor) of the eye (Fig. 18). There was extensive vacuolation in the brain involving the granular cell layers of the optic tectum and valva cerebelli, and the neurones at the outer edge of the olfactory lobe (Fig. 19) and torus longitudinalis of the

tegmentum. Single cells with basophilic cytoplasm were present in most areas where vacuolation was present (Fig. 20). Again degenerate cells were present in the vitreous humor. One fish had melanin-like deposition in areas of degeneration of the retina. Examination of surviving fish revealed nervous lesions in some of the silver perch that had been injected with the ITCS. In these fish the changes were restricted to the retina with lesions ranging from single scattered vacuoles in the inner and outer nuclear cell layers through to extensive vacuolation and degeneration with cells with basophilic intracytoplasmic inclusions and degenerate cells in the vitreous humor. In one fish there were aggregations of degenerating cells in the retina (Fig. 21). Typically there was some melanin-like deposition in the retinal lesions with extensive degeneration.

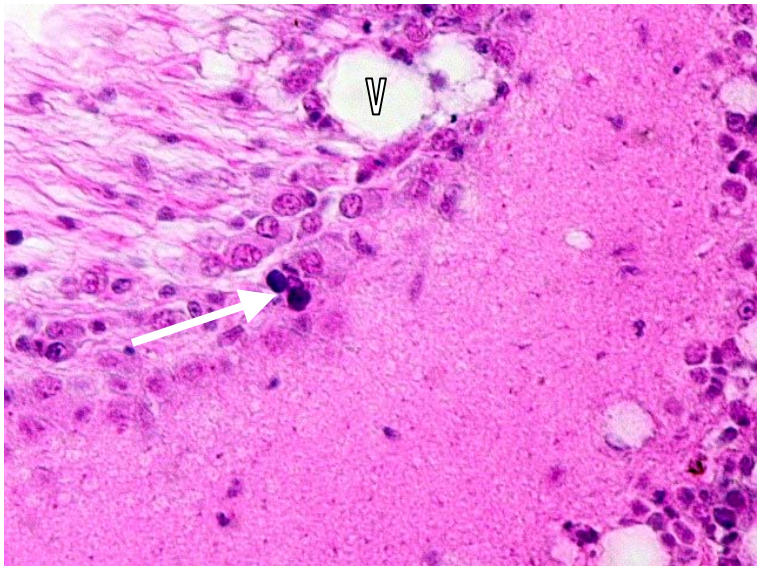

Figure 16: Silver perch 6 weeks. Vacuolation (**V**) in the ganglion cell layer with two cells with deeply basophilic cytoplasm (arrow) in the retina. x400, H&E.

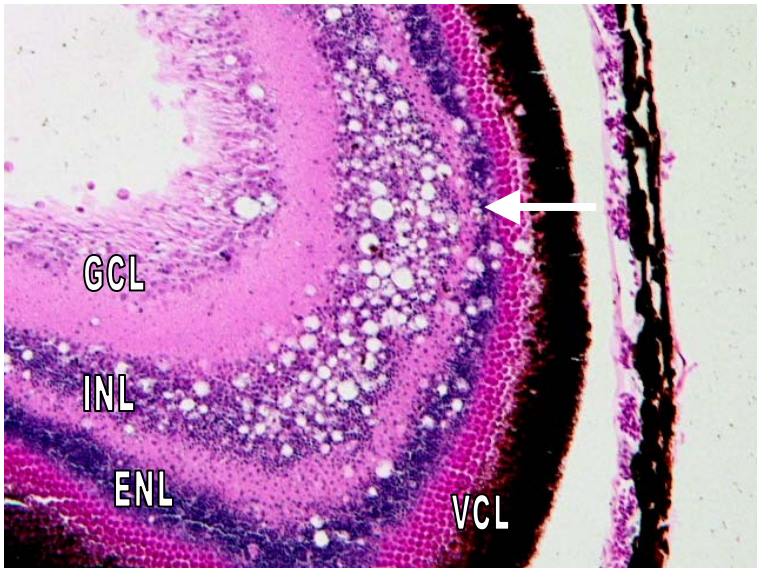

Figure 17: Silver perch 6 weeks. Retina with extensive vacuolation involving the ganglion cell layer (GCL), internal nuclear layer (INL), external nuclear layer (ENL) and the visual cell layer (VCL) with disruption of the external plexiform layer (arrow). x40, H&E.

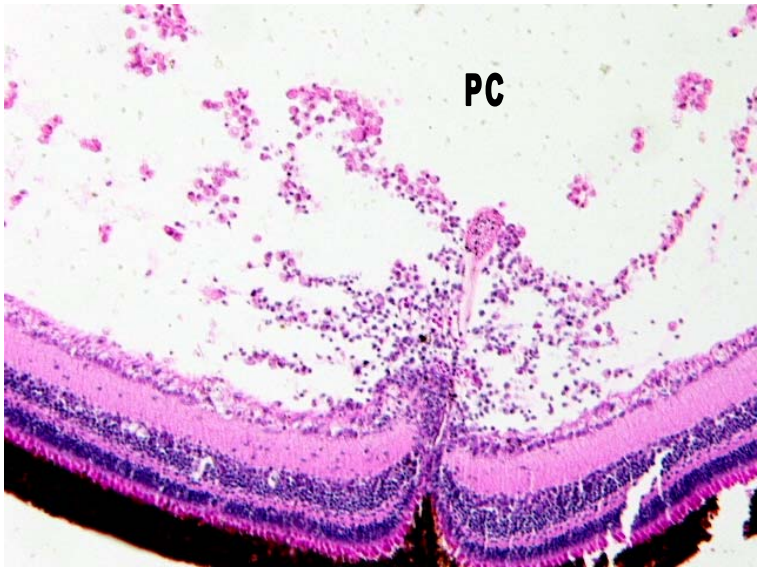

Figure 18: Silver perch 6 weeks. Degenerate cells in the posterior compartment (PC) of the eye. x40, H&E.

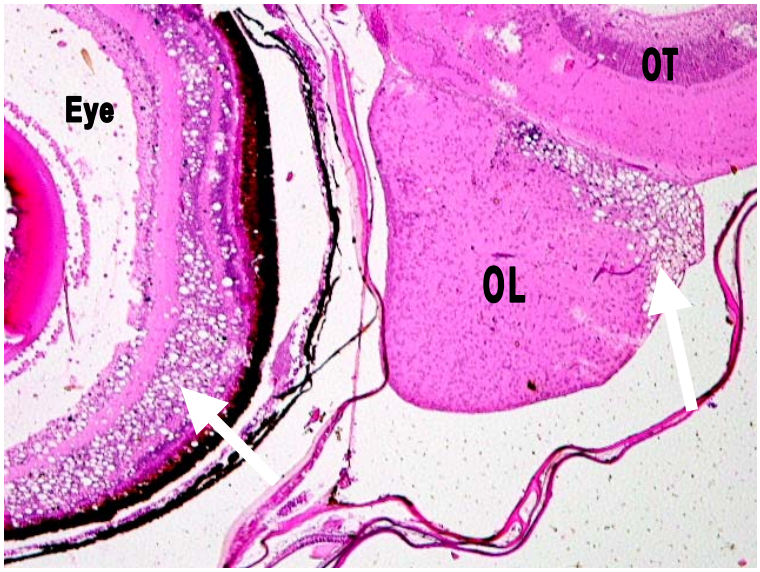

Figure 19: Silver perch 6 weeks. Extensive vacuolation (arrows) of the retina and the caudal edge of the olfactory lobe (OL). Optic tectum (OT) x40, H&E.

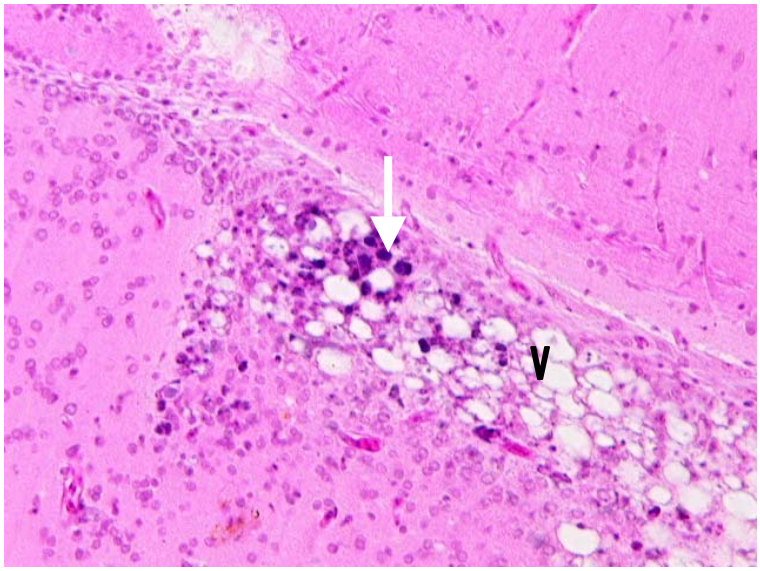

Figure 20: Silver perch 6 weeks. Caudal edge of olfactory lobe (enlargement of Figure 19) with vacuolation (V) and cells with basophilic cytoplasm (arrow). x200, H&E.

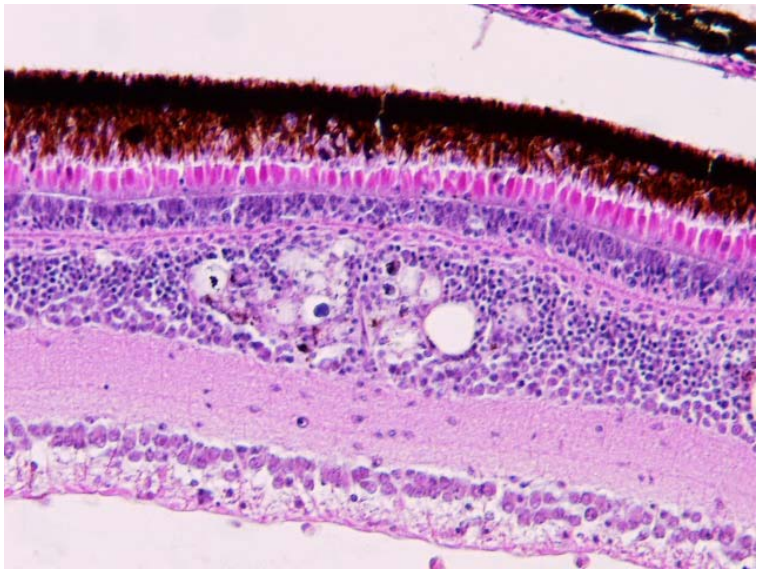

Figure 21: Silver perch 6 weeks. An aggregation of degenerate cells in the internal nuclear layer of the retina of a surviving fish. x200, H&E.

Silver perch 12 weeks

The initial moribund fish removed (>15 days after infection) had vacuolation of neuronal cells throughout the retina and brain. Multiple vacuoles and cellular degeneration were common in the internal nuclear layer of the retina, often forming aggregations of vacuolated necrotic cells. These aggregations were typically found in the ora serrata (the circumferential germinal zone at the ciliary margin of the retina) part of the retina (Fig. 22). Lower numbers of single vacuoles were present in the ganglion cell and external nuclear layers. Degenerate cells were present in the vitreous humor. Extensive vacuolation, aggregations of degenerate cells and cells with basophilic cytoplasms were common in the medulla oblongata (of the myeloncephalon) and tegmentum, and the granular cells of the periventricular area of the hypothalamic region (Fig. 23). Scattered single vacuoles were apparent in some of the fishes cerebellum and optic tectum (granular cell layers). Different from the younger silver perch where neuronal degeneration was seen in the olfactory lobe, optic tectum, valvula cerebelli and cerebellum, the older silver perch had the most significant lesions in the medulla oblongata, tegmentum and periventricular granular cells.

In the surviving fish there were few, if any, lesions in the brain, only the occasional single vacuoles in the granular cells of the cerebellum or optic tectum. There were a few fish with small cell aggregations in the medulla oblongata or upper spinal cord (Fig. 24). It is not clear if these aggregations are directly the result of the nodavirus infection, but they were not present in control fish. In those fish with lesions it was vacuolation and degenerate cell aggregations in the internal nuclear layer of the retina that were the most common lesion present. There were also scattered single vacuoles in the external nuclear and the visual cell layers. Melanin-like pigment could be present in both, single vacuoles and aggregated vacuoles (Fig. 25). A few degenerate cells were present in the vitreous humor. In two of the fish there was a pale cellular aggregation in the visual cell or epithelial layers of the retina (Fig 25).

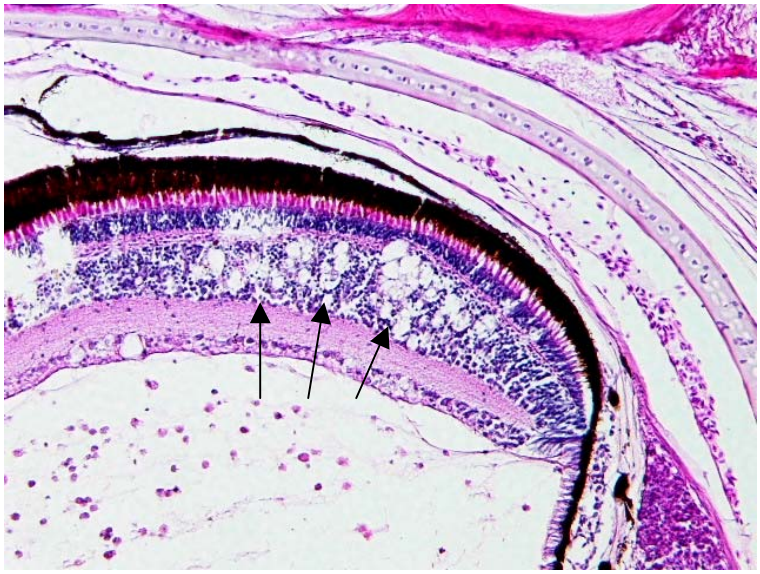

Figure 22: Silver perch 12 weeks. Aggregations of vacuolated, necrotic cells (arrows) in the internal nuclear layer of the in ora serrata part of the retina. x100, H&E.

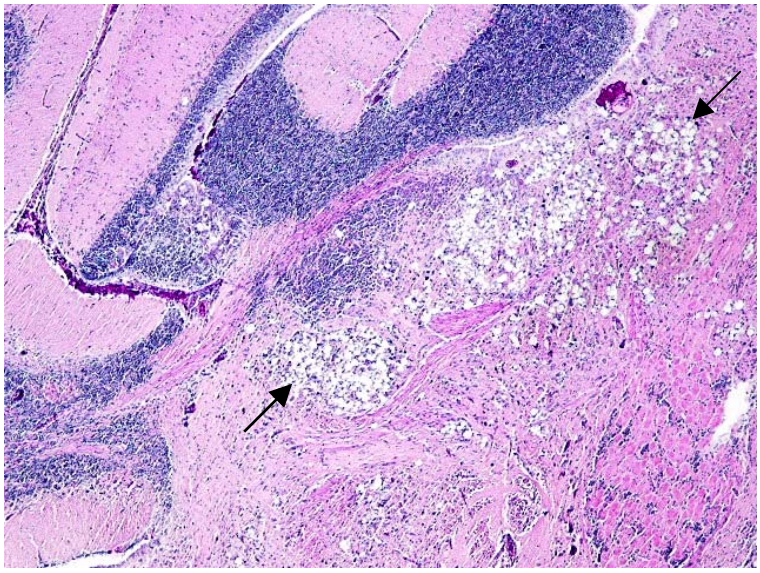

Figure 23: Silver perch 12 weeks. Extensive vacuolation through the tegmentum (extent indicated by arrows). x40, H&E.

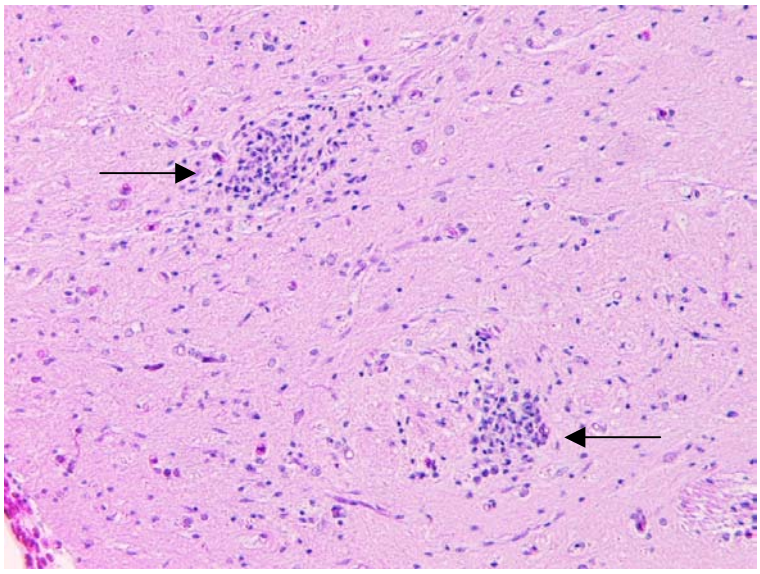

Figure 24: Silver perch 12 weeks. Small mononuclear cell aggregations (arrows) in the medulla oblongata region of the brain. x200, H&E.

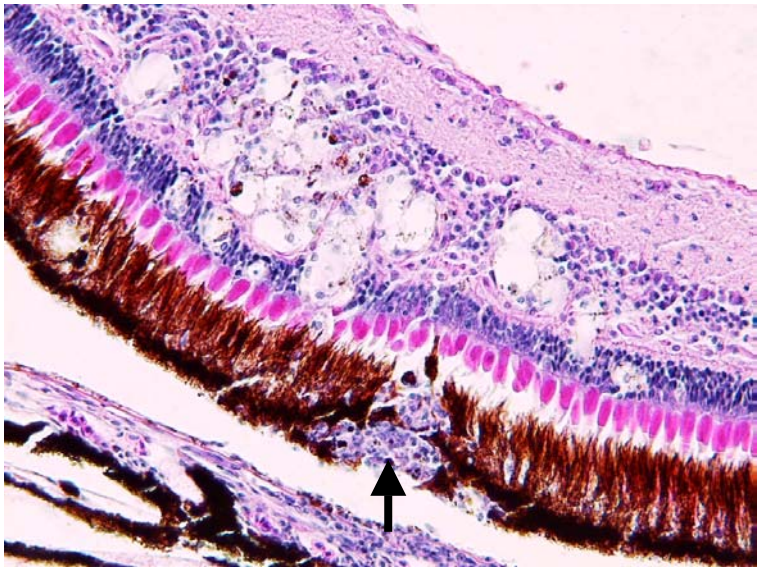

Figure 25: Silver perch 12 weeks. Aggregation of degenerated, vacuolated cells in the internal and external nuclear layers of the retina, with some melanin-like pigment deposits, and a cell aggregation (arrow) in the visual cell layer of a surviving fish. x200, H&E.

6.2.2.2. Golden perch

Golden perch 6 weeks

Only four fish were removed during the trial for histological examination. Changes in the retinas ranged from a few single vacuoles in the internal nuclear layer (Fig. 26) to extensive vacuolation and degeneration of the internal nuclear layer with scattered vacuoles in the external nuclear, ganglion cell and visual cell layers. In the severely affected retina the degeneration involved disruption of the external plexiform layer (Fig. 27). When ever retinal lesions were present there would also be some degenerate cells in the vitreous humor. Vacuolation in the brain involved the olfactory lobe and the granular cell layers of the optic tectum, cerebellum and the periventricular area of the hypothalamic region. In one fish scattered single vacuoles were also present in the molecular layer of the optic tectum and the tegmentum (Fig. 28). The vacuolar degeneration of the olfactory lobe affected many neuronal cells at the caudal edge of the lobe and some internally (Fig. 29). Occasional cells with basophilic cytoplasm were present in these areas of brain degeneration. One fish also had vacuolation in the spinal cord (Fig. 30). Two of the surviving fish had lesions in the retina (none in the brain). These involved single vacuoles and areas of continuous degeneration, with vacuoles, in the internal nuclear layer. Very occasional melanin-like pigment granules were present in these continuous areas of degeneration.

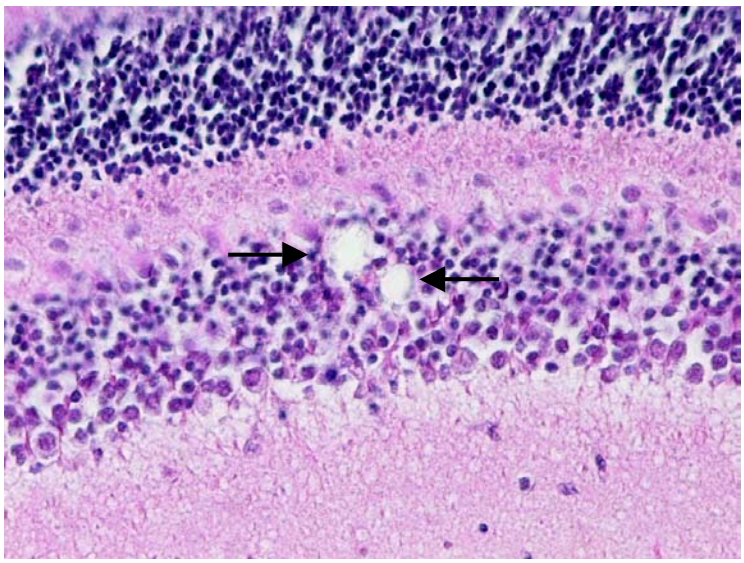

Figure 26: Golden perch 6 weeks. Two vacuoles (arrows) in the internal nuclear layer of the retina. x400, H&E.

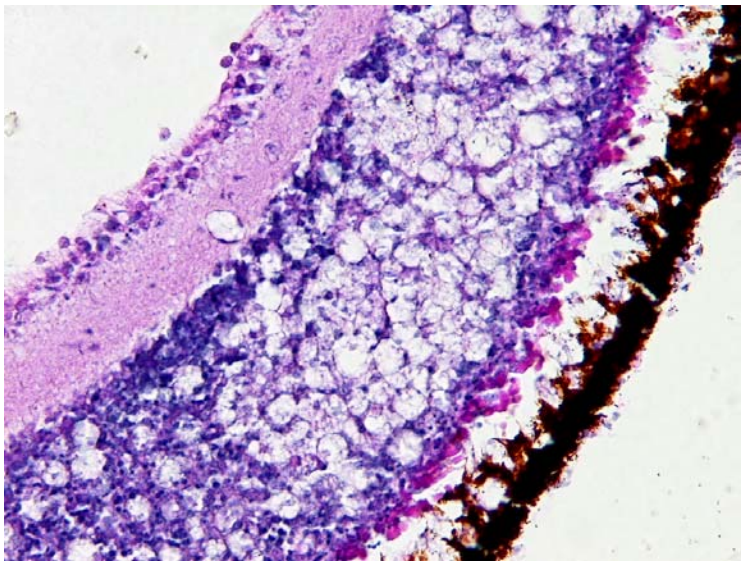

Figure 27: Golden perch 6 weeks. Extensive vacuolation in the internal nuclear layer, extending to involve the external nuclear layer and disrupting the external plexiform layer. x200, H&E.

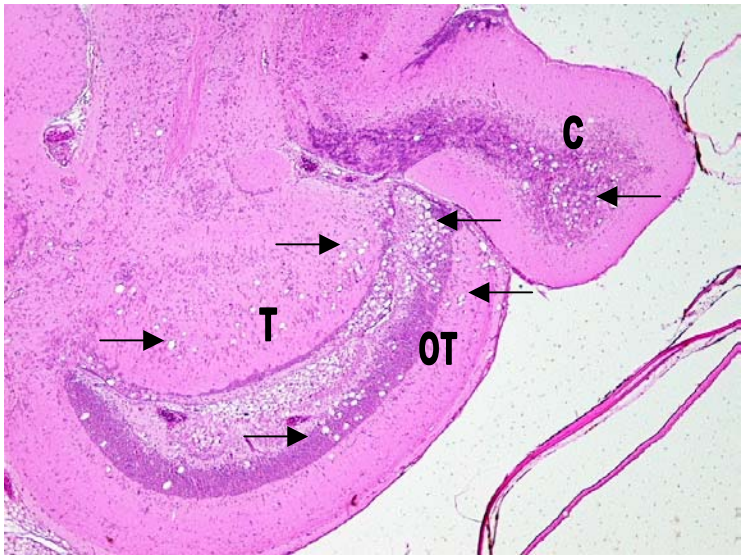

Figure 28: Golden perch 6 weeks. Note the distrubution of brain vacuolation with lesions in granular cell layers of the optic tectum (OT) and cerebellum (C), in the molecular layer of the optic tectum and the tegmentum (T) (arrows). x40, H&E.

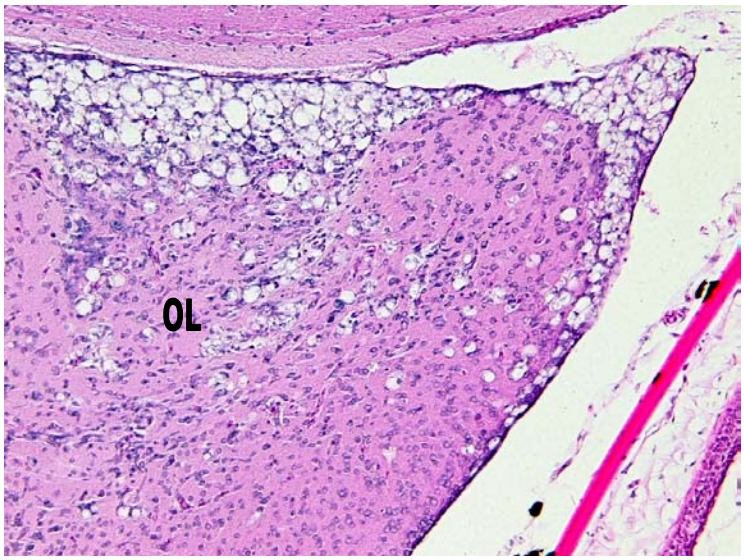

Figure 29: Golden perch 6 weeks. Extensive vacuolation at the edge of the olfactory lobe (OL) and single vacuoles internally. x100, H&E.

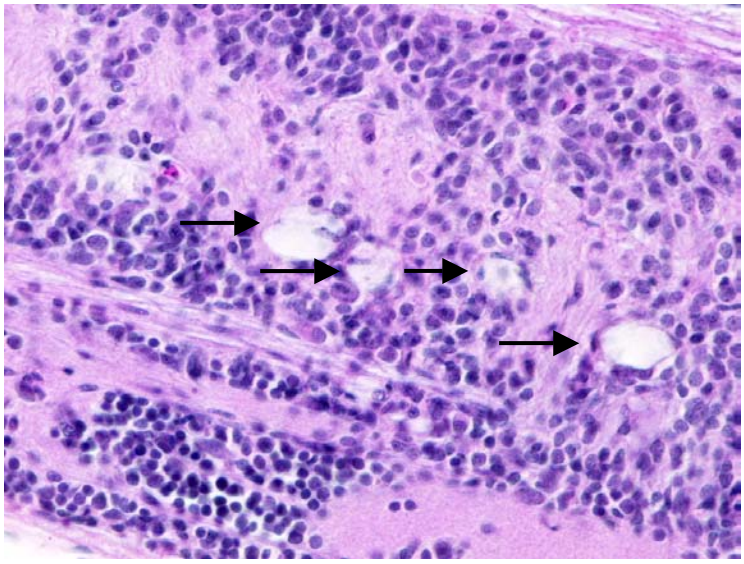

Figure 30: Golden perch 6 weeks. Single vacuoles (arrow) in the spinal cord. x400, H&E.

Golden perch 12 weeks

Four of the fish removed during the trial had severe brain lesions (>12 day after infection). The two fish removed at day 9 had ichthyobodosis. The changes in the retina involved multiple vacuolation of cells in the internal nuclear layer, often with 4-6 degenerated cells aggregated together (Fig. 31). Some melanin-like pigment was present in the degenerate cell aggregations and occasional cells with basophilic cytoplasm were present in the retina. In some fish the changes were present throughout the length of the retina, but in some the vacuolated, degenerated internal nuclear layer cells were only present towards the ora serrata. Occasional degenerate cells were in the vitreous humor. The brain vacuolation was extensive, affecting the olfactory lobe, optic tectum, tegmentum, hypothalamus and periventricular hypothalamic region (actually most of the diencephalon and mesencephalon was affected), and the cerebellum (Fig. 32). Large parts of these areas were comprised solely of vacuolated, degenerate cells (Fig 33). Cells with basophilic cytoplasm were scattered through these areas of vacuolation. The changes in the surviving fish were usually mild. In the retina all that was present could be a few vacuoles in the internal nuclear layer (Fig. 34) ranging up to quite extensive vacuolation involving the internal nuclear, ganglion cell and external nuclear layers. Again some scattered melanin-like pigment could be present in degenerate cells in the retina. Occasional cells with basophilic cytoplasm were present in the retina and in the areas of vacuolar degeneration in the brain. Often the only change in the brain were some vacuoles in the granular cell layer of the cerebellum (Fig. 35). In other fish, in addition to cerebellum lesions, there could be vacuoles at the edge and internally and/or areas of vacuolation at the outer edge of the olfactory lobe (Fig. 36). In three of the fish some vacuoles were also present in the hypothalamus and tegmentum.

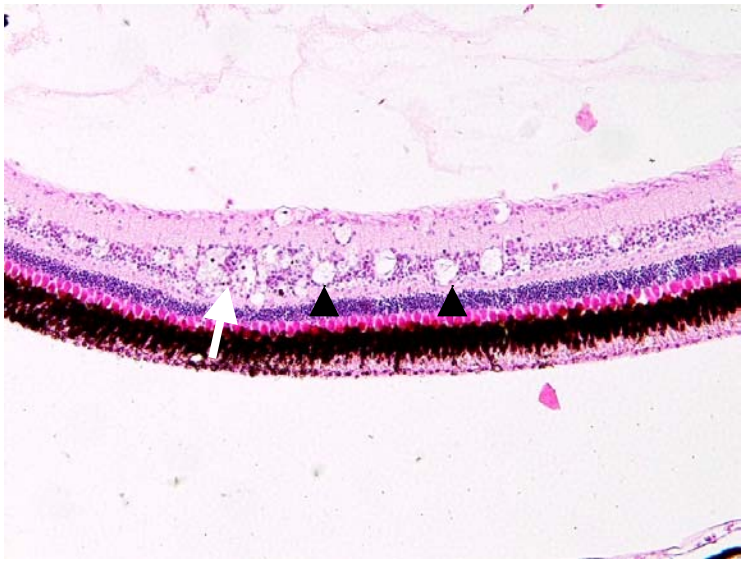

Figure 31: Golden perch 12 weeks. Single (arrow heads) and an aggregation of vacuolated cells (arrow) in the internal nuclear layer of the retina. Some vacuoles are also present in the ganglion cell layer. x100, H&E.

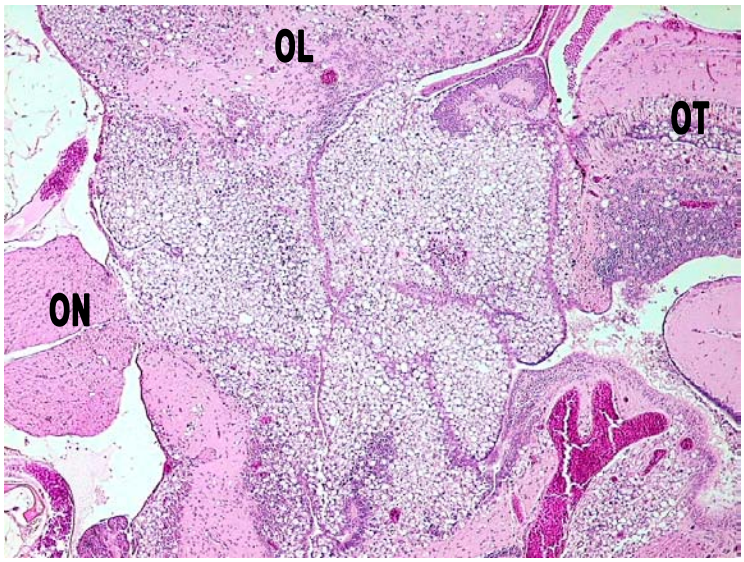

Figure 32: Golden perch 12 weeks. Extensive vacuolation of the brain involving most of the diencephalon and mesencephalon. Optic nerve (ON), olfactory lobe (OL), and optic tectum (OT). x40, H&E.

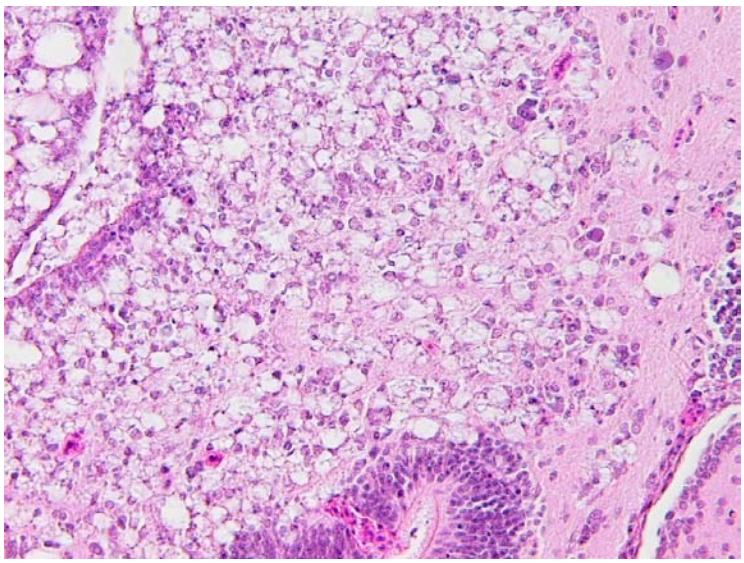

Figure 33: Golden perch 12 weeks. A higher magnification of Figure 32 in the periventricular area of the hypothalamic region showing vacuolation and pyknotic nuclei. x200, H&E.

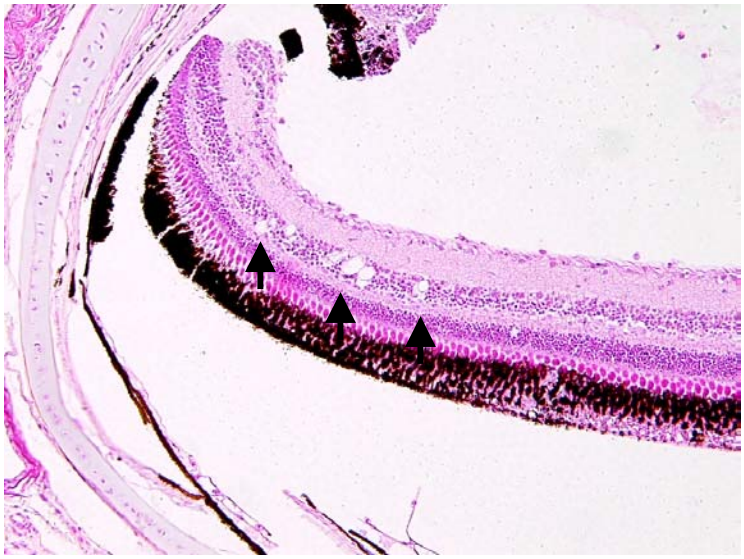

Figure 34: Golden perch 12 weeks. Some vacuoles (arrows) in the internal nuclear layer of the retina in a surviving fish. x100, H&E.

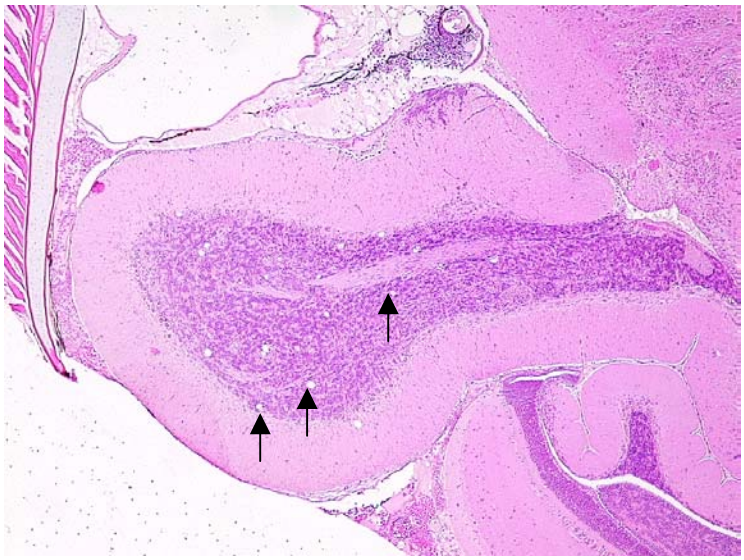

Figure 35: Golden perch 12 weeks. Some vacuoles (arrows) in the granular cell layer of the cerebellum of a surviving fish. x40, H&E.

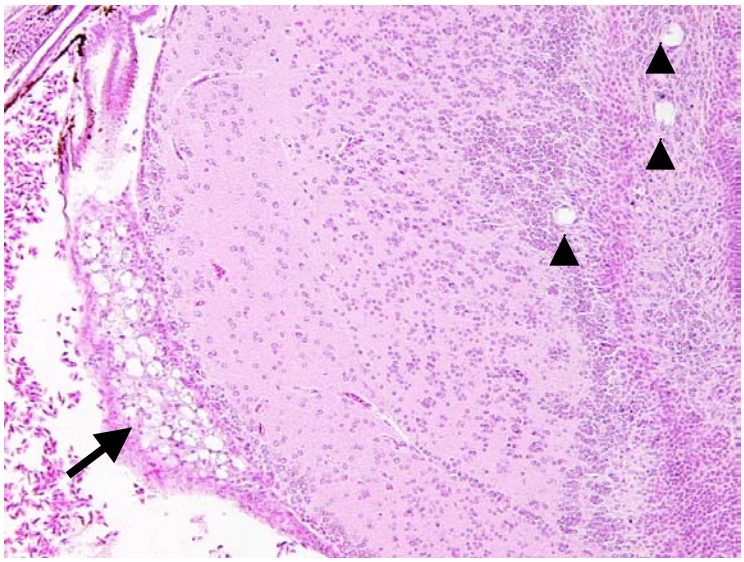

Figure 36: Golden perch 12 weeks. A focal aggregation of vacuolated cells at the edge of the olfactory lobe (arrow), and a few vacuoles internally (arrow heads), in a surviving fish x100, H&E.

6.2.2.3. Sleepy cod

Sleepy cod 6 weeks

The initial moribund fish removed during the trial (6 days after infection) had minor changes to the retina. Usually 1 to 4 vacuoles would be present in the internal nuclear layer, and occasionally the external nuclear layer (Fig. 37). A few degenerate cells would be present in the vitreous humor. More significant changes could be present in the brain, although some fish had only 5 or 6 vacuoles in the granular cell layers of the optic tectum, valva cerebelli, torus semicircularis and/or periventricular hypothalamic region, tegmentum and/or outer layer of the olfactory lobe (Fig. 38). The neuronal cell changes present in these fish were more degenerative and necrotic than simple vacuoles. Cell outlines with obvious fine debris in what was the cytoplasm, actual necrotic cells and pyknotic nuclei were present. Occasional cells with this vacuolar degeneration were present in the spinal cord. In those fish removed later in the trial (7 to 15 days after infection), the neuronal lesions included cells with basophilic cytoplasms and small inclusion bodies (Fig. 39). Again only a few single vacuoles could be present in the internal nuclear layer of the retina, although several fish had more extensive vacuolation of the internal nuclear, external nuclear and ganglion cell layers, and degenerate cells in the vitreous humor. The brain changes were more extensive in these fish. Often all the granular cell layers throughout the brain, the outer layer of the olfactory lobe, tegmentum and hypothalamus areas contained degenerating and necrotic cells, and cells with basophilic cytoplasms or inclusions (Fig. 40). These neuronal cells contained obvious fine cytoplasmic debris and the vacuoles were not always clear (Fig. 41). The areas of cell change in the brain were more necrotic (Fig. 42) as compared to that present in the silver and golden perch. Vacuolated, degenerating cells were often present in the rostral spinal cord (Fig. 43). The one surviving fish that had been injected with ITCS had no central nervous system tissue lesions.

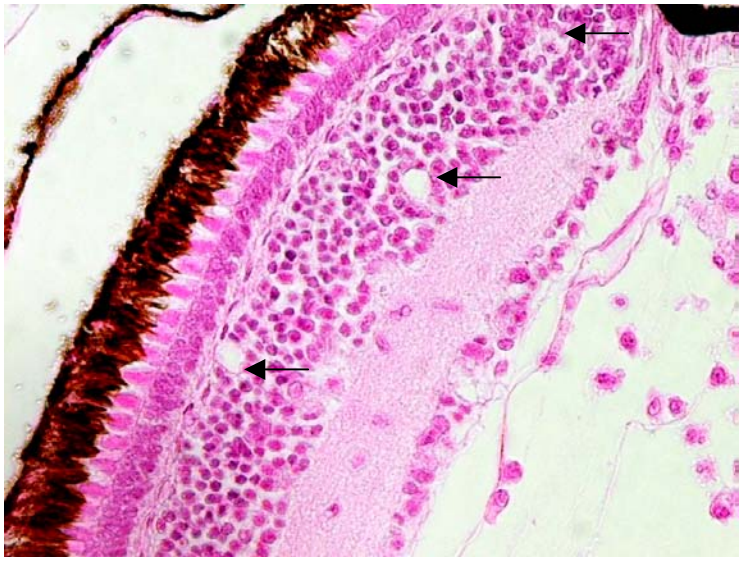

Figure 37: Sleepy cod 6 weeks. Occasional vacuoles (arrows) in the internal nuclear layer of the retina. x400, H&E.

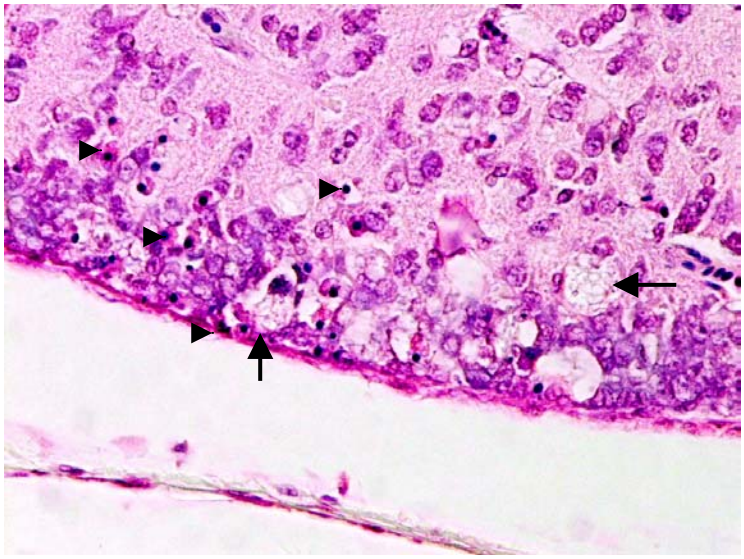

Figure 38: Sleepy cod 6 weeks. The edge of the olfactory lobe with vacuolar neuronal cell degeration (arrows) and pyknotic nuclei (arrow heads) in a fish removed 6 days after injection with infected tissue culture supernatent. x400, H&E.

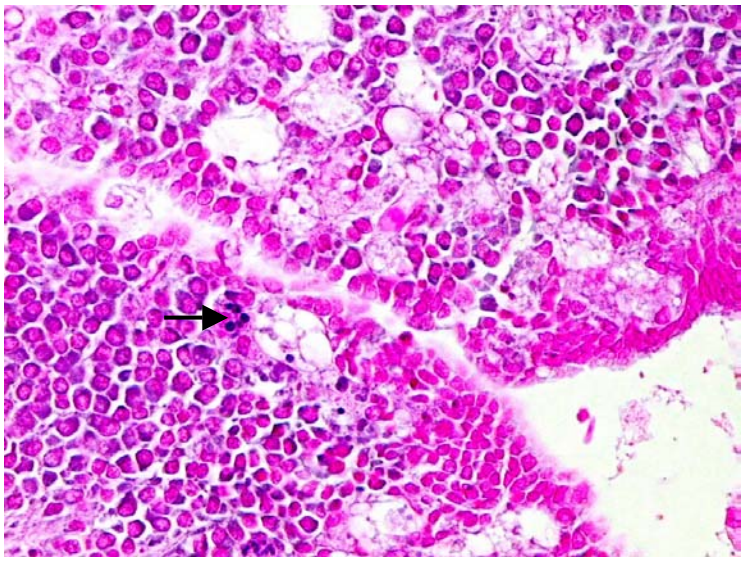

Figure 39: Sleepy cod 6 weeks. The periventricular hypothalamic region of the brain with vacuoles, vacuolar degeneration, cells with basophilic cytoplasm and basophilic inclusion bodies (arrow). X400, H&E.

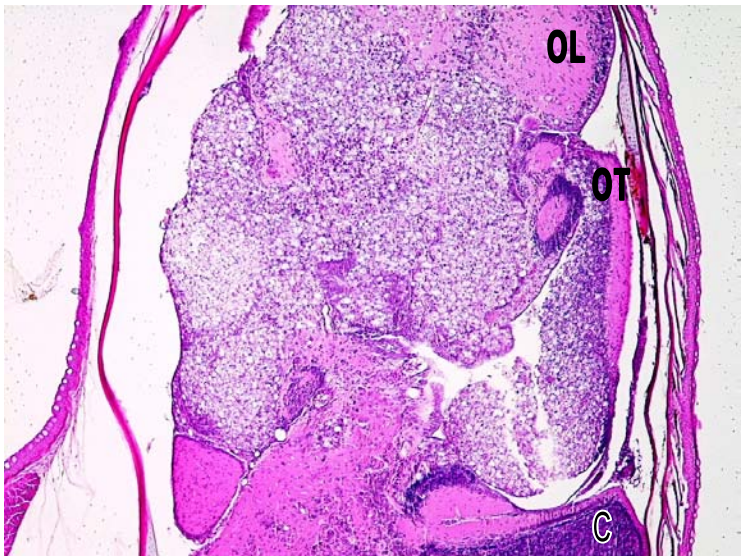

Figure 40: Sleepy cod 6 weeks. A brain with extensive vacuolation and cell necrosis. Optic tectum (OT), olfactory lobe (OL) and cerebellum (C). x40, H&E.

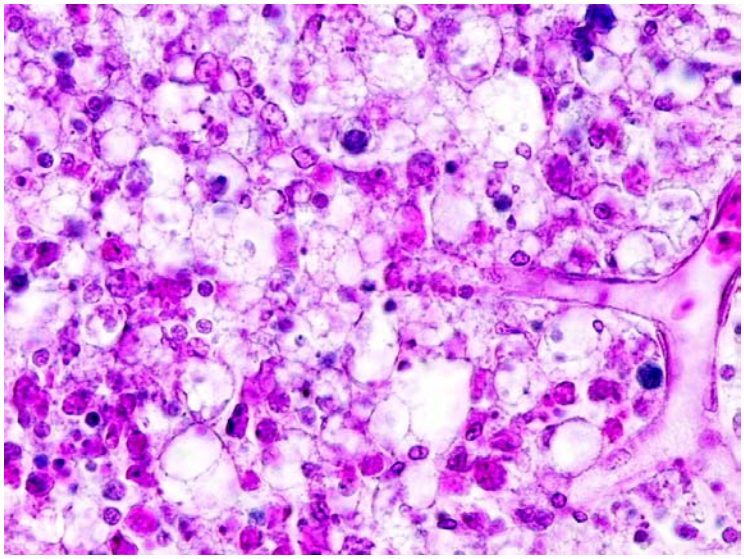

Figure 41: Sleepy cod 6 weeks. A higher magnification view from the middle of the necrotic brain area shown in the previous figure (Fig. 40). x400, H&E.

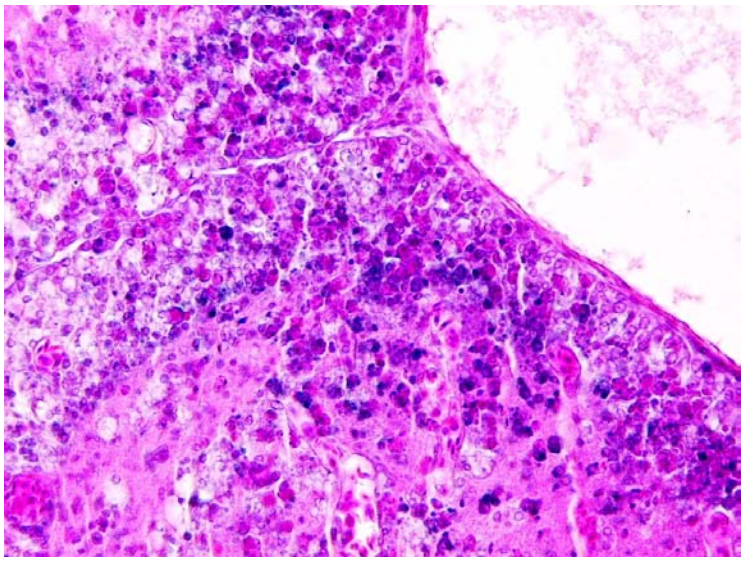

Figure 42: Sleepy cod 6 weeks. Extensive necrotic change at the edge of the olfactory lobe. x200, H&E.

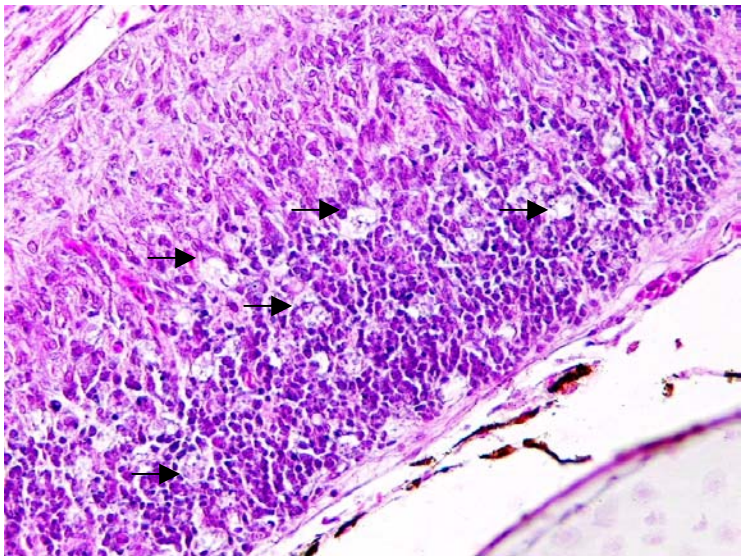

Figure 43: Sleepy cod 6 weeks. Multiple vacuolar degeneration (arrows) in the spinal cord. x200, H&E.

Sleepy cod 12 weeks.

The moribund fish removed before day 13 after infection had minor central nervous system tissue lesions. As with the 6 week sleepy cod group there were minimal changes to the retina. Usually 2 to 8 degenerating cells with vacuolar changes were present in the internal and external nuclear layers. A few degenerate cells would be present in the vitreous humor. Even in the fish removed after day 13 post infection there was never any more than moderate levels of vacuolated cells, often with obvious cytoplasmic debris remaining in the cells, in the internal and external nuclear layers of the retina. In the initial fish removed there would be cells with basophilic cytoplasm and degenerating neuronal cells in the brain (Fig. 44). These could be present in the granular cell layers of the cerebellum, periventricular hypothalamic region, valvula cerebelli and/or optic tectum, and the tegmentum, medulla oblongata and/or the outer layer of the olfactory lobe. There were never high numbers of affected cells, and the degenerating cells were hard to identify as vacuolated due to the fine cytoplasmic debris remaining within the cell outline (Fig. 45). In the spinal cord some scattered cells with basophilic cytoplasm and/or a vacuolar degeneration were apparent. As in the 6 week sleepy cod group, the neuronal cell changes were more degenerative and necrotic rather than an obvious vacuolation. More extensive areas of degeneration were present in those moribund fish removed after the day 13 post infection. The same areas of the brain were affected, but more severely (Fig. 46), with vacuoles most readily observed in the cerebellum (Fig. 47).

Five surviving fish from the injection infection group were examined. Three had extensive changes to the retina. In the internal nuclear layer there were several aggregations of pale cells (Fig. 48). These could contain a vacuole or cells with basophilic cytoplasms. Odd single vacuoles would be present in the retina. Large numbers of degenerating cells were present in the vitreous humor, some with basophilic cytoplasms. The brains of two fish had lesions consisting of scattered necrotic cells in the olfactory lobe (Fig. 49), periventricular hypothalamic region and granular cell layer of the cerebellum. In the cerebellum actual vacuoles would be present, but most of the degenerating cells had intracytoplasmic basophilic bodies and were probably necrotic (Fig. 50).

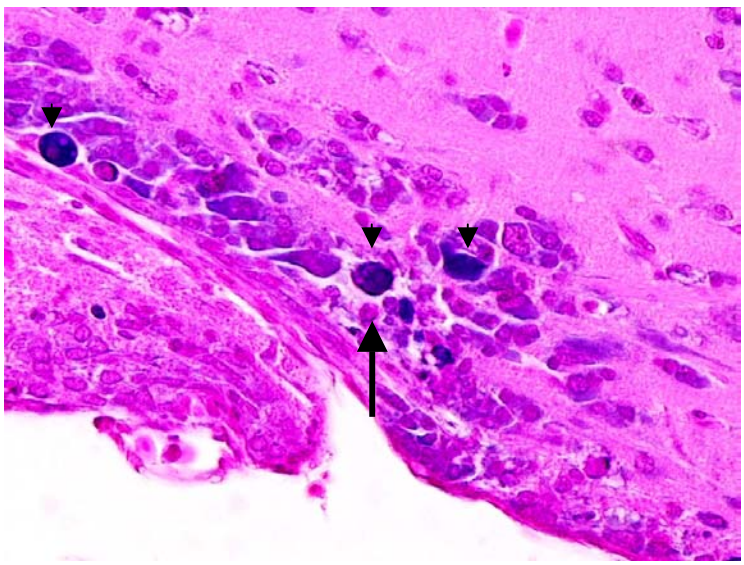

Figure 44: Sleepy cod 12 weeks. Cells with strongly basophilic cytoplasms (arrow heads) and neuronal cell degeneration (arrow) in the lower edge of the hypothalamus. x400, H&E.

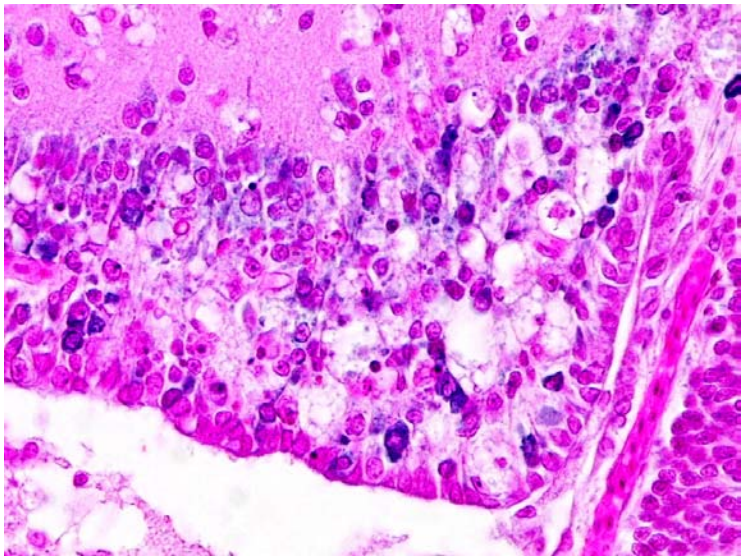

Figure 45: Sleepy cod 12 weeks. Cells with basophilic cytoplasms, neuronal cell degeneration and cell necrosis, with few obvious vacuoles, at the edge of the olfactory lobe. x400, H&E.

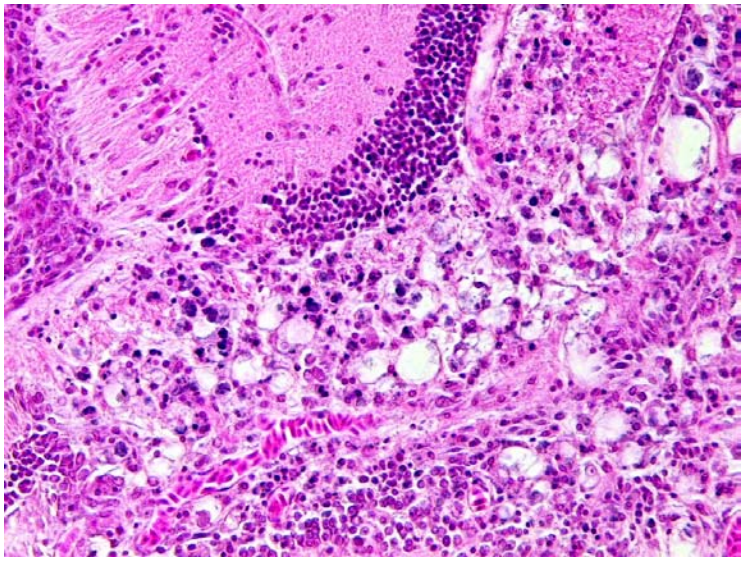

Figure 46: Sleepy cod 12 weeks. More extensive neuronal cell degeneration and necrosis with basophilic cells and vacuolation in the tegmentum. x200, H&E.

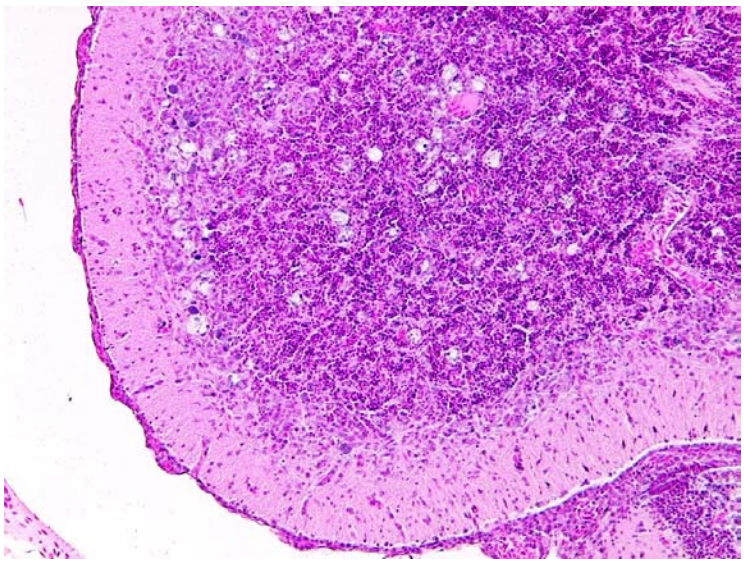

Figure 47: Sleepy cod 12 weeks. Vacuolation in the granular cell layer of the cerebellum. x100, H&E.

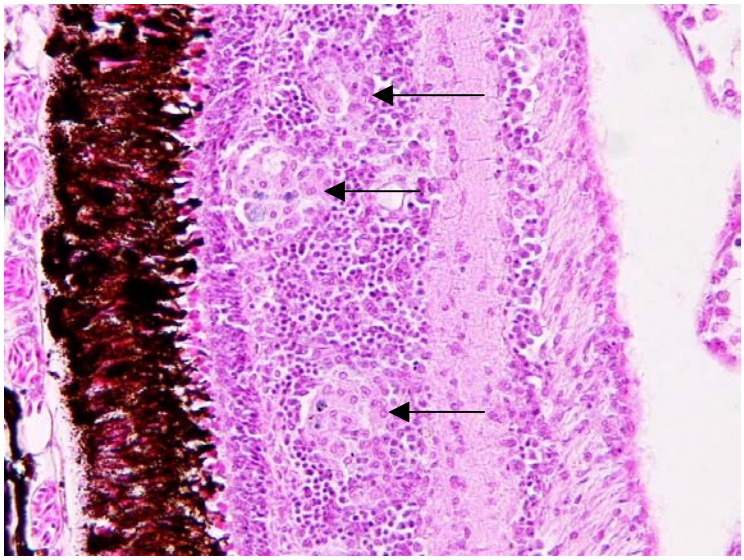

Figure 48: Sleepy cod 12 weeks. Pale cell aggregations (arrows) in the internal nuclear layer of the retina of fish surviving at the end of the trial. x200, H&E.

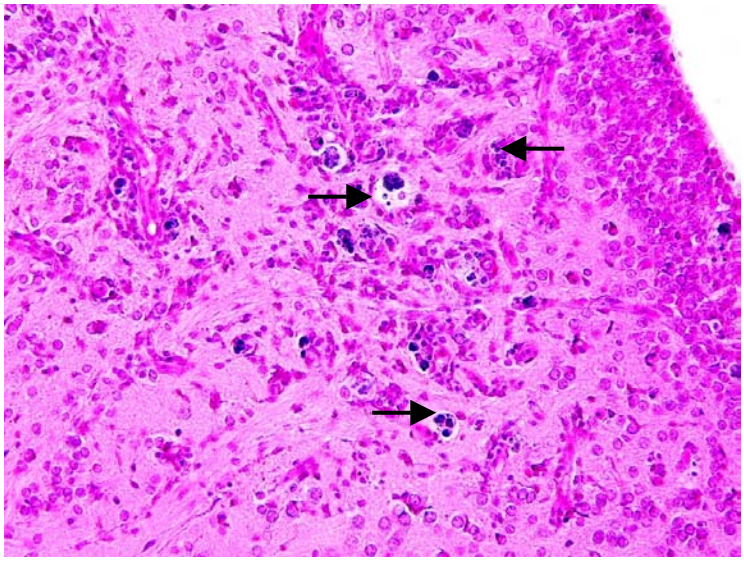

Figure 49: Sleepy cod 12 weeks. Basophilic inclusions (arrows) in necrotic cells in the olfactory lobe of a surviving fish. x200, H&E

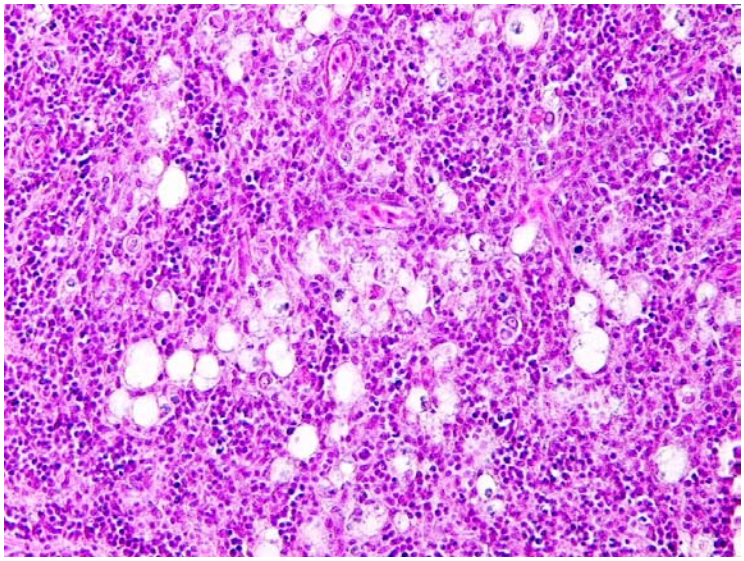

Figure 50: Sleepy cod 12 weeks. Vacuolation in the granular cell layer of the cerebellum of a surviving fish. x200, H&E.

6.2.2.4 Barcoo grunter

Barcoo grunter 6 weeks

Three moribund fish were removed during the trial for histological examination. There was an extensive vacuolation of neuronal cells in the internal nuclear layer of the retinas (Fig. 51). Vacuoles were present in the external nuclear, ganglion cell and visual cell layers. Scattered through this vacuolar degeneration there were cells with basophilic cytoplasms. A few degenerate cells were present in the vitreous humor. The brain lesions were not as extensive but vacuolation was present in the tegmentum, periventricular hypothalamic region, vagal lobe (Fig. 52) and/or the outer border of the granular cell layer of the cerebellum. Cells with basophilic cytoplasms and basophilic inclusions were scattered through these areas of vacuolar degeneration. Basophilic cells and inclusions were also present in the medulla oblongata and the outer edge of the olfactory lobe.

The changes in the surviving fish were mild. Usually the only lesion was 1 to 7 single necrotic cells in the internal and/or external nuclear layers in the retinas (Fig. 53). These cells would not be vacuoles as such but comprise a foci of pale staining cell debris with some melanin-like pigment deposits. Occasionally there would be a basophilic inclusion included in the necrotic cell debris. In a few fish there was a small aggregation of these pale, necrotic cells in the retina (Fig. 54). Only two of the surviving fish had lesions in the brain. In both fish this consisted of a focus of cells with basophilic cytoplasms at the edge of the olfactory lobe (Fig. 55).

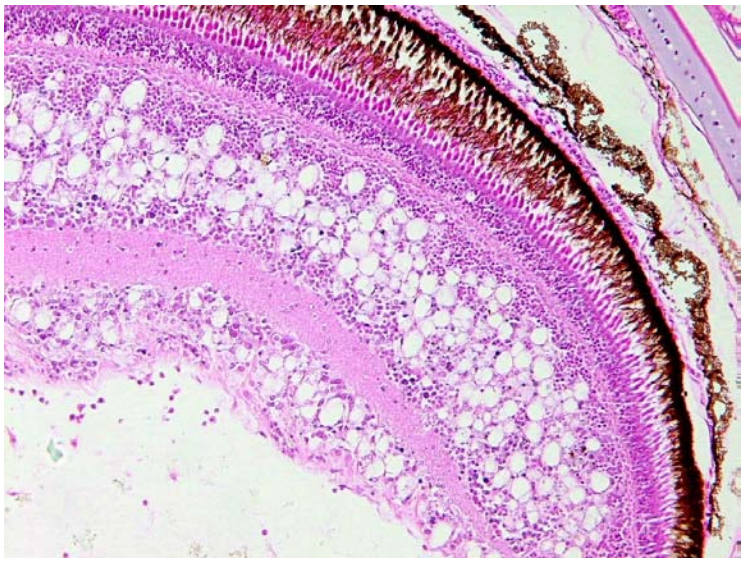

Figure 51: Barcoo grunter 6 weeks. Extensive vacuolation of the internal nuclear layer of the retina. Note the vacuoles in the ganglion cell and external nuclear layers, and a few degenerate cells in the vitreous humor. x100, H&E.

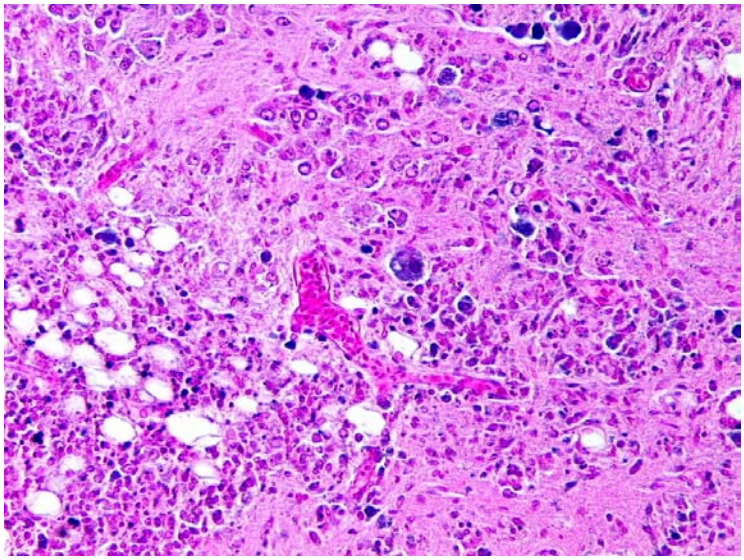

Figure 52: Barcoo grunter 6 weeks. The vagal lobe with vacuolation, cells with basophilic cytoplasms and basophilic inclusions. x200, H&E.

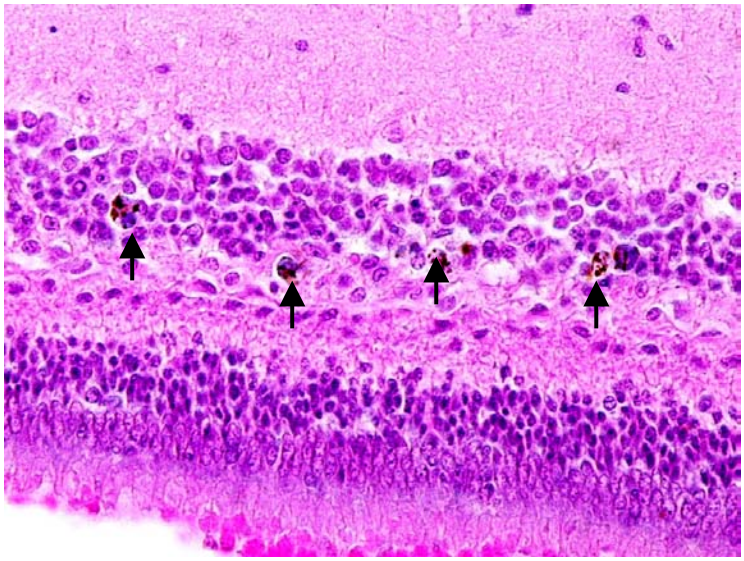

Figure 53: Barcoo grunter 6 weeks. Single necrotic cells (arrows) in the internal nuclear layer of the retina of a surviving fish. The individual necrotic foci include melanin-like pigment deposits and basophilic inclusions. x400, H&E.

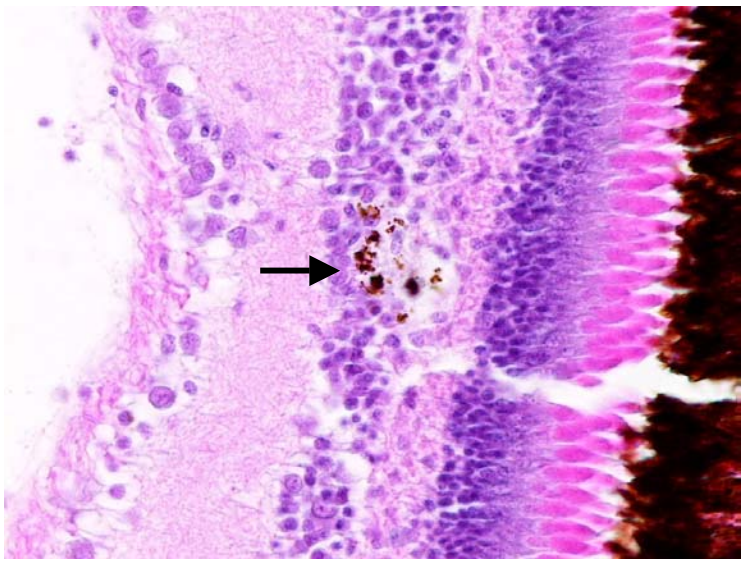

Figure 54: Barcoo grunter 6 weeks. A single aggregation of pale, necrotic cells (arrow), with melanin-like pigment, in the internal nuclear layer of the retina of a surviving fish. x400, H&E.

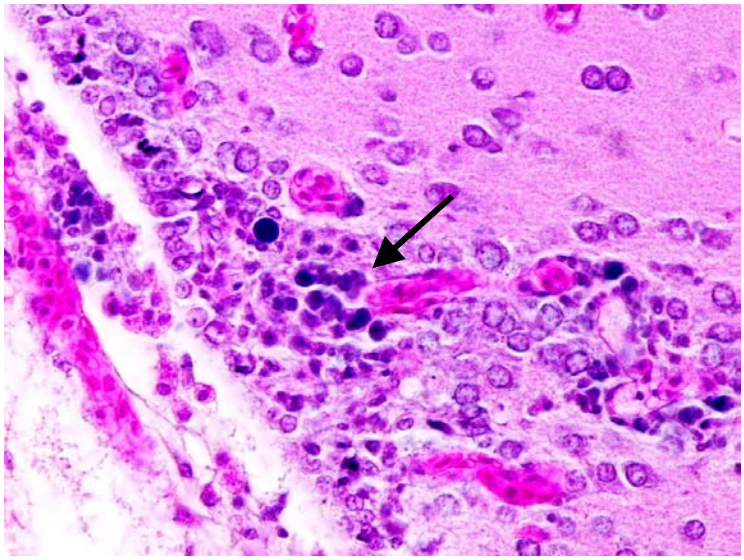

Figure 55: Barcoo grunter 6 weeks. Cells with basophilic cytoplasms (arrow) at the edge of the olfactory lobe of a surviving fish. x400, H&E.

Barcoo grunter 12 weeks

Four moribund fish were removed during the trial for histological examination. There was an extensive vacuolation in the retinas, similar to that seen in the moribund 6 week barcoo grunter fish (Fig. 56). Vacuolation of the internal nuclear and ganglion cell layers was most common, but vacuolated retinal cells were also present in the visual cell and external nuclear cell layers. Occasional cells with basophilic cytoplasms and melanin-like pigment was present in this vacuolar degeneration. Degenerate cells were present in the vitreous humor. Some vacuolated neuronal cells were present in the granular cell layers of the ventricular hypothalamic region, cerebellum (Fig. 57) and/or optic tectum, and/or the tegmentum. Cells with basophilic cytoplasms could be scattered through these areas of vacuolar degeneration (Fig. 58). In some of the fish only cells with basophilic cytoplasms would be present in the tegmentum, hypothalamus and/or medulla oblongata. The fish removed on day 20 after infection also had vacuoles along the spinal cord (Fig. 59).

The changes in the surviving fish were generally mild. As in the surviving 6 week barcoo grunter group, 1 to 4 individual pale, necrotic cells or a small aggregation of these cells were present in the internal and/or external nuclear layer of the retina, and occasionally in the visual cell layer (Fig. 60). Melanin-like pigment and basophilic inclusions could be present in the necrotic foci. The tendency for these pale necrotic cells to be close to the ora serrata was in evidence. Surprisingly, one of the surviving fish had an extensive vacuolation through the internal nuclear and ganglion cell layers (Fig. 61). Those fish with retinal lesions usually had a few degenerate cells in the vitreous humor. Some of the fish also had brain lesions. These comprised vacuoles in the granular cell layers of the optic tectum, periventricular hypothalamic region and/or cerebellum and/or the outer zone of the olfactory lobe. Six of the fish had small foci of cell aggregations in the tegmentum, medulla oblongata or spinal cord (Fig. 62). It was not clear if these related directly to the injected ITCS but the aggregations were not present in any of the control fish.

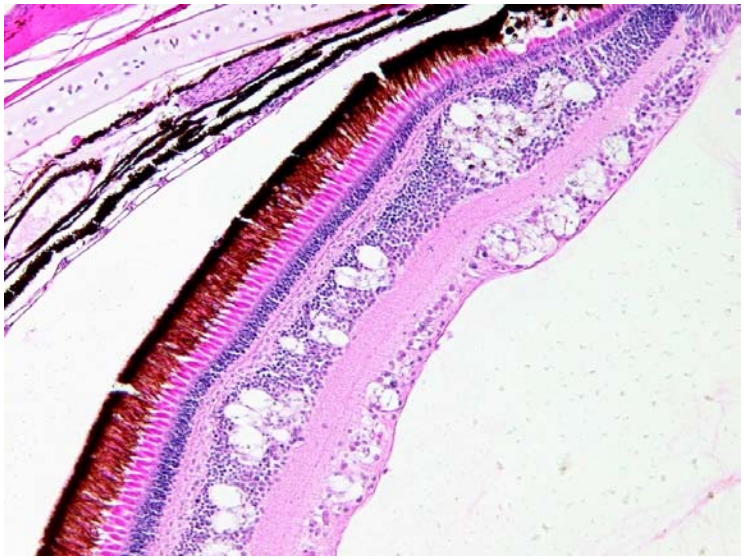

Figure 56: Barcoo grunter 12 weeks. Vacuolation mostly in the internal nuclear and ganglion cell layers of the retina. Note the melnanin-like pigment in some areas of vacuolation. x100, H&E.

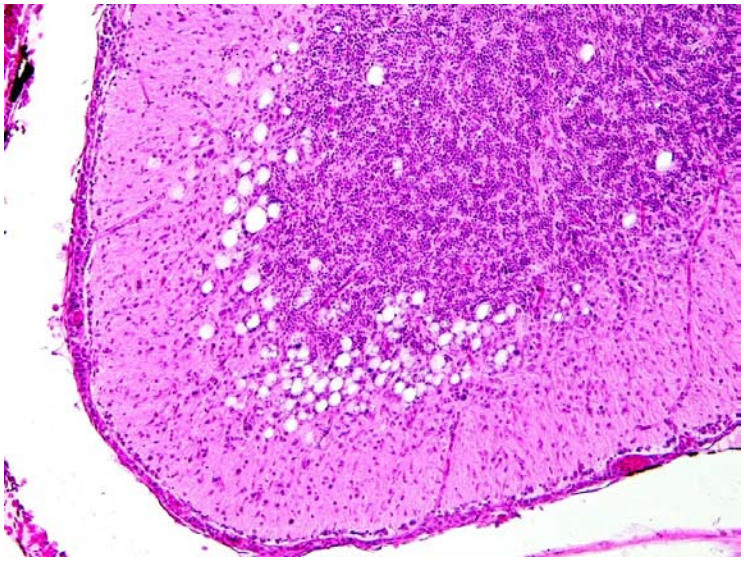

Figure 57: Barcoo grunter 12 weeks. A moderate level of scattered vacuoles in the cerebellum of a moribund fish removed on day 12 after infection. x100, H&E.

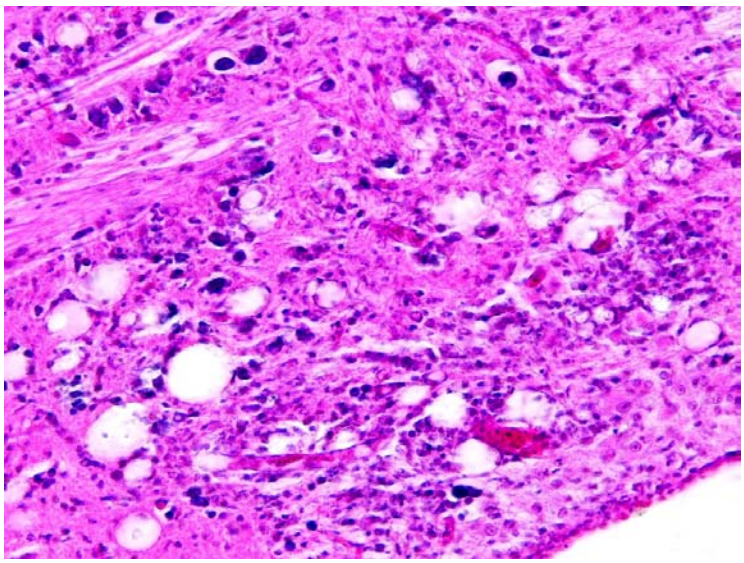

Figure 58: Barcoo grunter 12 weeks. Vacuolation and cells with basophilic cytoplasms in the tegmentum. x200, H&E.

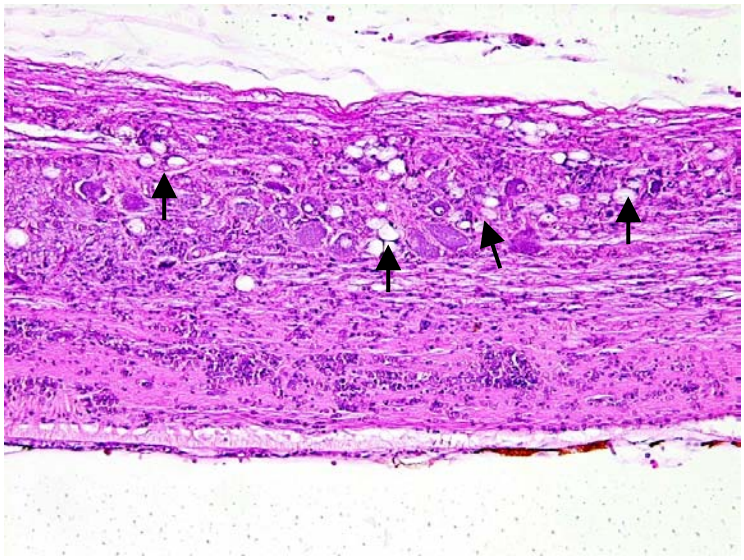

Figure 59: Barcoo grunter 12 weeks. Vacuolation (arrows) along the length of the spinal cord of a fish removed on day 20 after infection. x100, H&E.

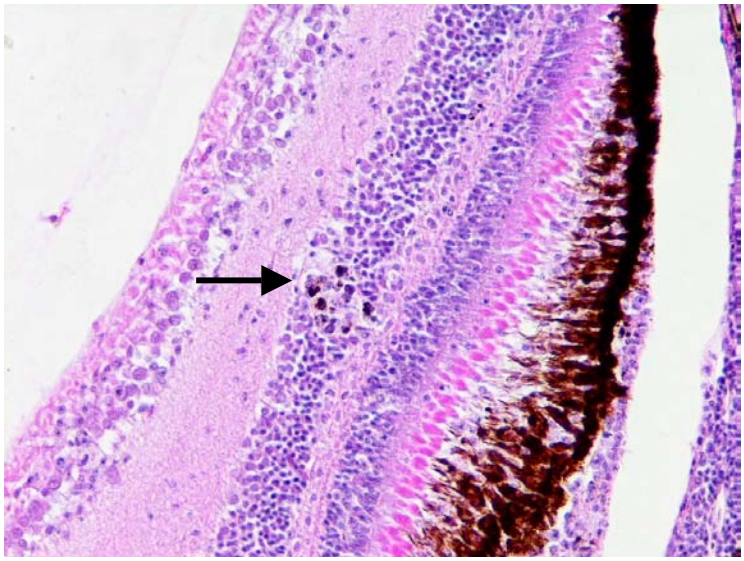

Figure 60: Barcoo grunter 12 weeks. A single, pale cell aggregation (arrow) in the internal nuclear layer of the retina of a surviving fish. x200, H&E.

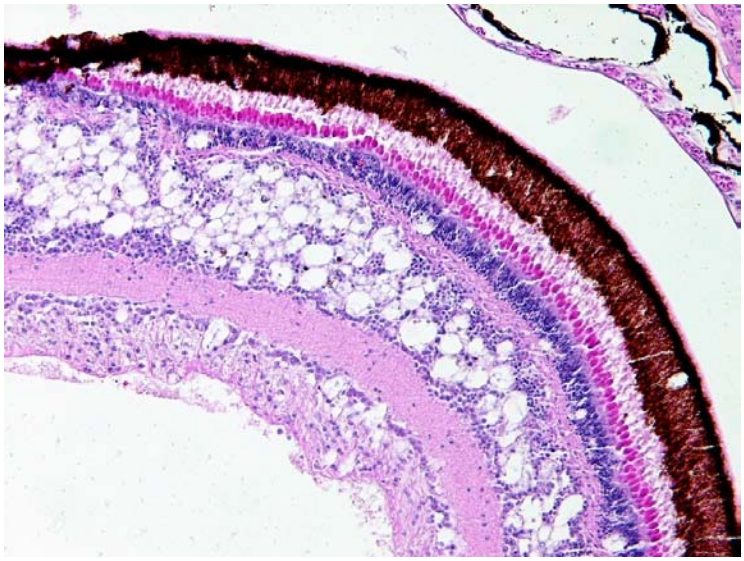

Figure 61: Barcoo grunter 12 weeks. Extensive vacuolation in the retina of a surviving fish. x100, H&E.

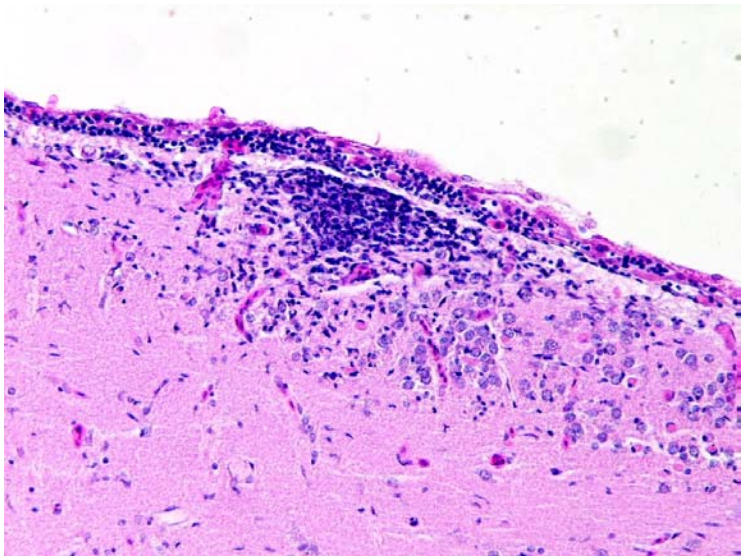

Figure 62: Barcoo grunter 12 weeks. A focus of cell aggregation at the edge of the spinal cord in a surviving fish. x200, H&E.

6.2.2.5. Discussion

Only some general comments will be made here on the histopathology observed in the different species of freshwater fishes as the sampling for histology was opportunistic. There was no systematic sampling to follow the development of the infection over time. Some comparisons between species and between clinically diseased fish and those lesions found in surviving fish can be commented on. It is also important to note the histopathology described here was only from fish that had intraperitoneal injections of nodavirus-infected tissue culture supernatent, not a route you would expect in natural infections. As a consequence, the lesions seen, or the distribution of these lesions, in these experimental fish may be different than those seen in a natural epizootic of viral nervous necrosis in these species.

Over all the range of lesions seen in this study are the same as those described from other species, including barramundi. Munday *et al* (2002) comment in their review of nodavirus infections of teleost fish "histopathological findings, characterised by vacuolation and necrosis of the central nervous system, are remarkably consistent between various species". The typical progression of the microscopic lesion described by Tanaka *et al* (2003) was evident in most of the fish examined in this study. That is the neuronal cells would show an intracytoplasmic vacuolation, followed by nuclear degeneration, cellular fragmentation and necrosis with the necrotic cell replaced by a space. The one exception to this was the development of obvious vacuoles and spaces in neuronal tissues in the sleepy cod. In this species, in moribund fish removed during the infection trials, the initial degeneration appeared as a foamy vacuolation of the cytoplasm or a more classic degeneration involving an increased cytoplasmic eosinophilia progressing to a necrosis where nuclear pyknosis and a loss of cell outline was present. The typical vacuolar degeneration was not always obvious with abundant cytoplasmic and nuclear debris remaining in the necrotic cell. This difference in the development of the neuronal degeneration may reflect the more lethal progression of the nodavirus infection seen in the sleepy cod. Of the four species infected, the sleepy cod had by far the highest mortality.

Also described from other fish species (Munday *et al* 2002) are the cells with basophilic cytoplasms (observed in all four species) and the intracytoplasmic, basophilic inclusions seen in the silver perch, golden perch and barcoo grunters. The presence of melanin-like pigment granules in vacuoles or foci of necrotic cells in the retinas of surviving silver perch, golden

perch and barcoo grunters has been reported by Johansen *et al* (2002) in persistently infected juvenile Atlantic halibut. The aggregations of large pale cells (probably macrophages) seen in some of the surviving silver perch and sleepy cod retinas were also seen in these persistently infected juvenile Atlantic halibut. Focal aggregates of cells (not described in detail) in experimentally infected European seabass retinas have also been reported at 42 days post-infection (Peducasse *et al* 1999). The implication is that these latter histopathological changes may be a consistent feature of non-lethal nodavirus infections which relate to the hosts repair of virus-damaged neuronal cells.

One lesion seen in the study, but at this time not proven to be specifically caused by nodavirus, was the focal cellular infiltrations present in the tegmentum, medulla oblongata and/or proximal spinal cord of the surviving fish from the 12 week silver perch and barcoo grunter groups. These foci of small mononuclear cells with scant cytoplasm were not detected in all fish but may represent a healing response to nodavirus damaged cells. This type of cell aggregation do not seem to have been reported previously in the literature on the histopathology of nodavirus infections.

One outstanding histological feature that was common to all species used in this study was the presence of degenerate and necrotic cells in the posterior compartment of the eye. While the number of the cells present varied from species-to-species and fish-to-fish, if there was at least one vacuolated retinal cell there would be, at least a few, cells in the vitreous humor. This was readily detected when scanning sections at low magnification and was a useful indicator of viral nervous necrosis in a specimen. While they did not show the cells, Husgard *et al* (2001) mention the presence of mononuclear inflammatory cells in the posterior chamber of the eyes of turbot surviving after experimental nodavirus infections. Increased protein content and foamy-appearing macrophages have been reported in the posterior chamber of the eye in Dover sole with VNN (Starkey *et al* 2001).

The distribution of central nervous system tissue lesions followed that already described from other species. In the retina the internal and external nuclear layers had the most extensive vacuolar degeneration, although vacuolation could be present in the ganglion cell layer and, less commonly, the visual cell layer. Brain lesions typically affected the outer zone of the olfactory lobe, the granular cell layers of the optic tectum, cerebellum and the periventricular hypothalamic region although the tegmentum, hypothalamus and medulla oblongata could also contain vacuolation or at least cells with basophilic cytoplasms. It is worth noting that not every individual fish of a specific species, experimentally infected in this study, would have the same parts of the brain affected. In severe, extensive vacuolar degeneration and necrosis of the brain most all of the mesencephalon and diencephalon would be affected. There does not seem to be any difference in the way a nodavirus affects a freshwater fish species as compared to marine species. A vacuolar degeneration of neuronal cells in the spinal cords was seen in some of the golden perch, sleepy cod and barcoo grunter. This is consistent with reports from other species where, except for larval infections, vacuolation of the spinal cord is less common than in the brain. In the surviving fish (fish we assume that are not going to die), if there were central nervous system tissue lesions, they were usually mild and infrequent and had the same distribution as the moribund fishes. The lesions were always present in the retina but not always in the brain. The surviving fish also had the only examples of the pale macrophage-like cell aggregations, the small mononuclear cell infiltrations and more commonly the vacuolar lesions containing the melanin-like pigment.

Currently further analysis of the fish tissues collected in the transmission trials is underway. The sheep polyclonal anti-BNNV coat protein antibody developed under FRDC project 2001/626 “Aquatic Animal Health Subprogram: Development of diagnostic tests for the

detection of nodaviruses" has been applied in an IFAT and IHC format to detect nodavirus in tissue sections. Histological sections made from paraplast blocks prepared in this project will be immuno-stained to better understand the distribution of nodavirus in the experimentally infected freshwater fishes.

6.2.3. Electronmicroscopy of diseased fish

With the implementation of the nested RT-PCR, despite the transformation of the LCB cell line, we were able to confirm the infection by barramundi nodavirus with the PCR methodology. Selected moribund fish from the transmission trials were sampled for electronmicroscopy to just confirm nodavirus was present in central nervous system tissue lesions (see Figs. 63, 64, 65 and 66). Only a small number of fish had brain and retina tissue sampled, and as with the sampling for histological examination, this sampling was opportunistic. All the fish sampled had been injected with ITCS. No comments can be made here on the development of nodavirus infections in individual cells nor on the actual type of cell infected by the injected nodavirus.

The size (around 25nm) and morphology of the virions (Fig. 63) is consistent with that described previously for nodavirus in different species (Glazebrook *et al* 1990; Grotmol *et al* 1995; Johansen *et al* 2002). Typically the nodavirus was apparent in paracrystalline arrays (Figs. 65 and 66) and free in the cytoplasm of all four freshwater fish species. This is also consistent with that described from barramundi (Glazebrook *et al* 1990; Munday *et al* 1992), European seabase (Breuil *et al* 1991) and Atlantic halibut (Grotmol *et al* 1995). Occasionally, the necklace-like chains of virions reported by Johansen *et al* (2002) in persistently infected Atlantic halibut were seen in this limited electronmicroscope study (Fig. 64).

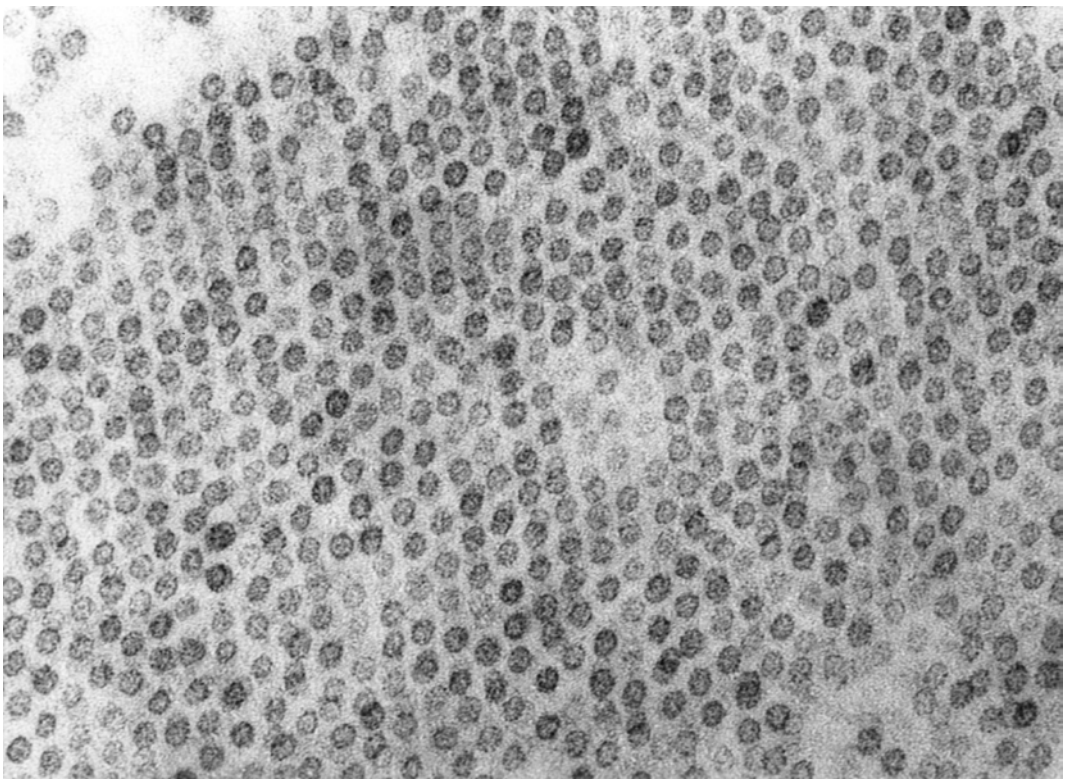

Figure 63: Sleepy cod 6 weeks. Nodavirus virions in a retinal cell. (105,000X magnification)

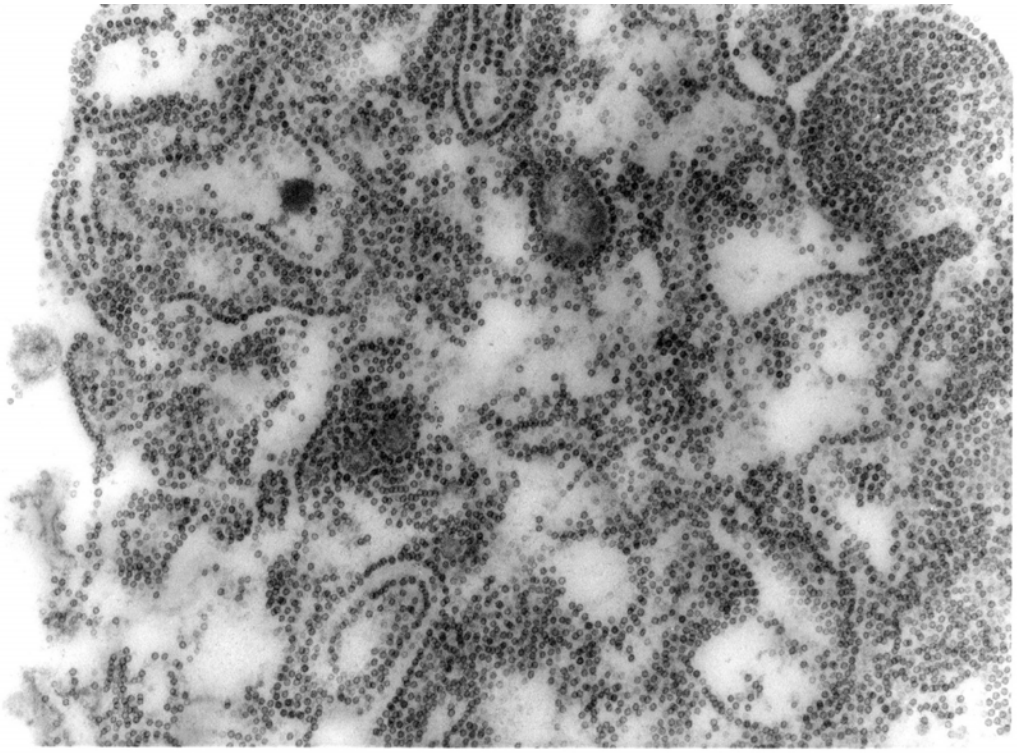

Figure 64: Sleepy cod 6 weeks. Individual nodavirus virions free in the cytoplasm and forming necklace-like chains in a retinal cell. (28,500X magnification)

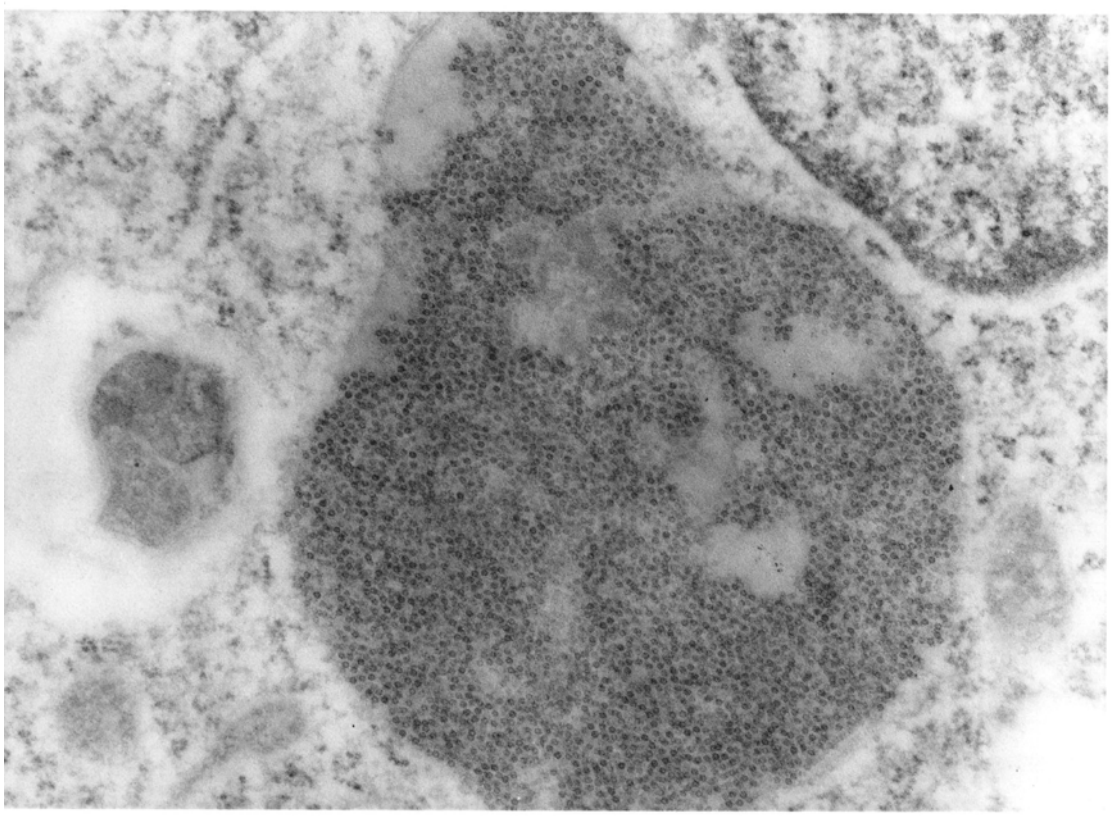

Figure 65: Barcoo grunter 12 weeks. An intracytoplasmic nodavirus virion paracrystalline array in a brain cell. (28,500X magnification)

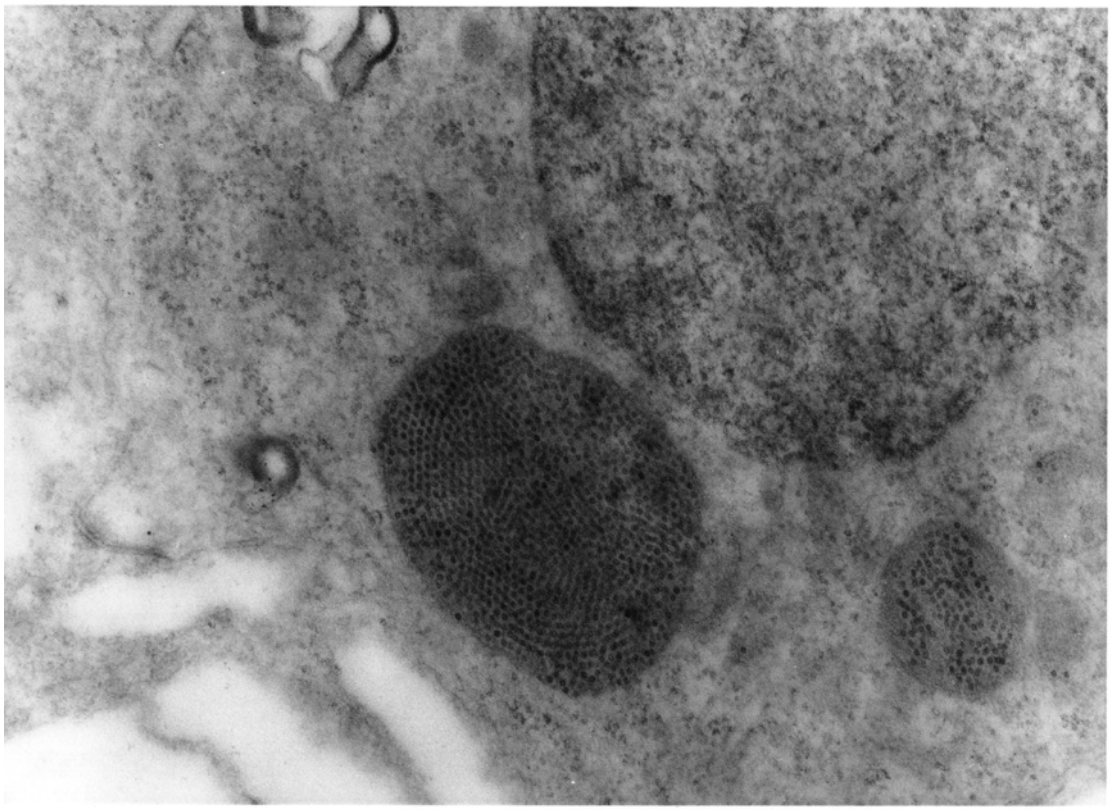

Figure 66: Golden perch 12 weeks. An intracytoplasmic nodavirus virion paracrystalline array in a brain cell. (28,500X magnification)

6.2.4. RT-PCR results on diseased fish and in survivors

The absence of a cell culture isolation system susceptible to BNNV at the end of the freshwater fish infection trials meant that confirmation of nodavirus, or more specifically BNNV RNA, depended on PCR testing.

The absence of CNS tissue histopathology in the bath infected fish was matched by the absence of any nested RT-PCR positive pools of fish sampled at the end of the 28 day trial period (see Table 3). This supports the conclusion inferred from the absence of CNS histopathology that bath infection attempts were not successful.

In contrast there was a high prevalence of PCR positive tests when ITCS injected fish, as moribund fish removed during the trial or as survivors at the end of the trial, were analysed (see Table 3). Typically the moribund fish tissues were positive at the first step, the RT-PCR, while the positive test result was only observed with amplification at the second step or nested PCR in the surviving fishes. This indicates the level of virus present in the surviving fish tissues was probably lower with BNNV being removed as the viral lesions were being resolved through healing.

Table 3: The effect of barramundi nodavirus on the histopathology of freshwater fishes and nested RT-PCR results of fish dying during and euthanased 28 days post-infection after experimental transmission trials

				Number of fish with nodavirus-related histopathology or positive to the nodavirus PCR test			
				Moribund fish		Survivors	
Infection route	Fish species	Age (weeks)	Mortality (%)	CNS[a] pathology	nested RT-PCR	CNS pathology	nested RT-PCR (pools)[b]
Bath	Silver perch	6	3.8	0/3	-	0/27	0/7
		12	8.3	0/3	-	0/44	0/11
	Golden perch	6	2.5	0/1	-	0/40	0/7
	Sleepy cod	6	0	-	-	0/40	0/12
	Barcoo grunter	6	0	-	-	0/40	0/10
Inject	Silver perch	6	46.2	8/8	3/8	5/18	4/4
		12	25.0	6/6	-	23/44	11/11
	Golden perch	6	6.4	4/4	4/6	2/37	5/8
		12	14.5	4/6[c]	-	19/36	10/10
	Sleepy cod	6	98.7	29/29	10/10[d]	0/1	-
		12	92.3	51/51	8/8[e]	3/5	2/2
	Barcoo grunter	6	4.8	3/3	1/1	18/35	10/10
		12	7.3	4/4	-	12/36	10/10

[a] Central nervous system (brain, retina and spinal cord).
[b] Fish collected at the end of the trials were pooled into groups of 1 to 4 individual fish head tissues.
[c] Two moribund fish sampled on Day 9 post-infection had Ichthyobodosis.
[d] Ten pools of 4 fish in each pool.
[e] Eight pools of 2 or 3 fish in each pool.

7. The sensitivity of barramundi nodavirus to a range of environmental conditions and disinfectants.

Under Objective Three "determine the sensitivity of the barramundi nodavirus to a range of environmental conditions and to disinfectants".

7.1. The project failed to complete this objective.

The project application outlines methods that would have evaluated the effect of temperature, salinity, drying and disinfectants (chlorine, formalin, PVP-iodine and a quaternary ammonium compound) on infected tissue culture supernatent (ITCS). The measurement of viral deactivation as a decrease in $TCID_{50}$ (tissue culture infective dose) using reisolation in the then existing cell culture isolation system – the LCB cells, was planned. Following production of sufficient ITCS for transmission trials, and at around passage 35-45 of the LCB cells, we found the cells stopped being susceptible to barramundi nodavirus. As a consequence we were unable to assess virus deactivation *in vitro*.

Further attempts to enhance the susceptibility of LCB cells to nodavirus were made (see section 8.1). No effect was observed. New barramundi brain cell lines were initiated on three occasions (LCB-2, LCB-3 and LCB-4). The three new cell lines produced were not susceptible to the barramundi nodavirus. The importance of *in vitro* culture and replication of nodavirus cannot be underestimated. The loss of susceptible cells in this project prevented completion of this objective. Further, while the newer molecular detection technology provides an increasing important aid in the diagnosis of viral infections, classical isolation of viruses in cell culture systems remains the gold standard for the diagnosis of viral disease in fishes. The subsequent funding of a diagnostic test development project for nodaviruses, with the DAFF Commonwealth Government's New Budget Initiative, 'Building a National Approach to Animal and Plant Health' funds, included a significant component on establishing a cell line which could be used to grow nodaviruses. FRDC Project 2001/626 "Aquatic Animal Health Subprogram: Development of diagnostic tests for the detection of nodaviruses" is ongoing.

7.2. Current literature on nodavirus sensitivity.

There have been two publications that specifically looked at the inactivation of nodaviruses (Arimoto *et al* 1996 and Frerichs *et al* 2000), the table following summarises the findings (Table 4).

Nodaviruses are transmitted vertically but viable eggs may be rendered virtually free of virus by surface disinfection with ozone – recommendations are 0.1µg mL^{-1} residual ozone for 2.5 minutes for striped jack (Arimoto *et a.* 1996), 4µg mL^{-1} for 30 seconds for halibut (Grotmol and Totland 2000) and 1µg mL^{-1} for 1 minute for striped trumpeter (S. Battaglene, DPIWE, Tasmania, Australia pers comm).

Table 4: The effect of various physical and chemical conditions on the viability of fish nodaviruses.

Tested on purified striped jack nodavirus from infected larvae then resuspended in phosphate buffered saline at 20°C (except for temperature treatments).

		Virus detection assay in hatched striped jack larvae.	
Treatment	**Concentration or level**	**Time of exposure**	**Inactivation**
Heat	60°C	<10 minutes	Yes
	50°C	>10 minutes	No
pH	7	>10 minutes	No
	12	<10 minutes	Yes
Chlorine	50 mg/l	<10 minutes	Yes
Iodine	50 mg/l	<10 minutes	Yes
Formalin	>1,600 mg/l	>10 minutes	No
Benzalkonium chloride	50 mg/l	<10 minutes	Yes
Ozone TRO (total residual oxidants)	0.1 mg/l	<2.5 minutes	Yes
	0.5 mg/l	<0.5 minutes	Yes

Aramoto *et al* 1996.

Tested on sea bass nodavirus infected tissue culture supernatant at 15°C (except for temperature treatments).

		Virus detection assay in cell culture reisolation	
Treatment	**Concentration or level**	**Time of exposure**	**Inactivation**
Temperature	15°C	>1 year	No
	25°C	<3 months	Yes
	37°C	<4 days	Yes
	60°C	<1 hour	Yes
pH	2	<42 days	Yes
	7	>42 days	No
	11	<15 days	Yes
Salinity	0 ppt	<6 months	Yes
	20 ppt	>6 months	No
	37 ppt	>6 months	No
Formalin	2%	>6 hours	No
Iodine	25 mg/l	<5 minutes	Yes
Chlorine	50 mg/l	<5 minutes	Yes
	25 mg/l	<30 minutes	Yes
Iodine (when virus protected by protein)	100 mg/l	>30 minutes	No
Chlorine (when virus protected by protein)	100 mg/l	>30 minutes	No

Frerichs *et al* 2000.

8. Methods to detect nodavirus in asymptomatic carrier fish.

Under Objective Four "evaluate the cell culture isolation system as a method of detecting nodavirus in asymptomatic carrier fish".

8.1. Cell culture isolation system

8.1.1 Methods

8.1.1.1 Culture of Lates calcarifer *Brain (LCB) cells*

The stock of LCB cells was in continuous culture prior to this project starting. Specifically these were the LCB cells passaged less than 29 times and cells that had never been stored in liquid nitrogen. Stock LCB cells were cultured in 150cm^2 tissue culture flasks using Earle's modification of Eagle's medium (EMEM) supplemented with 20% foetal bovine serum (FBS), 100 IU/ml benzylpenicillin, 0.1 mg/ml streptomycin sulphate and 2 µg/ml amphotericin B. Cell monolayers were checked microscopically and passaged twice weekly. For passaging, the medium was removed, the monolayer was washed with phosphate buffered saline containing phenol red (PBS-PR) and 10 ml of 0.05% (w/v) trypsin solution, pre-warmed to 37ºC, was added. The cells were monitored and the flask gently rocked until the cells had dissociated from the substrate. Repeated pipetting of the cell suspension produced a single cell suspension then all but 1 ml of the trypsinised cells was removed from the flask. Forty millilitres of growth medium was added and the cells were incubated at 25°C.

8.1.1.2 Sample preparation

For samples containing a large amount (>3 fry) of tissue, whole larvae, or heads from fry, were homogenised in an equal volume of Viral Transport Medium, VTM, (Medium 199 supplemented with 1000IU/ml benzylpenicillin, 1mg/ml streptomycin sulphate and 2µg/ml amphotericin B) using a Diax 900 homogeniser. The homogenate was transferred to 50ml centrifuge tubes and clarified by centrifugation at 2000 rpm for 20 minutes at 5°C. The clarified supernatant was stored in 0.9 ml aliquots at -80°C. Prior to use, aliquots were thawed and clarified by centrifugation at 10,000 rpm for 10 minutes at 5°C. Multiple aliquots were stored to avoid multiple freeze-thaw cycles whenever possible.

For individual fry, the head was removed and homogenised in 1ml of VTM in a stomacher bag using a 2lb hammer and the homogenate transferred to a 1.5ml O-ring screw cap microcentrifuge tube. The homogenate was stored in 0.9 ml aliquots at -80°C. Prior to use, aliquots were thawed and clarified by centrifugation at 10,000 rpm for 10 minutes at 5°C.

8.1.1.3 Virus isolation and amplification

Prior to the commencement of the project it had been determined that no cytopathic effect (CPE) would be observed if the cells were inoculated when the monolayer was more than 60% confluent and maintenance media contained less than 10% FBS. A reduction in FBS concentration in the growth media from 20% to 10% led to a significant reduction in the growth rate of the cells and failure of the development of typical BNNV CPE (Fig. 68) after inoculation of the cells with known positive samples. Therefore the standard protocol of

inoculating cells when the monolayer was no more than 24 hours old, between 50% and 60% confluent and using maintenance medium containing 10% FBS was used for all subsequent optimisation, virus isolation and amplification work.

Virus isolation was conducted on monolayers of LCB cells, grown to 50%-60% confluency in 25cm^2 tissue culture flasks. The growth medium was removed and 1ml inoculum was added and the flasks were incubated at 25°C for 60 minutes. The inoculum was removed and 10ml EMEM, supplemented with 10% FBS, was added. Flasks were incubated at 25°C and monitored daily for the presence of CPE. When typical BNNV CPE had occurred, cells and media were harvested from the flasks and stored in 0.9 ml aliquots at -80°C until required.

8.1.1.4 Optimisation of the LCB cell line and virus isolation

8.1.1.4.1. Comparison of growth rates at 25°C and 20°C

Comparison of the growth rate of LCB cells at 25°C and 20°C was determined by seeding thirty two 25cm^2 tissue culture flasks with 10ml of cells from a 300ml stock in EMEM supplemented with 20% FBS. Fifteen flasks were incubated at 20°C and the remaining 15 flasks were incubated at 25°C. For five days, three flasks were removed from each incubator and trypsinised into 5ml as described above. The total cells per flasks were counted using a haemocytometer. The two extra flasks were inoculated with BNNV infected LCB cell culture supernatant as described above and development of CPE was monitored daily.

8.1.1.4.2. Growth in 24 and 96 well plates

Prior to the commencement of the project, all virus isolations were conducted in 25cm^2 tissue culture flasks. As samples requiring virus isolation were received as one sample and the requirement for virus isolation was not excessive, this was considered the most sensitive and economical way to conduct virus isolation work. However, as it was anticipated that the virus isolation requirements would significantly increase after the infectivity trials, experiments were undertaken to optimise the seeding density of LCB cells in 24-well tissue culture plates. The use of 24-well tissue culture plates would increase efficiency as more samples could be tested in a smaller volume of reagents. Similarly, in order to optimise titrations to quantify the amount of virus present, experiments were also undertaken to optimise the seeding density of LCB cells in 96-well tissue culture plates. LCB cells were adjusted to 1×10^4, 2×10^4, 4×10^4, 6×10^4, 8×10^4, 1×10^5, 2×10^5, 4×10^5, 6×10^5, 8×10^5 and $1x10^6$ cells/ml in EMEM supplemented with 20% FBS. One millilitre of each cell concentration was added to each well of a 24-well plate, with one plate used per dilution and 100µ of each dilution was added to each well of a 96 well plate, with one plate used per dilution. Plates were incubated in an airtight box at 25°C with CO_2 produced by addition of Eno to a beaker of distilled water in the box. Monolayer development was monitored and recorded daily.

8.1.1.4.3. Gelatin pre-treatment (GPT)

During passaging of the LCB cells into new tissue culture flasks variability in cell attachment was observed. Essentially two passages were required in a new flask before repeatable cell attachment and monolayer development was achieved. In an effort to remove variability and increase efficiency, the method of Schor *et al* (1996), was trialed. Flasks were pre-treated by addition of 2ml of 0.5% (w/v) tissue culture grade gelatin in PBS and the flasks were

incubated at 37°C overnight. Prior to use the gelatin solution was poured from the flask. To quantify any increase in plating efficiency, 10ml from a stock of LCB cells in suspension was passaged into each of three new 25cm^2 tissue culture flasks which had been gelatin pre-treated, and into three new 25cm^2 tissue culture flasks which had been left untreated. After 7 hours, cells were photographed and the number of cells per photograph determined.

8.1.1.5 Transformation of LCB

At between passage 35 to 45 the LCB cells transformed and were no longer susceptible to infection with nodavirus. In order to determine the exact passage when the loss of susceptibility occurred, LCB cells, which had been stored in liquid nitrogen at passage 18 were thawed and cultured as above until the growth rate had stabilised and cell could be used for virus isolation and amplification work. Whenever cells underwent routine passaging, cells were also passaged into three 25cm^2 tissue culture flasks that were incubated at 25°C. When the cells were 60% confluent two flasks were inoculated with BNNV as above and a third was mock-infected as a negative control. Flasks were incubated at 25°C and monitored daily for the appearance of CPE.

8.1.1.6 Enhancing susceptibility of LCB

The polycations, DEAE-Dextran and polybrene, have been added to virus inocula to increase the infectivity of samples during virus isolation attempts (Table 5). Experiments were therefore undertaken to determine whether these polycations would be useful in increasing the susceptibility of the transformed LCB cells to BNNV. The following method was used to expose the LCB cells to dilutions of each polycation to determine the concentration where the polycations were toxic. Passage 51 LCB cells were cultured in 96 well tissue culture plates in an airtight box at 25°C with CO_2 produced by addition of Eno to distilled water in the box. When cells were 60% confluent, 50µl of serial 2-fold dilutions of DEAE-Dextran and polybrene, prepared in EMEM from 640 to 2µg/ml, were added to quadruplicate wells of the 96-well plate and the cells were incubated at 25°C for 60 minutes. Each well was washed three times with EMEM, 100µl of EMEM supplemented with 10% FBS was added to each well and the plates were incubated incubated overnight at 25°C with CO_2 produced by addition of Eno to distilled water in the box. The following day, the viability of the cells was assessed by MTT Cytotoxicity Assay (Promega) as per the manufacturer's instructions.

A reduction in viability of the LCB cells was not observed until concentrations of DEAE-dextran and polybrene higher than 40µg/ml and 160µg/ml were used. From the literature and the tocixity results is was decided to use both polycations at a concentration of 10µg/ml. To determine the usefulness of the polycations, virus amplification of four known BNNV positive samples were conducted in 25cm^2 tissue culture flasks as described previously, but each polycation was added to the clarified inoculum, to a final concentration of 10µg/ml, and mixed well. Virus isolation then conducted according to the standard protocol.

Table 5: Cell lines and viruses used in conjunction with DEAE and polybrene.

Cell Line	Polycation	Virus	Concentration used	Incubation Time	Reference
BHK	DEAE	Rabies	50 μg/ml	60 minutes	Kaplan *et al* (1967)
CK	DEAE	LTV	160-320 μg/ml	60 minutes	Rossi and Watrach (1970)
NRK	DEAE	MSV	25 μg/ml	60 minutes	Hesse *et al* (1978)
NRK	Polybrene	MSV	25 μg/ml	60 minutes	Hesse *et al* (1978)
XC	DEAE	MuLV	25 μg/ml	60 minutes	Hesse *et al* (1978)
XC	Polybrene	MuLV	25 μg/ml	60 minutes	Hesse *et al* (1978)
FHM	Polybrene	IHNV	5 μg/ml	30 minutes	Leong *et al* (1981)
CHSE	Polybrene	IHNV	5 μg/ml	30 minutes	Leong *et al* (1981)
Cf2Th	Polybrene	SSV	5 μg/ml	60 minutes	Coelen *et al* (1983)
PMC9	Polybrene	BaEV	5 μg/ml	60 minutes	Coelen *et al* (1983)
Vero	Polybrene	SNV	600 μg/ml	60 minutes	Mastromarina et al., (1991)
BHK	DEAE	VSV	10 μg/ml	60 minutes	Bailey et al., (1984)

8.1.1.7 New cell line development (LCB-2, LCB-3 and LCB-4)

Barramundi brain cell lines were initiated on three occasions from barramundi brain tissue using the following methods. Pre-warm the trypsin solution and EMEM to 25°C. After euthanasia, the brain was aseptically removed from the barramundi and placed into a petri dish containing antibiotic-enriched EMEM (Ae EMEM; EMEM supplemented with 1000IU/ml benzylpenicillin, 1mg/ml streptomycin sulphate and 2μg/ml amphotericin B). Any fatty tissue was trimmed away and the tissue finely chopped into small (<1mm^3) pieces in a biohazard cabinet. The tissue was washed three times in Ae EMEM by pelleting and resuspension at 1,000 × g for 10 minutes at room temperature. After removing as much media as possible, 2ml of pre-warmed 0.05% (w/v) trypsin solution was added and the tissue pieces were transferred to a sterile universal bottle containing a small stirring flea. A further 10ml of 0.05% (w/v) trypsin solution was added and the tissue incubated on a magnetic stirrer for 45 minutes at 25°C. The cells were pelleted at 1,000 × g for 10 minutes at room temperature and the trypsin solution was removed.

For the LCB-2 development, the cells were resuspended in 80ml EMEM supplemented with 20% FBS. Ten millilitres was added to eight 25cm^2 tissue culture flasks with the cells incubated at 25°C. Flasks were monitored daily, and media changes and routine passaging was conducted as required. For the LCB-3 development, the cells were resuspended in 2ml EMEM , with 1 ml added to 40ml EMEM supplemented with 10% FBS and the remaining 1ml added to 40ml EMEM supplemented with 20% FBS. Ten millilitres was added to each of eight 25cm^2 tissue culture flasks with the cells incubated at 25°C. Flasks were monitored daily, and media changes and routine passaging was conducted as required. For the LCB-4 development, the pelleted cells were resuspended in 2ml EMEM and 1ml was added to 9ml EMEM supplemented with 10% FBS and the remaining 1ml was added to 9ml EMEM supplemented with 20% FBS. Each 10ml suspension was added to one gelatin pre-treated 25cm^2 tissue culture flask and the cells were incubated at 25°C. Flasks were monitored daily, and media changes and routine passaging was conducted as required. When growth rates of the cell lines were appropriate, susceptibility trials were undertaken using the amplification method described in section 8.1.1.3.

8.1.1.8 Cloning of the LCB cells.

Passage 18 LCB cell cultures were thawed from liquid nitrogen and at passage 29 the cells were at the stage where passaging was required weekly. Cloning of these cells was undertaken using the following methods. The cells were trypsinised using the standard method and were pelleted by centrifugation at 1,000 rpm for 5 minutes at room temperature. The trypsin was removed and the cells were resuspended in 50ml EMEM. An aliquot of the cell suspension was placed in a sterile petri dish in a laminar flow cabinet. The petri dish was placed on the stage of an inverted microscope in the laminar flow cabinet and individual cells were collected into a sterile gel loading tip using a 2-20µl pipette. The single cells were then transferred to a well of a 96 well plate containing 100µl of EMEM and 20% FBS. The wells of ten 96 well plates were seeded in this way and were incubated at 25°C in a 5% CO_2 environment. Attachment and inspection of the wells was conducted every two days.

8.1.2 Results and discussion

8.1.2.1 Culture of LCB cells

Culture of the LCB cells in EMEM, supplemented with 20% FBS, produced an evenly distributed, rapidly growing monolayer (Fig. 67) with the stock culture flasks requiring passaging twice weekly. Monolayer formation was variable during the first passage into a new flask, however once cells had been cultured once in a new flask, growth and time to monolayer formation was predictable and repeatable. Seeding into 25cm^2 flasks consistently resulted in the formation of a 60% confluent monolayer within 24 hours.

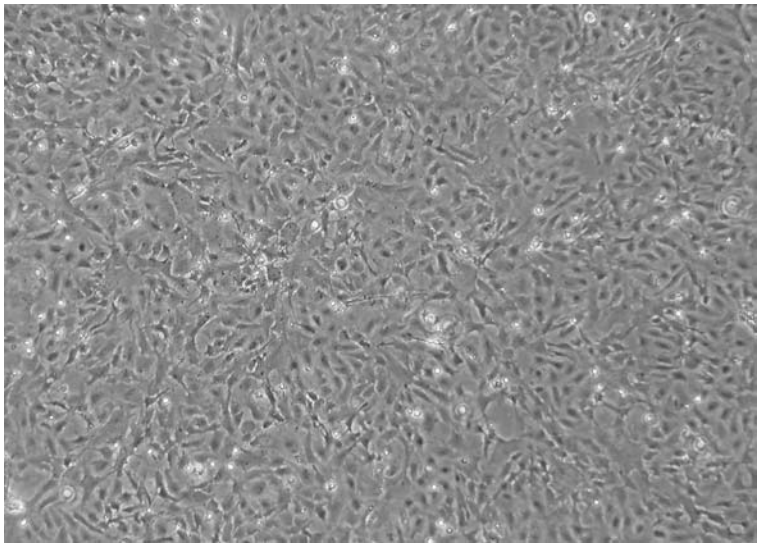

Figure 67: Uninfected LCB cells prior to passage.

8.1.2.2 Sample preparation

The sample preparation procedure used resulted in repeatable virus isolation (see Section 8.1.2.3). This procedure is different to that recommended by the Office International des Epizooties (OIE) for the isolation of nodavirus (OIE, 2003), where samples are homogenised in nine volumes of Hanks' Balanced Salt Solution and filtered through a 0.22µm filter.

However, no sample toxicity was ever observed after inoculation of LCB cells and isolation rates from clinically infected material were excellent (see section 8.1.2.3). Due to resource constraints dilution in an equal volume of VTM, followed by clarification by centrifugation at 10,000 x g, is routinely used in the virology section of Oonoonba Veterinary Laboratory and has been used to isolate a number of aquatic and terrestrial animal viruses (N Moody, personal observation; Moody, 1992; 1997) and in our opinion is at least as good as the OIE recommended procedure.

8.1.2.3 Virus isolation and amplification

Typical BNNV CPE (Fig. 68) development from clinical material occurred within 24 to 48 hours post infection. There was a 100% correlation between production of CPE after inoculation of LCB cell cultures, indicating presence of infectious virus, when virus isolation was undertaken from samples where typical nodavirus lesions were observed after histological analysis of fixed material (Table 6).

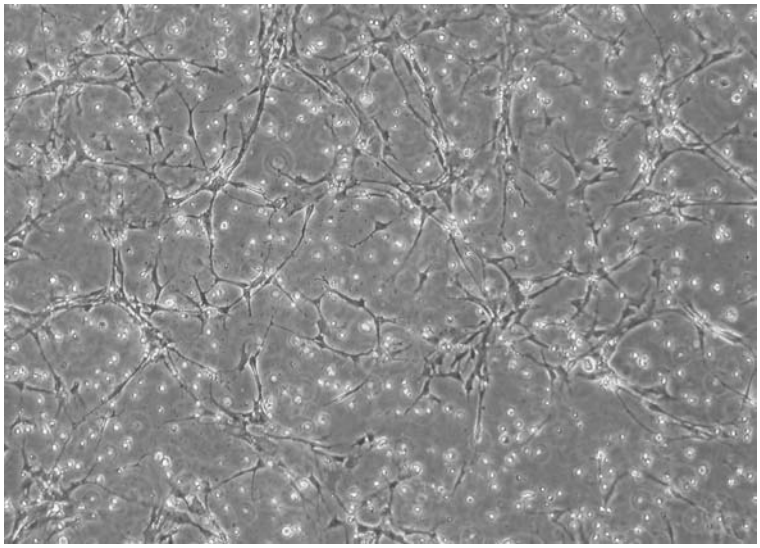

Figure 68: BNNV-infected LCB cells, showing typical BNNV CPE

Repeatable amplification of stock virus was achieved using the standard protocol and typical CPE development occurred within 48 to 72 hours post infection.

Table 6: Comparison of histology and virus isolation in LCB cells for detection of BNNV from clinical material.

Accession	Disease (Clinical/Histo)	Isolation in LCB cells	Accession	Disease (Clinical/Histo)	Isolation in LCB cells
1	✓	✓	10	✓	✓
2	✓	✓	11	✓	✓
3	✓	✓	12	✓	✓
4	✓	✓	13	✓	✓
5	✓	✓	14	×	×
6	✓	✓	15	×	×
7	✓	✓	16	×	×
8	✓	✓	17	×	×
9	✓	✓			

8.1.2.4 Optimisation of the LCB cell line and virus isolation

8.1.2.4.1 Comparison of growth rates at 25°C and 20°C

Growth of the LCB cell line was significantly reduced when the temperature was reduced from 25°C to 20°C (Fig. 69). After inoculation and incubation at 25°C, CPE developed as usual. After inoculation and incubation at 20°C, no CPE developed at all and it was apparent that nodavirus would only replicate in rapidly dividing cell cultures. Therefore, the incubation temperature of the stock cultures and cultures used for virus isolation and amplification was kept at 25°C.

8.1.2.4.2 Growth in 24 and 96 well plates

The optimal density seeding of LCB cells for both 24 well plates and 96 well plates was $8x10^4$ cells/ml. The concentration resulted in the development of a 50% to 60% confluent monolayer after 24 hours in the 24 well plates and maintained the required rapid growth rate of the cells. A consistent observation was the uneven distribution of the cells in the wells. The cells appeared to preferentially settle in the middle of the well and become too confluent to inoculate, while the cells around the outside of the well were only about 20% confluent. This did not occur with the other mammalian and piscine cell lines routinely used in the laboratory. The concentration of $8x10^4$ cells/ml, when used with 96 well plates resulted in the production of a 100% confluent monolayer after five days. This was considered desirable as it would give viral dilutions containing a lower amount of virus more time for viral replication and expression of CPE to occur. However, titration experiments, conducted according to the standard method of Reed and Muench (1938), failed to produce CPE in any dilution of the sample that was greater than 10^{-1}. It is unknown why this occurred. All the samples had produced CPE after virus isolation in the LCB cells grown in 24 well plates and, as there were marked differences in the intensity of lesions after histological analysis, there was strong evidence to suggest the viral load was in excess of $10^{1.5}$ $TCID_{50}$/50µl.

Unfortunately, before additional experiments could be conducted to try and resolve this lack of repeatability when cells were passaged into tissue culture plates, the LCB cells transformed, so this work was not completed.

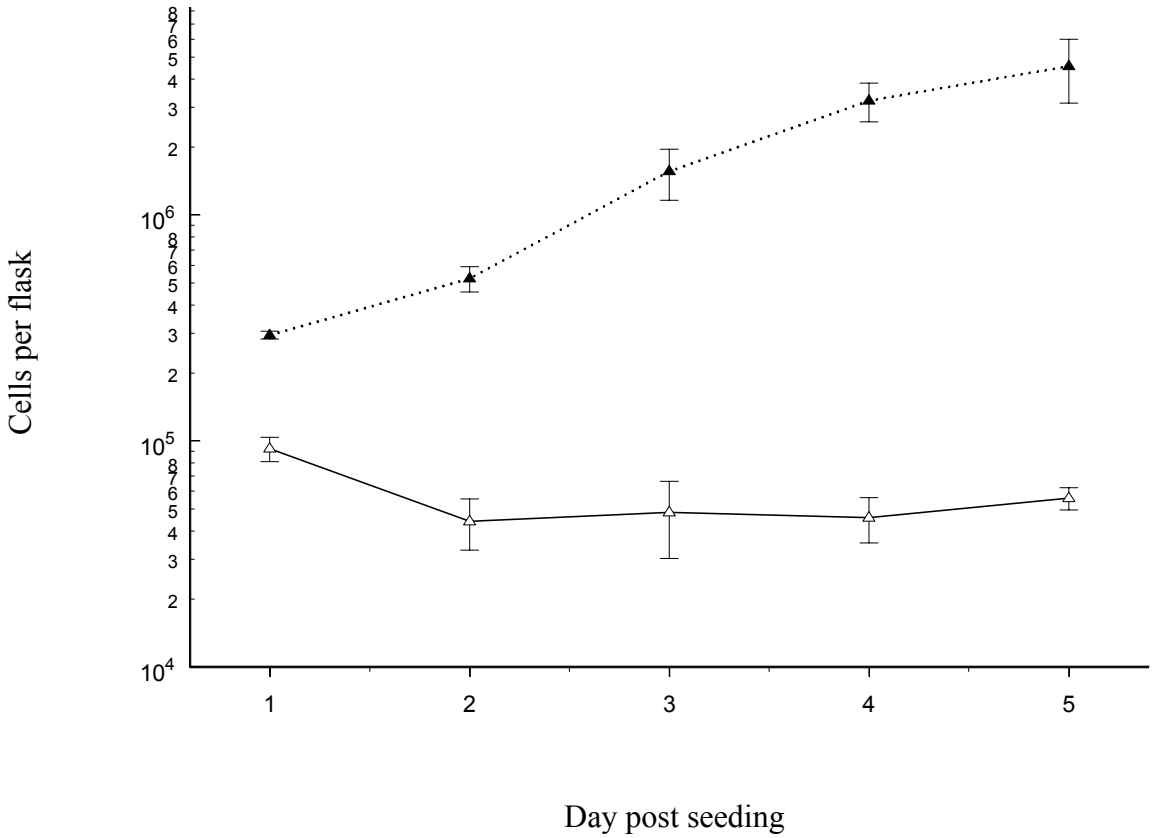

Figure 69: Comparison of growth of LCB cells at 25°C (▲) and 20°C (Δ).

8.1.2.4.3 Gelatin pre-treatment

Adhered cell counts from untreated flasks were significantly lower (mean = 13.5 cells per photograph, sd = 2.19, n = 10) than adhered cell counts from the gelatin pre-treated (GPT) flasks (mean = 176, sd = 7.85, n = 10). The gelatin pre-treatment had led to a 13-fold increase in plating efficiency of the LCB cells, greatly reducing the time between passaging of cells and use for either viral culture or isolation (Figs. 70a and 70b).

The use of the gelatin pre-treatment was adopted for routine use with the LCB cell cultures at the laboratory. A system was developed where gelatin pre-treated 25cm^2 flasks were seeded with LCB cells in the morning and the monolayer was between 50% and 60% confluent in the afternoon. After inoculation with clinical samples the same day as seeding, CPE had developed by the next morning, confirmatory RT-PCR, and later nested RT-PCR testing could be conducted that day and agarose gels to visualise any product completed by noon on the third day. If the case was urgent, the isolation and confirmation of nodavirus as the agent producing CPE could be completed within 48 hours.

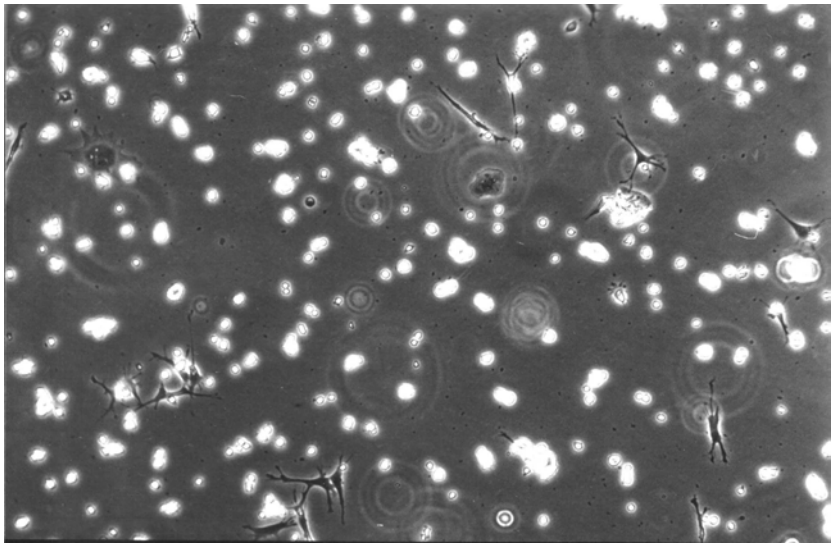

Figure 70a: LCB cells 7 hours after passage into an untreated 25cm^2 flask

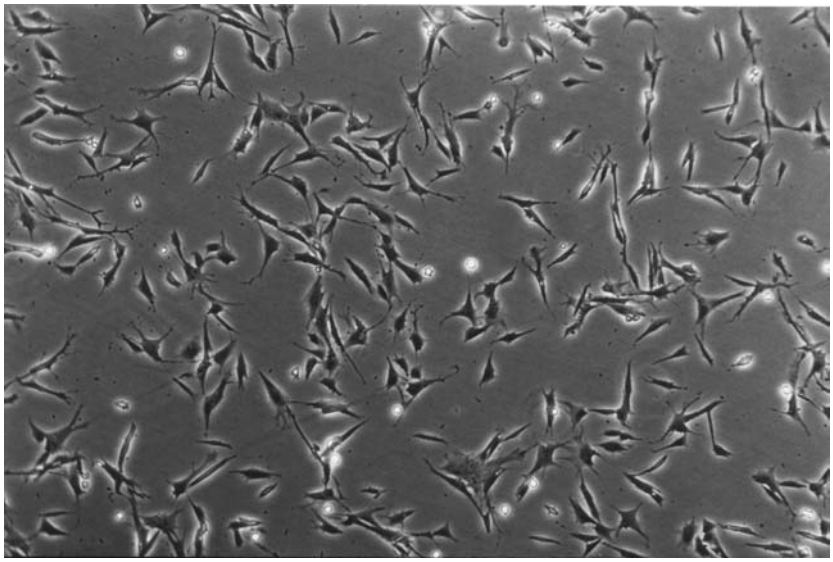

Figure 70b: LCB cells 7 hours after passage into a gelatin pre-treated 25cm^2 flask

8.1.2.5 Transformation of LCB

LCB cells were stored in liquid nitrogen at passage 18, passage 24 and passage 32, using standard procedures. No virus isolation or amplification was conducted on cells between passage 29 and passage 62. Attempts to culture BNNV in cells from passage 62 to 75 were unsuccessful.

From passage 62 onwards, a very unusual bacterial contaminant was identified in the cultures. This contaminant was in the stock cells in the clean Cell Culture laboratory. However, the bacterial growth was not overt and could not be detected by observation of the stock cultures using standard light microscopy. Expression of the bacteria only occurred approximately three days after inoculation of the LCB cells when the nutrient concentration had been

reduced through cell metabolism. Attempts to grow the bacteria on standard bacteriological agar plates and a modified EMEM-FBS agar failed to result in any bacterial growth. Troubleshooting identified very small holes in the plastic surrounding the individually wrapped disposable 10ml pipettes used during the passaging of the stock cell cultures. This was traced back to a fault during the manufacturing process at the plant in England. The use of re-usable glass pipettes was implemented for all cell culture and stored LCB cells were thawed from liquid nitrogen and passaged.

The growth rate of LCB cells removed from liquid nitrogen storage was not appropriate for culture of BNNV until passage 39, which took 2.5 months after the cells had been thawed. However, infection with known positive BNNV samples failed to produce any CPE when cells were infected using the standard protocol described above.

While there were no visible morphological changes, the LCB cells were no longer susceptible to infection with BNNV. A further three aliquots of passage 24 cells and one aliquot of passage 18 cells were removed from liquid nitrogen, thawed and cultured, but growth rates were not appropriate for culturing BNNV until after passage 39 and exposure to known positive BNNV samples consistently failed to produce reproducible and complete CPE. CPE was occasionally observed in some areas of the monolayer after 24 hours, however by 48 hours post inoculation, surrounding cells had regrown into the areas where cells had detached from the substrate and the appearance of the monolayer was not different to that of control cell cultures (Figs. 71a and 71b).

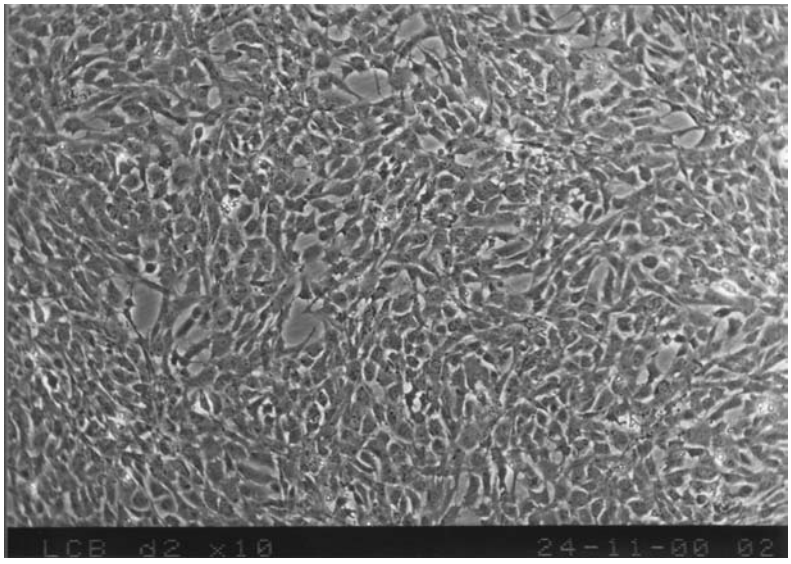

Figure 71a: Uninfected LCB cells, at passage 49

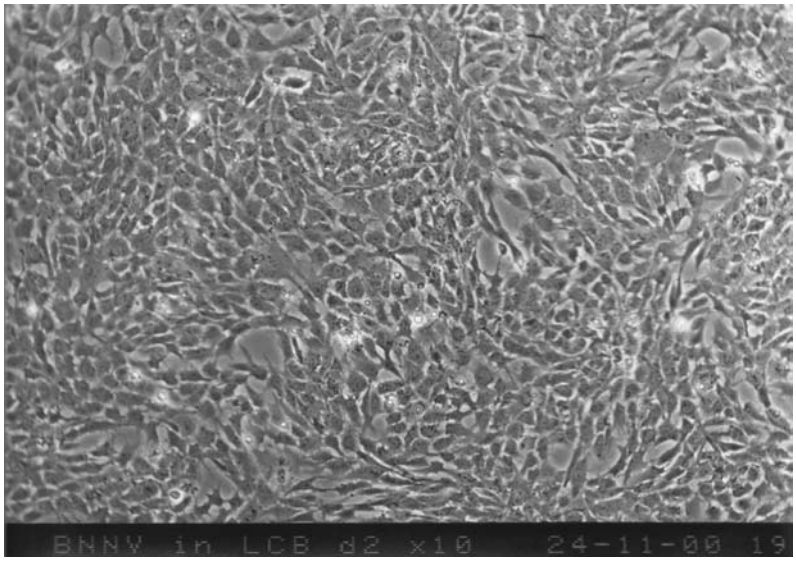

Figure 71b: Passage 49 LCB cells, 2 days post exposure to BNNV.

Due to the length of time taken for the cells to reach the stage that their growth rate was considered acceptable for BNNV culture i.e., more than 2 months after removing from liquid nitrogen storage, no further stored cell aliquots could be assessed before the project ended.

8.1.2.6 Enhancing susceptibility of LCB

Addition of polycations did not result in susceptibility of the transformed LCB cells to infection with BNNV. There were no distinguishable morphological changes between treated and infected monolayers and control monolayers.

8.1.2.7 New cell line development (LCB-2, LCB-3 and LCB-4)

Initiation of new cell lines was attempted on three occasions from barramundi brain tissue. All attempts resulted in the establishment of passageable cell lines in each of the seeded 25cm^2 flasks. The flasks containing the cells from each separate attempt were designated LCB-2, LCB-3 and LCB-4.

LCB-2 was a cell line that required passaging twice weekly by passage 5 and these cells were cryopreserved, using standard procedures, at passage 7. Inoculation of 60% confluent LCB-2 cell cultures at passage 9, with BNNV positive material, failed to result in the appearance of CPE. Cultures were inoculated a further two times, using different known BNNV-infected material but on each occasion, CPE failed to develop.

The LCB-3 cell line development resulted in the production of cell lines in each of the eight flasks that were seeded. Adherence was significantly better in the cells grown in EMEM supplemented with 10% FBS, however growth was significantly slower. Inoculation of the cell lines grown in EMEM supplemented 20% FBS, when 60% confluent at passage 19, with BNNV positive material, failed to result in the appearance of CPE. The LCB-3 cell cultures were inoculated a further two times, using different known BNNV-infected material, but again on each occasion CPE failed to develop. Due to resource and time constraints these cell lines were not retained in storage.

The LCB-4 cell line development resulted in excellent adherence of cells to the GPT flasks compared to the previous attempts, where cells were seeded into untreated flasks. However, before the cells lines could be tested for susceptibility to BNNV, contamination occurred at passage 5 and the cultures were lost.

Due to resource and time constraints and the time taken for the cultures to become established and reach a suitable growth rate before the end of the project, no further new cell line development was attempted.

8.1.2.8 Cloning of the LCB cells.

No attached cells were seen in any of the 96 well plates after cloning and no clones were produced. The same cells used to produce the cell suspension used for cloning continued to grow so the failure of the clones to grow was not due to a failure in the viability of the cells. Due to time constraints, no further attempts to clone LCB cells were made.

8.2. Molecular detection tests

8.2.1. Methods

8.2.1.1. Implementation of the nodavirus RT-PCR and nested RT-PCR protocol

Implementation of the RT-PCR and nested RT-PCR was ongoing through the project as new knowledge was generated after evaluation and application of the different experimental protocols. Generally, protocol development occurred in three phases; i) optimisation of RNA extraction methods, ii) optimisation of the RT-PCR and iii) optimisation and refinement of the RT-PCR and Nested RT-PCR.

All reagents used in the molecular test implementation were either purchased and certified RNase-free or rendered RNase-free as follows; plastic ware was incubated overnight in 0.1% (w/v) diethypyrocarbonate (DEPC) in deionised water and autoclaved for 15 minutes on liquid cycle; non-Tris-based solutions were made to 0.1% (w/v) diethypyrocarbonate (DEPC), incubated overnight at 37°C and autoclaved for 15 minutes on liquid cycle and glassware was incubated at 200°C for at least 4 hours (Sambrook *et al* 1989). All material used for the protocol development was obtained from submissions that had had a sub-sample processed for histological examination and lesions observed in central nervous system tissue.

RNA extraction

Three different RNA extraction methods were evaluated; SDS/Proteinase K phenol/chloroform extraction and ethanol precipitation, which is a standard nucleic extraction technique; TRIZOL which was a newer product and utilised guanidinium thiocyanate, a potent inhibitor of RNAses (Chomczynski and Sacchi, 1987); and a commercially-available spin column kit, as these types of kits were becoming a lot more common and were also based on the ability of guanidinium thiocyanate to act as a potent RNAse inhibitor. Extractions were conducted on replicated, passage two, LCB cell supernatants, from six clinical submissions to the laboratory. After testing cell culture supernatant, homogenised, clarified, infected barramundi material was tested.

i) SDS/Proteinase K phenol/chloroform extraction: RNA was extracted according to the method of Sambrook *et al* (1989). Clarified cell culture supernatant or barramundi larvae were incubated with 1% SDS, 250µg/ml Proteinase K, 2mM 2-β-mercaptoethanol and 40U RNase inhibitors for 60 minutes at 37°C. An equal volume of acid phenol:chloroform:isoamyl alcohol (125:24:1) pH 4.2, pre-warmed to room temperature, was added to each sample. The tubes were shaken vigorously for 15 seconds and centrifuged at 12,000×*g* for 30 seconds at room temperature. The upper aqueous phase was removed to a new tube and an equal volume of chloroform added to each tube and, after shaking vigorously for 15 seconds, the tubes were centrifuged at 12,000×*g* for 30 seconds at room temperature. Chloroform extractions were continued until no protein was visible at the interface between the aqueous and organic phases. The aqueous phase was then removed to a new tube and nucleic acids precipitated by addition of $1/10^{th}$ volume of sodium acetate and 2.5 volumes of ice-cold absolute ethanol followed by incubation at -20°C for at least 60 minutes. Precipitated nucleic acids were pelleted by centrifugation at 12,000×*g* for 15 minutes at 5°C, washed by addition of 500µl 75% (v/v) absolute ethanol in deionised water and centrifuged at 12,000×*g* for 15 minutes at 5°C. The supernatant

was carefully removed and the tubes air-dried, uncovered, at 37°C. Nucleic acids were resuspended in 50µl deionised water and stored at -80°C until tested using the RT-PCR protocol described in the following section.

ii) TRIZOL extraction: All virology samples are homogenised and stored as aliquots of clarified supernatant tested by these samples were also tested by virus isolation in the LCB cell line. Therefore, as TRIZOL is designed for use on solid tissue samples the extraction protocol (Life Technologies) required slight modification of the manufacturer's instructions as follows. Two hundred microlitres of clarified homogenised barramundi or LCB cell culture supernatant was incubated with 1ml TRIZOL for 5 minutes at room temperature. After addition of 200µl chloroform the tubes was shaken vigorously for 15 seconds and incubated for 3 minutes at room temperature. The phases were separated by centrifugation at 12,000 x g for 15 minutes at 5°C and the upper, aqueous phase was removed to a new tube and nucleic acids precipitated by the addition of either 500µl isopropanol followed by incubation for 10 minutes at room temperature, or addition of $1/10^{th}$ volume of sodium acetate and two volumes of ice-cold absolute ethanol. For both precipitation methods, after centrifugation at 12,000 x g for 10 minutes, the supernatant was removed and pelleted nucleic acids washed, dried, resuspended in deionised water and stored as above until tested using the RT-PCR protocol described in the following section.

iii) ROCHE High Pure Viral RNA Kit: RNA was extracted according to the manufacturer's instructions as follows. Two hundred microlitres of clarified homogenised barramundi or LCB cell culture supernatant was incubated with of 400µl poly(A) carrier RNA supplemented binding buffer and shaken for 10 minutes at room temperature. The sample was loaded into High Pure filter tube and collection tube and centrifuged for 15 seconds at 8,000xg at room temperature. The High Pure filter tube was transferred to another collection tube, 500µl Inhibitor Removal Buffer was added and the tube centrifuged for 60 seconds at 8,000x*g* at room temperature. The High Pure filter tube was transferred to another collection tube, 450µl Wash Buffer was added to the upper reservoir and the tube centrifuged for 15 seconds at 8,000xg at room temperature followed by a further centrifugation at 13,000xg for 10 seconds at room temperature. The High Pure filter tube was transferred to another collection tube and the RNA eluted by addition of 50µl Elution buffer and centrifuge for 60 seconds at 8,000xg at room temperature. RNA was removed from the collection tube and stored at -80°C until tested using the RT-PCR protocol described in the following section.

Initial RT-PCR protocol

The RT-PCR testing was conducted according to the method of Nishizawa *et al* (1994) with some previous modifications. The R3–F2 primer set (Table 7) amplifies a 426bp sequence of the nodavirus coat protein gene on the viral RNA2. RNA was incubated at 90°C for 5 minutes and cDNA was transcribed by addition of 9.5µl RNA template to 20µl RT reaction mix containing 1x M-MuLV RT buffer (Promega), 20U M-MuLV reverse transcriptase (Promega), 1.0U RNase inhibitor (Promega), 1µM reverse primer (R3), 1mM of each dNTP (dGTP, dCTP, dATP, dTTP) (Promega), 5mM $MgCl_2$ (Promega) and DEPC-treated water and incubation at 42°C for 60 minutes. The cDNA reaction was incubated at 99°C for 10 minutes and 20µl RT reaction mix was added to an 80µl PCR reaction mix containing 1x *Tth* PCR buffer, 0.2µM forward primer (F2), 2.5U *Tth* DNA polymerase (Promega), 2mM $MgCl_2$

(Promega) and deionised water. PCR amplification was achieved by incubation as follows: 1 cycle at 72°C for 10 minutes and 95°C for 2 minutes, 30 cycles of 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 40 seconds with a final extension step of 72°C for 10 minutes. Products were analysed by electrophoresis through 2% (w/v) agarose in TAE (40mM Tris-acetate, 1mM EDTA), staining with ethidium bromide and visualisation using a UV transilluminator.

Table 7: Primers and primer sequences for the nested RT-PCR

Primer name	Sequence	Reference
R3	5'-CGAGTCAACACGGGTGAAGA-3'	Nishizawa *et al* (1994)
F2	5'-CGTGTCAGTCATGTGTCGCT-3'	
NR'3	5'-GGATTTGACGGGGCTGCTCA-3'	Thiery *et al* (1999)
NF'2	5'-GTTCCCTGTACAACGATTCC-3'	

Initial nested RT-PCR protocol

Initially, the nested RT-PCR protocol was conducted according to the method of Thiery *et al* (1999), with some modifications. The NR'3-NF'2 primer set (Table 7) amplifies a 295bp region within the 426bp RT-PCR product. RT-PCR was performed as described above, except the number of amplification cycles was reduced to 25. Five microlitres of the RT-PCR reaction was added to a 95µl Nested PCR reaction mix containing 1x *Tth* PCR buffer (Promega), 2.5U Tth DNA polymerase (Promega), 15.0µM of each primer (NF'2 and NR'3), 200mM of each dNTP (dGTP, dCTP, dATP, dTTP) (Promega), 2mM $MgCl_2$ (Promega) and deionised water. PCR amplification was achieved by incubation as follows: 1 cycle at 94°C for 2 minutes, 25 cycles of 94°C for 40 seconds, 50°C for 40 seconds and 72°C for 40 seconds with a final extension step at 72°C for 10 minutes. Products were analysed by agarose gel electrophoresis as described previously.

Optimisation of RT-PCR and nested RT-PCR reaction conditions

As the quantity of viral RNA could not be determined after RNA extraction from the samples, the initial template volume added was not varied and all optimisation experiments were conducted using 9.5µl undiluted RNA in the RT reaction. The concentration of primers was optimised for the RT-PCR and the concentration of primers and $MgCl_2$ were optimised for the nested PCR. This was followed by the refinement of the entire nested RT-PCR protocol.

Nested RT-PCR optimisation

The RT-PCR positive product was purified using the High Pure PCR Purification Kit (ROCHE) according to the manufacturer's instructions. Purified DNA was diluted to 100ng, 10ng, 1ng and 0.1ng and the purity was determined by visualisation after electrophoresis through a 2% agarose gel.

Primer concentration was optimised as follows: Five microlitres of each dilution was added to five 0.2ml PCR tubes and 95µl of reaction mixture, containing 1x PCR buffer (Promega), 2mM $MgCl_2$, 200µM dNTPs (Promega), 2.5U *Tth* DNA polymerase (Promega) and NR'3 and NF'2 primers at one of the following concentrations (10µM, 8µM, 6µM, 4µM, or 2µM), was added to each tube. PCR amplification was achieved by incubation as

follows: 1 cycle at 94°C for 2 minutes, 25 cycles of 94°C for 40 seconds, 50°C for 40 seconds and 72°C for 40 seconds with a final extension step at 72°C for 10 minutes. Products were analysed by agarose gel electrophoresis as described previously.

Magnesium chloride concentration was optimised as follows: Five microlitres of each dilution was added to five 0.2ml PCR tubes and 95µl of reaction mixture, containing 1x PCR buffer (Promega), 4µM each primer, 200µM dNTPs (Promega), 2.5U Tth DNA polymerase (Promega) and $MgCl_2$, primers at one of the following concentrations (1mM, 1.5mM, 2mM, 2.5mM and 3mM), was added to each tube. PCR amplification and analysis was conducted as described previously.

Reverse Transcriptase, DNA Polymerase and One Step RT-PCR Kit evaluation

Experiments evaluating a newer reverse transcriptase (Omniscript RT), polymerase (Hot Star*Taq* Master Mix) and test format (One-Step RT-PCR Kit) were conducted in parallel on replicated samples. The Hot Start enzyme was evaluated as this type of enzyme was becoming more commonly referred to in the literature and the particular enzyme chosen was based on evaluation of different manufacturer's promotional material. The RT enzyme used was also chosen based on evaluation of different manufacturer's promotional material. The One-Tube RT-PCR format was evaluated to see how it compared with the two tube format that was currently in use. The production of amplicons, over a range of diluted templates, for each of the two steps of the nested RT-PCR (RT-PCR and nested PCR) was used to compare three sets of reverse transcriptase and DNA polymerase combinations; a) M-MuLV and *Tth* DNA polymerase; b) Omniscript RT and HotStarTaq Master Mix; and c) One Tube RT-PCR Kit and HotStarTaq Master Mix. RNA target template was extracted from a known BNNV-positive LCB cell culture supernatant sample using the High Pure Viral RNA extraction kit (ROCHE). Serial ten-fold dilutions of template, from undiluted to 10^{-7}, were prepared in deionised water in 500µl PCR tubes. Aliquots (12µl) were transferred from each dilution to 200µl PCR tubes to produce replicated sets of the diluted RNA template.

a) One set of dilutions (undiluted to 10^{-7}) were tested by nested RT-PCR using M-MuLV (RT) and *Tth* DNA polymerase as follows:

i. RNA target template was incubated at 90°C for 5 minutes and cDNA was transcribed by addition of 9.5µl RNA template to 10.5µl RT reaction mix containing 1x M-MuLV RT buffer (Promega), 100U M-MuLV reverse transcriptase (Promega), 1.0U RNase inhibitor (Promega), 1µM reverse primer (R3), 2mM of each dNTP (dGTP, dCTP, dATP, dTTP) (Promega), 2.5mM $MgCl_2$ (Promega) and DEPC-treated water and incubation at 42°C for 60 minutes.

ii. The cDNA reaction was incubated at 99°C for 10 minutes and 20µl RT reaction mix was added to an 80µl PCR reaction mix containing 1x *Tth* PCR buffer, 0.2µM F2, 2.5U *Tth* DNA polymerase (Promega), 2.5mM $MgCl_2$ (Promega) and deionised water.

iii. PCR amplification was achieved by incubation as follows: 1 cycle at 72°C for 10 minutes and 95°C for 2 minutes, 30 cycles of 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 40 seconds with a final extension step of 72°C for 10 minutes.

iv. For the nested PCR, 20µl of the RT-PCR reaction was added to a 95µl nested PCR reaction mix containing 1x *Tth* PCR buffer (Promega), 2.5U Tth DNA polymerase (Promega), 4µM of each primer (NF'2 and NR'3), 200µM of each dNTP (dGTP, dCTP, dATP, dTTP) (Promega), 2.5mM $MgCl_2$ (Promega) and deionised water.

v. PCR amplification was achieved by incubation as follows: 1 cycle at 94°C for 2 minutes, 25 cycles of 94°C for 40 seconds, 50°C for 40 seconds and 72°C for 40 seconds with a final extension step at 72°C for 10 minutes.
vi. Products were analysed by agarose gel electrophoresis as described previously.

b) Another replicate set of dilutions were tested using Omniscript RT (RT) and HotStarTaq Master Mix (DNA polymerase) as follows:
i. RNA target template was incubated at 90°C for 5 minutes and cDNA was transcribed by addition of 9.5µl RNA template to 20µl RT reaction mix containing 1x RT-PCR buffer (QIAGEN), 4U Omniscript RT (QIAGEN), 10U RNase inhibitor (Promega), 1.0µM reverse primer (R3), 2mM of each dNTP (dGTP, dCTP, dATP, dTTP) (Promega), and DEPC-treated water and incubation at 30°C for 60 minutes.
ii. The cDNA reaction was incubated at 99°C for 10 minutes and 5µl RT reaction mix was added to a 45µl PCR reaction mix containing 1x HotStarTaq Master Mix (QIAGEN), 0.2µM F2, 0.1µM R3, 2.5mM $MgCl_2$ (Promega) and deionised water.
iii. PCR amplification was achieved by incubation as follows: 1 cycle at 72°C for 10 minutes and 95°C for 2 minutes, 30 cycles of 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 40 seconds with a final extension step of 72°C for 10 minutes.
iv. For the nested PCR, 5µl PCR reaction mix was added to a 45µl PCR reaction mix containing 1x HotStarTaq Master Mix (QIAGEN), 4µM each primer (NF'2 and NR'3), 2.5mM $MgCl_2$ (Promega) and deionised water.
v. PCR amplification was achieved by incubation as follows: 1 cycle at 94°C for 2 minutes, 25 cycles of 94°C for 40 seconds, 50°C for 40 seconds and 72°C for 40 seconds with a final extension step at 72°C for 10 minutes.
vi. Products were analysed by agarose gel electrophoresis as described previously.

c) A third set of dilutions were tested using the One Step RT-PCR Kit (QIAGEN) (RT), according to the manufacturer's instructions, and HotStarTaq Master Mix (DNA polymerase) as follows:
i. RNA target template was incubated at 90°C for 5 minutes and 9.5µl RNA template was added to a 40.5µl RT-PCR reaction mix containing 1x One Step RT-PCR buffer (QIAGEN), One Step RT-PCR Enzyme Mix (QIAGEN), 10U RNase inhibitor (Promega), 0.6µM of each primer (F2, R3), 400µM of each dNTP (dGTP, dCTP, dATP, dTTP) (Promega), and DEPC-treated water.
ii. Incubation of the reaction was as follows: 50°C for 30 minutes, 95°C for 15 minutes, 30 cycles of 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 40 seconds with a final extension step of 72°C for 10 minutes.
iii. Nested PCR amplification was achieved using the HotStarTaq Master Mix (QIAGEN) follows: 1 cycle at 72°C for 10 minutes and 95°C for 2 minutes, 30 cycles of 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 40 seconds with a final extension step of 72°C for 10 minutes. For the nested PCR, 5µl PCR reaction mix was added to a 45µl PCR reaction mix containing 1x HotStarTaq Master Mix (QIAGEN), 4µM each primer (NF'2 and NR'3), 2.5mM $MgCl_2$ (Promega) and deionised water.
iv. PCR amplification was achieved by incubation as follows: 1 cycle at 94°C for 2 minutes, 25 cycles of 94°C for 40 seconds, 50°C for 40 seconds and 72°C for 40 seconds with a final extension step at 72°C for 10 minutes.
v. Products were analysed by agarose gel electrophoresis as described previously.

Barramundi broodstock spawning fluid testing by nested RT-PCR

The BNNV nested RT-PCR required further refinement when experimental testing of barramundi broodstock spawning fluids commenced. When barramundi spawning fluids were first tested using the nested RT-PCR, multiple amplicons were produced. Reduction in cycle number for each PCR, and in the template volume for the nested RT-PCR, were evaluated to reduce or eliminate the non-specific banding.

Due to the difficulty in obtaining samples, testing was performed on the cDNA that had been prepared from the extracted RNA for the first nested RT-PCR.

The effect of reducing cycling conditions for the RT-PCR and nested PCR, and the effect of reducing template volume added to the nested PCR was determined by:

i. The cDNA reaction was incubated at 99°C for 10 minutes and 5µl RT reaction mix was added to replicate 45µl PCR reaction mixes containing 1x HotStarTaq Master Mix (QIAGEN), 0.2µM F2, 0.1µM R3, 2.5mM $MgCl_2$ (Promega) and deionised water.
ii. RT-PCR amplification was achieved by incubation as follows:
    a) 1 cycle at 72°C for 10 minutes and 95°C for 2 minutes, **25 cycles** of 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 40 seconds with a final extension step of 72°C for 10 minutes **or**
    b) 1 cycle at 72°C for 10 minutes and 95°C for 2 minutes, **30 cycles** of 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 40 seconds with a final extension step of 72°C for 10 minutes.
iii. The nested PCR was determined by adding **1µl or 5µl** from the replicated RT-PCR reaction mixes to replicated 49µl or 45µl PCR reaction mixes containing 1x HotStarTaq Master Mix (QIAGEN), 4µM each primer (NF'2 and NR'3), 2.5mM $MgCl_2$ (Promega) and deionised water.
iv. Nested PCR amplification was achieved by incubation as follows:
    a) 1 cycle at 94°C for 2 minutes, **25 cycles** of 94°C for 40 seconds, 50°C for 40 seconds and 72°C for 40 seconds with a final extension step at 72°C for 10 minutes **or**
    b) 1 cycle at 94°C for 2 minutes, **30 cycles** of 94°C for 40 seconds, 50°C for 40 seconds and 72°C for 40 seconds with a final extension step at 72°C for 10 minutes.
v. Products were analysed by agarose gel electrophoresis as described previously.

Final nested RT-PCR protocol

The following protocol, incorporating the reduced PCR cycles and reduced template volume for the nested PCR, was evaluated on clinical samples and infected tissue culture supernatants.

i. RNA was extracted using the ROCHE High Pure Viral RNA extraction kit as described previously.
ii. RNA was incubated at 90°C for 5 minutes then reverse transcription was conducted at 37°C for 60 minutes in a 20µl reaction volume containing 9.5µl template, Omniscript RT buffer (QIAGEN), 4U Omniscript RT (QIAGEN), 0.5mM each dNTP, 1U ribonuclease inhibitor (Promega) and 1.0µM reverse primer (R3).
iii. PCR amplification was carried out in a 50µl reaction mix containing 5µl of the reverse transcription reaction, 0.2µM of each primer (R3, F2), 2.5mM $MgCl_2$ and 25µl HotStarTaq Master Mix (QIAGEN).

iv. Thermal cycling was conducted in an Eppendorf Mastercycler programmed as follows: 1 cycle at 95°C for 15 minutes, 25 cycles at 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 40 seconds and 1 cycle at 72°C for 10 minutes.
v. Nested PCR amplification was carried out in a 50µl reaction mix containing 1µl of the PCR reaction, 4.0µM of each primer (NR'3, NF'2), 2.0mM $MgCl_2$ and 25µl HotStarTaq Master Mix (QIAGEN).
vi. Thermal cycling was conducted in an Eppendorf Mastercycler programmed as follows: 1 cycle at 95°C for 15 minutes, 25 cycles at 94°C for 40 seconds, 50°C for 40 seconds and 72°C for 40 seconds and 1 cycle at 72°C for 10 minutes.
vii. Products were analysed by agarose gel electrophoresis as described previously.

8.2.1.2. Comparison of cell culture isolation system and RT-PCRs

Comparisons of cell culture isolation, RT-PCR and nested RT-PCR testing were conducted on clinical samples submitted to the Oonoonba Veterinary Laboratory or generated during the infectivity trial work. Nodavirus infection of these samples had been determined by either appearance of clinical signs in fish, direct exposure to the virus or histological analysis.

8.2.2. Results and discussion

8.2.2.1. Implementation of the nodavirus RT-PCR and nested RT-PCR protocol

RNA extraction

Viable RNA (i.e. resulting in a positive RT-PCR amplification) was obtained using all three extraction methods. When SDS/Proteinase K phenol/chloroform and TRIZOL were compared better yields were obtained using SDS/Proteinase K phenol/chloroform (Fig. 72). TRIZOL is designed for use on tissue and had very little scope to vary the concentrations used when the samples were clarified tissue culture supernatant. SDS/Proteinase K phenol/chloroform extraction was easier to perform as concentrations could be varied to suite the samples size. The other advantage of the SDS/Proteinase K phenol/chloroform extraction was the ability to remove almost all the aqueous phase for subsequent chloroform extraction. This was not possible with TRIZOL and if more than 75% of the aqueous phase was removed excessive co-precipitation of salts during the precipitation occurred. These salts inhibited the RT-PCR reaction. This co-precipitation of salts increased when homogenised barramundi samples were used. Changing from isopropanol to absolute ethanol/sodium acetate precipitation reduced the co-precipitation of salts when using TRIZOL but due to the variability in the quality of the extracted RNA, SDS/Proteinase K phenol/chloroform extraction was chosen as the method of choice.

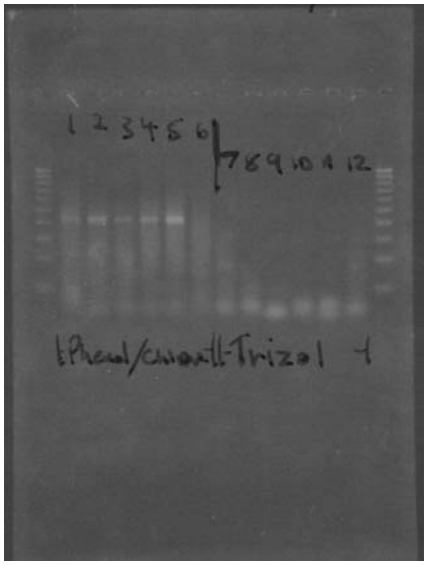

Figure 77: An example of a comparison between Phenol/Chloroform (Lanes 1-6) and TRIZOL (Lanes 7-12) for RNA extraction followed by RT-PCR.

To assess the applicability of a spin-column based kit, the ROCHE High Pure Viral RNA Kit was evaluated as, at the time, this was the only kit available designed for use on liquid samples. The quantity and quality of the RNA produced by the spin column-based kit was significantly greater than the RNA produced by both of the precipitation-based methods. Quantity and quality were arbitrarily determined by observing the presence or absence of amplicons and by the intensity of the amplicon after testing of the known BNNV-positive samples. With the precipitation-based methods, visual inspection of the precipitated RNA was undertaken, with co-precipitation of salts occasionally observed. These salts would often lead to false-negative results, as they inhibited the activity of either the reverse transcriptase and/or the DNA polymerase. While no salt contamination could be observed with the spin column-based kit, the kit had an inhibitor removal step and no false-negative results were observed. The kit was also better with regard to time and Workplace Health and Safety issues, resulted in greater confidence in the integrity of the product and was a much simpler method explain to train new staff as all the variables (e.g., volumes, spin speed and time, and reagent preparation) were standardised. The precipitation-based methods were more subjective to use and required a lot more operator expertise to obtain RNA of adequate quality and quantity. For this reason, the ROCHE High Pure Viral RNA Kit was chosen for all subsequent RNA extractions.

Optimisation of RT-PCR and nested RT-PCR reaction conditions

Nested RT-PCR optimisation

The published methods used produced amplicons in the tests run in the laboratory, optimisation further enhanced the quality of the results. While all the different primer and magnesium chloride concentrations tested resulted in the production of amplicons of the required size, the combination of primers at 4μM and magnesium chloride at 2mM led to the production of brighter, clearer amplicons. Therefore, these concentrations were used in subsequent nested PCR testing.

Reverse Transcriptase, DNA Polymerase and One Step RT-PCR Kit evaluation

Omniscript RT and HotStarTaq Master Mix consistently resulted in a more sensitive test than when the same samples were tested using either of the enzymes originally described in the methods of Nishizawa *et al* (1994) and Thiery *et al* (1999) or the One Step RT-PCR Kit. Table 8 shows the typical comparative results of nested RT-PCR of BNNV-infected cell culture supernatant. Testing using the original enzymes and the One-Step RT-PCR Kit produced a false-negative result in the RT-PCR. This was not an uncommon finding when cell culture supernatant was tested using the RT-PCR, however, as Omniscript RT and HotStarTaq Master Mix did produce a positive amplification it was concluded these were the most appropriate enzymes to use in the PCRs.

Table 8: Amplification of a PCR product (after the RT-PCR and after the second or nested PCR) when using the different reverse transcriptases (RT), DNA polymerases and a One Step RT-PCR Kit in the BNNV nested RT-PCR test on BNNV-infected cell culture supernatant.

	Presence of amplicon with three different enzymes or test format					
	RT: MuLV **Polymerase:** *Tth*		Omniscript HotStarTaq		One Step RT-PCR Kit HotStarTaq	
DNA dilution	PCR	nPCR	PCR	nPCR	PCR	nPCR
Undiluted	-	+	+	+	-	+
10^{-1}	-	+	+	+	-	+
10^{-2}	-	-	-	+	-	-
10^{-3}	-	-	-	+	-	-
10^{-4}	-	-	-	+	-	-
10^{-5}	-	-	-	+	-	-
10^{-6}	-	-	-	+	-	-
10^{-7}	-	-	-	+	-	-

Nested RT-PCR testing of the One Step RT-PCR Kit products resulted in the same level of detection as that when Omniscript RT and HotStarTaq Master Mix were used for the nested RT-PCR. This may simply be due to the increased sensitivity of the HotStarTaq Master Mix used for the nested PCR step. It was decided to use Omniscript RT and HotStarTaq Master Mix for the entire nested RT-PCR as this was more cost-effective, as fewer reagents needed to be purchased and stored, allowed greater freedom when it came to optimisation of individual test components, was a simpler test format and was significantly more sensitive at the RT-PCR stage.

Barramundi broodstock spawning fluid testing by nested RT-PCR

Reducing the number of cycles in each PCR to 25 and reducing the volume of the RT-PCR product, added to the nested PCR, from 5µl to 1µl eliminated the non-specific bands seen when broodstock spawning fluids were initially tested by the Nested RT-PCR. The changes significantly improved the quality of the result, as positive bands were much clearer and intense.

Final nested RT-PCR protocol

The final modification of the nested RT-PCR was the optimisation of the test when used to test broodstock spawning samples. The changes had no effect on the ability of the test to detect BNNV in infected larvae or tissue culture supernatants.

8.2.2.2. Comparison of cell culture isolation system and RT-PCRs

The LCB cell line was at least as sensitive as the nested RT-PCR at detecting BNNV in clinical material submitted to the laboratory. While the RT-PCR test alone never produced a false negative result when larval barramundi were tested in parallel with virus isolation in the LCB cell line, this is probably related more to the viral load in the larvae than to the sensitivity of the test. When the cell line was available for routine use, all the samples tested were associated with typical nodavirus clinical signs and obvious lesions in histological sections.

When the RT-PCR was used as a confirmatory test, false-negative results did occur (Table 9) and the nested RT-PCR was implemented for all subsequent nodavirus molecular testing requirements.

Table 9: Comparison of RT-PCR and nested RT-PCR for confirmation of BNNV identity after isolation in the LCB cell line.

Accession	Disease (Clinical/Histo)	Isolation in LCB cells	RT-PCR on LCB cells	Nested RT-PCR on LCB cells
1	✓	✓	✓	✓
2	✓	✓	✓	✓
3	✓	✓	✓	✓
4	✓	✓	✓	✓
5	✓	✓	✓	✓
6	✓	✓	×	✓
7	✓	✓	✓	✓
8	✓	✓	✓	✓
9	✓	✓	✓	✓
10	✓	✓	×	✓
11	✓	✓	×	✓
12	✓	✓	×	✓
13	✓	✓	✓	✓
14	×	×	×	×
15	×	×	×	×
16	×	×	×	×
17	×	×	×	×

8.3 Sensitivity of the cell culture isolation system to exotic nodaviruses at AAHL-FDL

Cells transformed before the cells could be sent to AAHL.

9. Benefits and adoption

Several significant findings from the project confirmed, at least experimentally, that freshwater fishes could be lethally infected by barramundi nodavirus. Further, the nodavirus appears to persist in the central nervous system tissue in some of those freshwater fish that survive the initial infection. This has the benefit of supporting current regulatory controls, required by state and territory fisheries authorities, on the movement of barramundi fry and aquaculture outside their natural range. The project outputs provide fisheries managers with further specific scientific information to justify these regulatory controls. As outlined in the project application, maintenance of these controls provides a level of protection from disease for endemic freshwater fishes. This will benefit the natural fauna and fishers who utilise the freshwater habitat for recreation.

The diagnostic benefits of cell culture isolation and nodavirus detection using RT-PCR, particularly nested RT-PCR, were demonstrated using clinical specimens with VNN and broodstock gonad samples. But the project also gave an insight into the difficulties of developing sustainable *in vitro* cell culture isolation systems that will support the growth of BNNV. One of the benefits has been national recognition that more R&D is required to refine diagnostic tests for nodaviruses and to develop reagents in Australia for use in Australian aquatic animal health laboratories. Following this project a diagnostic test development project on nodavirus was funded under the Commonwealth Government's new budget initiative, 'Building a National Approach to Animal and Plant Health', to improve diagnostic techniques for important aquatic animal diseases. The results of the infection experiments were presented to the National Fish Pathologists meeting in Tasmania, 2002. Histological sections showing the VNN lesions produced in the freshwater fishes were shown to that meeting. This provided an opportunity to fish pathologists from around Australia to familiarise themselves with the range of lesions associated with VNN in different species of fish.

Information on the susceptibility of native freshwater fishes has been presented at several Australian Barramundi Farmers Association annual meetings and to fish stocking committee meetings. The evidence that a barramundi viral disease may be able to affect other fishes of recreational significance has meant, at least in Queensland, an increased awareness of the disease risks associated with fish translocations. Routine histological examination to detect disease in batches of barramundi and other hatchery produced freshwater fish fry has been adopted as a risk management strategy before fry are stocked into lakes and impoundments.

10. Further Development

The need for further development and refinement of diagnostic and detection tests for BNNV and other nodaviruses, the need for validation of these tests across a range of clinical materials in Australia and the need to produce reagents locally was recognized nationally. This lead to in a new project, FRDC 2001/626 "Aquatic Animal Health Subprogram: development of diagnostic tests for the detection of nodavirus". The nodavirus diagnostic test development project is funded under the DAFF Commonwealth Government's New Budget Initiative, 'Building a National Approach to Animal and Plant Health' to improve diagnostic techniques for important aquatic animal diseases. The objectives of FRDC 2001/626 cover three areas. To optimize and validate the nested RT-PCR for the detection of endemic and exotic nodaviruses from a range of tissue samples and fish species. To attempt to establish a cell line which can be used to isolate endemic and exotic nodaviruses from a range of fish species. To produce immunodiagnostic tests capable of localizing nodaviruses in fish tissues and cell cultures. As part of FRDC 2001/626 the developed detection test technology will be distributed to aquatic animal health laboratories around Australia and a draft Australian and New Zealand Standard Diagnostic Procedure for the detection of nodaviruses will be prepared.

In the continued absence of a readily available cell culture isolation system for nodaviruses in Australia, the sensitivity of the nested RT-PCR to detect BNNV in a range of tissues offered an applicable method to investigate options in commercial barramundi hatcheries to screen fish to produce nodavirus-free fry. Industry supported this concept as it had the potential to remove the risk of VNN outbreaks and the resulting lack of fry for on-growing. With the findings that freshwater fishes were experimentally susceptible to BNNV, there remained the question of spread of BNNV to fish in natural ecosystems. QDPI has been releasing hatchery-reared barramundi fry in impoundments and rivers in northern Queensland to enhance recreational fishing opportunities for some time now. The nested RT-PCR is a detection test that could be used to determine the natural distribution of BNNV in wild barramundi and freshwater fishes, and if the release of hatchery-reared barramundi had influenced the prevalence of BNNV in these wild populations. A project, FRDC 2002/043 "Aquatic Animal Health Subprogram: the production of nodavirus-free fish fry and the nodaviruses natural distribution", which addressed these two aspects has been funded by the Fisheries Research and Development Corporation.

11. Conclusion

The translocation of any live fish, particularly if the fish are to be released into open water systems or on-grown in culture facilities, carries with it the risk of also transferring a disease or pathogen to the new aquatic environment. That culture of barramundi outside its natural range could expose native freshwater fishes to lethal barramundi nervous necrosis virus (BNNV) infections was a specific concern for fisheries authorities faced with licensing of barramundi aquaculture operations. Glazebrook (1995) apparently demonstrated lethal BNNV infections in 2-3 month old Macquarie perch, silver perch and Murray cod. The development of the barramundi brain cell line that supported the *in vitro* growth of BNNV provide the opportunity to do further infection studies to determine species susceptibility.

The first objective of the project was to establish a standard infection model for barramundi nodavirus in barramundi larvae and fry. It was concluded a bath exposure to BNNV by adding clarified infected tissue homogenate or infected tissue culture supernatant (ITCS) to a reduced volume of aquarium water containing fish for one hour, then refilling the aquarium, was an effective infection model. Direct injection of ITCS was, as expected, an effective model to deliver BNNV into the experimental fish. The infectivity of BNNV in tissue culture supernatant was shown to be the same as BNNV extracted from diseased barramundi fry. An experimental bath transmission was achieved in 10 week old barramundi fry, although the pathogenicity was reduce as compared to younger barramundi. A bath infection was successful in freshwater, indicating BNNV may be able to spread from fish to fish in freshwater environments. A standard bath and injection infection method was established and used in the BNNV infection experiments on four species of freshwater fishes.

The project demonstrated that the infection of four species of freshwater fishes silver perch (*Bidyanus bidyanus*), golden perch (*Macquaria ambigua*), sleepy cod (*Oxyeleotris lineolatus*) and Barcoo grunter (*Scortum barcoo*) by a nodavirus from barramundi with viral nervous necrosis (VNN), grown in a barramundi brain cell line, was possible. The virus caused mortalities when ITCS was injected into the peritoneal cavity of the experimental fishes. The mortality over 28 days for six and 12 week old silver perch was 46.2% and 25.0%; for golden perch, 6.4% and 14.5%; for sleepy cod, 98.7% and 92.3%; and for Barcoo grunter 4.8% and 7.3% respectively. This compared to 44.3% for six week old and 22.5% for 12 week old barramundi fry in similar trials. The difference in the level of lethal effects of BNNV in different fish species is not unusual. Strain differences between nodavirus isolates have been defined by nucleotide sequence and serotype differences. Strain differences from cross-infectivity studies have also been reported where a nodavirus isolate can be a non-lethal infection in one fish species but pathogenic to another species. It can be concluded that the effect of BNNV on fishes can range from being limited (for example in Barcoo grunter) to being highly lethal (for example sleepy cod). This will be significant when management of disease risk on fish translocation is considered.

The histopathology of central nervous system tissues observed in moribund freshwater fishes and survivors was very similar to that described from other species with clinical VNN. Vacuolation and necrosis of neuronal cells was seen in the retina, brain and spinal cord. Of note was the consistent presence of degenerate mononuclear cells in the posterior compartment of the eye. Even if there were only one or two vacuolated retinal cells, there would be a few cells in the vitreous humor. Some differences were noted in the sleepy cod fry where the neuronal lesions were not always typically vacuolar. A more acute necrotic process, with cytoplasmic and nuclear debris remaining in the space previously occupied by

the necrotic cell, was apparent in the brain of moribund fish. Melanin-like pigment granules in vacuoles or foci of necrotic cells, aggregations of large pale cells and foci of small mononuclear cells with scant cytoplasm were present in some of the surviving freshwater fishes. These lesions are similar to those described from other species of fishes that had non-lethal nodavirus infections. Fish appear to be able to repair neuronal damage caused by nodavirus. The persistence of BNNV at Day 28 post-infection (PI) was confirmed when all four species of the surviving fishes were tested with the nodavirus nested RT-PCR.

While BNNV infections were demonstrated experimentally, the transmission of infection by a bath challenge was not achieved in the freshwater fishes. The bath infection method would have been closer to the natural route of infection. This failure may be due to the age of the fish and, or the concentration of the BNNV used in the trials. It was clear from the barramundi infection model studies in this project, and other published research, that the older the fish the less pathogenic the nodavirus infection. It would be desirable to repeat the bath infection trials on freshwater fishes less than 6 weeks of age. With the transformation of the barramundi brain cell line (LCB), and its loss of susceptibility to BNNV infection, we were not able to titrate the virus to standardise infectious dose. This meant that the experimental infections were not directly comparable. The differences in mortality of six and 12 week old fishes at 28 days PI probably demonstrated this dose effect, for example 6 week old golden perch had a 6.4% mortality while at 12 weeks the measured mortality was higher at 14.5%. Again it would be desirable to have a nodavirus susceptible cell line so that titration of BNNV could be done to determine dose effects. Despite the failure to demonstrate a bath transmission, the fact that BNNV can replicate and persist in the freshwater fishes, and that experimental infection of barramundi fry was possible in a freshwater bath, implies there is a continuing need to consider the risk BNNV can pose to freshwater fishes when barramundi fry are moved to environments outside their natural range.

The LCB cells allowed the production of BNNV for the infection trials and gave a 100% isolation of BNNV when fish samples with VNN were tested. The transformation of the LCB cells meant that the third objective to determine BNNV susceptibility to environmental and disinfectants could not be completed. Similarly the complete evaluation of the LCB cells to isolate BNNV in asymptomatic carrier fish and exotic nodaviruses was not achieved (objective four). It was clear that the development of another susceptible cell line would be highly desirable. This was recognized nationally and further research is now underway in FRDC project 2001/626 “Aquatic Animal Health Subprogram: development of diagnostic tests for the detection of nodavirus”. Significant progress was made in the implementation and refinement of the RT-PCR and nested RT-PCR detection tests for nodavirus. In this work it was concluded the nested RT-PCR was at least as sensitive as virus isolation on LCB cells but more sensitive than the one step, RT-PCR. The use of the nested RT-PCR also confirmed the persistence of BNNV in surviving experimental freshwater fishes, a correlation with histopathological findings. The use of the nested RT-PCR is recommended for all nodavirus molecular detection testing in the future. It was concluded further refinement of the nested RT-PCR is required with regard to sensitivity of detection of other nodavirus isolates, the type of tissue analysed and the mass of the sample tested.

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Appendix 1: Intellectual Property

There is no specific intellectual property arising from this project. All information generated is in the public domain.

Appendix 2: Staff List

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