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aquatic animal health subprogram

The production of nodavirus-free fish fry and the nodaviruses natural distribution

I G Anderson and H J Oakey

September 2008

FRDC Project No. 2002/043

Australian Government
Department of Agriculture, Fisheries and Forestry

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Department of Primary Industries and Fisheries

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Fisheries Research and Development Corporation

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This publication has been compiled by I G Anderson and H J Oakey of Biosecurity Queensland, DPI&F.

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Table of Contents

2002/043 The production of nodavirus-free fish fry and the nodaviruses natural distribution

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OBJECTIVES:

1. To determine the natural level of nodavirus infection in wild barramundi.
2. To determine the presence or absence of nodavirus infections in freshwater fish species.
3. To describe the relatedness of any nodaviruses isolated from freshwater fishes.
4. To define the best testing protocol in barramundi hatchery production systems to ensure nodavirus-free fry production.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED TO DATE

This project has created the knowledge that inapparent nodavirus infections exist naturally in wild barramundi populations in north Queensland with, on average, 23.9% of adult barramundi carrying the virus. This baseline information will assist future nodavirus survey design and allow future evaluation of impacts on barramundi populations in regard to nodavirus infections, one measure of population health. The project has shown that stocking hatchery-reared barramundi fry in the Johnstone River has not resulted in any inapparent nodavirus infections in freshwater fishes; neither did stocking increase the natural level of inapparent nodavirus infections in wild barramundi. This knowledge supports previous opinions that it is likely the presence of stocked barramundi have minimal disease effects on freshwater fishes in open water systems.

The project failed to confirm a testing protocol that would ensure the production of nodavirus free barramundi fry. The successful application of a sensitive molecular detection test for nodavirus to screen captive barramundi broodstock and their progeny would have created a protocol to give a high assurance that barramundi fry had no nodavirus at all. The project has created useful knowledge that will help to direct alternative detection test application and sampling protocols with the aim to produce nodavirus-free barramundi fry.

Need

Nodavirus infections of finfish have become a significant issue in marine aquaculture in recent years. Viral nervous necrosis, the lethal disease caused by nodavirus, was first reported in a barramundi hatchery in 1990. Viral nervous necrosis outbreaks can cause the death of up to 100% of fish larvae. Outbreaks continue to be reported in barramundi from hatchery larval rearing tanks or ponds in Queensland, Western Australia, Northern Territory and South Australia. There is a need to reduce the economic impact of this

disease to commercial barramundi hatcheries. Archer fish, striped trumpeter, snapper, barramundi cod, estuary cod and flowery cod in Australian hatcheries have also been reported with nodavirus infections and affected by viral nervous necrosis. The movement of barramundi fry produced in hatcheries outside of the natural range of barramundi became a concern in the 1990s. There was a concern that the barramundi nodavirus could be carried in apparently healthy, asymptomatic barramundi fry as an inapparent viral infection. The risks that this nodavirus could affect the local freshwater fishes led to restrictions on movement of barramundi fry outside the natural range and strict aquaculture license conditions. Even in north Queensland, where authorities consider nodavirus an endemic natural infection of wild barramundi, there was concern that stocking hatchery-reared barramundi fry could affect the level of nodavirus in wild fishes in the receiving waterways. There was a need to determine if stocking had any effect on natural nodavirus levels (prevalence) in wild fish populations.

As there had been several nodavirus diagnostic test development projects in recent years this project had access to a sensitive molecular detection test (a nested RT-PCR) that could detect very low numbers of nodavirus in tissue samples. This technology utilises a precise molecular key (two primers) to recognise the nodavirus genetic material and two stages of the polymerase chain reaction (PCR) to amplify a specific molecular product so that a positive sample can be easily determined in the laboratory. The use of this test allowed us to determine the natural level of nodavirus in wild barramundi then compare this nodavirus prevalence in barramundi from pristine rivers and those in a river where stocking of hatchery-reared barramundi had occurred. Similarly there was a need to determine if there had been any spread of barramundi nodavirus to freshwater fishes in the stocked river. Finally the molecular detection test was to be used to screen captive barramundi broodstock and their progeny in hatcheries. As the current approach for prevention of viral nervous necrosis has been to exclude nodavirus from larval cultures, the aim was to define a testing protocol that would detect the virus in broodstock and exclude them and their progeny from the hatchery. This would ensure nodavirus-free fry production. This would meet the need to reduce hatchery losses due to nodavirus and remove any concern with regard to nodavirus spread to freshwater fishes when the barramundi fry were moved.

Wild barramundi and freshwater fish nodavirus prevalence

Gonad tissue (testes and ovaries) from adult wild barramundi were collected from three pristine rivers in the Gulf of Carpentaria (Mitchell River, Staaten River and Flinders River) and from the Johnstone River on the east coast of north Queensland. The Johnstone River has been stocked with hatchery-reared barramundi fry. At no time were barramundi killed just for this project, the fish were commercially caught or collected in conjunction with other Queensland fisheries resource monitoring or fisheries research activities. As many of the samples were collected in remote locations and stored frozen until delivered to the laboratory selected samples were checked for degradation. On each occasion we found sample quality was sufficient to ensure accurate test results. At the end of the project 94 barramundi from the Mitchell River, 85 from Staaten River, 41 from Flinders River and 64 from Johnstone River had been sampled and the gonad tissue tested. 17.2% of the Johnstone River barramundi were nodavirus test positive while the average nodavirus test prevalence for Gulf barramundi from the three rivers was 25.9%. Nodavirus prevalence varied widely from river to river in the Gulf. The reason for this is unknown, perhaps there were differences between the different rivers or there was an affect from the year the samples were taken.

Live bait fish were collected with Queensland fisheries resource monitoring teams using electro-fishing in three rivers in north Queensland; the Daintree, Johnstone and Herbert Rivers. The smaller bait fish were targeted as we wanted to lethally sample brain and eye tissue from 150 individuals of at least four different species. The project met its objectives by testing five species; mouth almighty, silver biddy, carp gudgeon, rainbow fish and bassies. All the fish tested negative to nodavirus, including all the fish collected from the Johnstone River. It does not appear that the stocking of hatchery-reared barramundi fry caused an increase in nodavirus prevalence in barramundi or that nodavirus was transmitted to the wild freshwater fishes.

Production of nodavirus free barramundi fry

Barramundi larvae most commonly become infected by nodavirus via their parents. The nested RT-PCR was used to detect nodavirus in gonad material or reproductive tract fluid obtained by cathetization of the vent of broodstock barramundi at the start and end of the breeding season. The project directly involved the captive broodstock populations of three commercial hatcheries and one government centre (until the end of the summer of 2003/2004). The hatcheries sent live samples of larvae after hatching and fry when they were harvested from the larval rearing tanks or ponds. This part of the project was not intended as a close investigation of nodavirus biology in barramundi. The aim was to apply a sensitive molecular detection test to samples collected in a manner that was likely to be applicable and acceptable to a commercial barramundi hatchery. The aim was that interpretation of the test results would indicate those points where screening would allow identification and elimination of the source(s) of nodavirus for the fry.

No trend for increased test prevalence was seen from the start of the breeding season to the end. Of the 438 individual broodstock tested 9.8% were test positive at one or more tests. While many of the broodstock were consistently test negative, the sequential testing of individual broodstock revealed that 18 fish were test negative on the first test then test positive at a subsequent test. Neither the test status of the spawned broodstock nor the test status of the broodstock tank population predicted the test status of either the larvae or fry. Neither was there any correlation between the test status of the progeny as larvae and as fry. Tank or pond rearing did not appear to have any effect on the fry nodavirus test status. Over all the nodavirus nested RT-PCR test has not revealed consistent results which could be the basis of recommendations for an effective testing protocol for nodavirus-free fry production. More research is required on the biology of nodavirus infections in barramundi, particularly the fate of the virus in asymptomatic adults. A detailed study on the pathogenesis of nodavirus infections would provide information to support the timing of sample collection and tissues to target in screening programs.

KEY WORDS: Barramundi, betanodavirus, nodavirus, viral nervous necrosis, viral encephalopathy and retinopathy, barramundi, freshwater fishes, virus testing, nested RT-PCR, prevalence, viral screening, blood, gonad material, larvae, fry, hatchery.

2. Background

Nodavirus infections of finfish have become a significant issue in marine aquaculture in recent years. Viral Nervous Necrosis (VNN) or Viral Encephalopathy and Retinopathy (VER), the disease caused by nodaviruses, has now been recorded in more than 25 different species, across all continents except Africa (see the reviews; Munday and Nakai, 1997, Munday, Kwang and Moody, 2002 and also the papers of Curtis *et al.* 2001; Starkey *et al.* 2001; Barker *et al.* 2002; Johnson *et al.* 2002; Oh *et al.* 2002; Chi *et al.* 2003; Gomez *et al.* 2004). Typically the disease caused by nodaviruses affects intensively reared larvae and fry of marine finfish. The clinical signs recorded vary somewhat depending on species but relate to the damage to the brain and retina, that is to say anorexia (an effect of blindness), abnormal swimming, over-inflation of the swim bladder and changes in body colour. In larval epizootics, mortalities of up to 100% cause problems for hatchery productivity and the regular supply of fingerlings for on-growing. If disease is seen in fingerlings total mortalities are lower. The severity of the disease has resulted in VNN being listed as a reportable disease on the Australian "National List" and any disease in Australia is reported quarterly.

While high level mortalities in intensive larval cultures are the common effect of fish nodaviruses, 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 seabass (*Dicentrarchus labrax*) due to VNN. This significant mortality was associated with elevated seawater temperatures. Year old European seabass averaging 154 grams were reported with VNN in a sea cage when water temperatures were 12.5°C (Mladineo 2003). A syndrome of VNN where there was also over-inflation of the swim bladder was reported in 170-1,850 gram sevenband grouper (*Epinephelus septemfasciatus*), with one farm having a cumulative mortality of about 40% over six weeks (Fukuda *et al.* 1996). These reports emphasise 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.

A nodavirus from barramundi (*Lates calcarifer*) larvae was first described in Australia in 1990 (Glazebrook, Heasman and de Beer 1990) following several years where high mortalities were seen in the first few weeks of experimental, intensive larval culture. VNN epizootics had a significant effect on the one commercial barramundi hatchery operating at that time, but were rare in government hatcheries. Changes in husbandry and improved hygiene practices in the hatchery 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 no longer 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. DNA sequence analysis of amplicons suggested that the sleepy cod nodavirus was slightly different than barramundi nodavirus (Moody *et al.* 2008). 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. Outbreaks of VNN continue to be reported in barramundi from tank and pond larval rearing systems in Queensland, with two or three cases in 2003, 2004, 2005 and 2006, and also in Western Australia, Northern Territory and South Australia during 2004 (NACA 2004a, b, c and d).

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/VNN. As a result the Australian Barramundi Farmers Association (ABFA) had raised concerns with the Queensland Department of Primary Industries and Fisheries (DPI&F) about the risk of hatchery-reared barramundi being stocked into impoundments and rivers. This industry sector still requires access to the barramundi fishery to collect replacement adult males for their breeding programs at times. There is an assumption that some of the stocked barramundi fingerlings could have asymptomatic nodavirus infections and the stocking could increase the prevalence of nodavirus in wild populations of barramundi. This may impact the nodavirus-status of collected broodstock or provide a source of virus in the hatchery/pond intake water and subsequently increase the prevalence of VNN in cultured barramundi larvae.

During the 1990s there was an expansion of barramundi aquaculture to South Australia and New South Wales. At that time the risk of nodavirus translocation and the viruses' effect on endemic freshwater fishes became an issue. Glazebrook (1995) apparently demonstrated that two to three month old Macquarie perch (*Macquaria australasia*), silver perch (*Bidyanus bidyanus*) and Murray cod (*Maccullochella peelii peelii*) could be lethally infected by barramundi nodavirus. 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 licence 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. State fisheries managers and recreational fishers have concerns about the risk barramundi nodavirus poses to fishes in the Murray-Darling catchment.

In 1999 FRDC funded a project (Project No. 99/205) on nodavirus at the Tropical and Aquatic Animal Health Laboratory (TAAHL), DPI&F Townsville (Anderson and Moody 2005). This followed the development of a barramundi cell line which supported the *in vitro* growth of barramundi nodavirus. The cell line provided, amongst other benefits, the opportunity to amplify and produce single virus preparations for transmission studies. Transmission trials at TAAHL using freshwater fishes at 6 and 12 weeks of age showed that, while bath transmission was not possible, silver perch, golden perch (*Macquaria ambigua*), sleepy cod (*Oxyeleotris lineolatus*) and barcoo grunter (*Scortum barcoo*) were

susceptible to VNN if nodavirus was injected. Interestingly, sleepy cod were highly susceptible with up to 99% dead at 28 days post-infection. Nested RT-PCR analysis of surviving fish tissues indicated viable infections were established without disease in some of these experimental fishes. Other trials also showed that short-term bath exposure of 20 day old barramundi in freshwater, with cell culture nodavirus added to the freshwater, successfully transmitted VNN. That is to say, at least experimentally, barramundi nodavirus can be transmitted in freshwater. While these results do not confirm natural transmission of barramundi nodavirus to freshwater water fishes is possible (bath transmission of nodavirus was not successful), the nodavirus does replicate in their tissues and fish younger than 6 weeks may still be susceptible to infection via virus-contaminated water.

The VNN epizootic in sleepy cod and the results from FRDC Project 99/205 continue to support the need for an ongoing consideration of translocation of nodaviruses and possible effects on freshwater fishes. New species records of VNN in Australia continue to increase as more marine finfish are used in experimental aquaculture. VNN has now been recorded in archer fish (*Toxotes jaculatrix*) (TAAHL case no. 01-41913), striped trumpeter (*Latris lineate*) (Munday, Kwang and Moody 2002), snapper (*Pagrus auratus*) (R. Reuter, pers. comm.), barramundi cod (*Cromileptes altivelis*), Australian bass (*Macquaria novemaculeata*), estuary cod (*Epinephelus coioides*), mulloway (*Argyrosomus hololepidotus*) and flowery cod (*Epinephelus fuscoguttatus*) larvae and fry (NACA 2004b; NACA 2004c; NACA 2005a; NACA 2006). PCR testing of fish tissues for nodavirus have returned positive results for a further six species; trumpeter whiting (*Sillago maculate*), sea mullet (*Mugil cephalus*), sea bream (*Acanthopagrus australis*), flat-tailed mullet (*Liza argentea*), luderick (*Girella tricuspidata*) and yellowtail kingfish (*Seriola lalandi*) (Moody, pers. comm.; NACA 2004b; NACA 2005b). The lack of epidemiological information on the prevalence of nodavirus in wild populations remains a constraint on the development of appropriate policy for managing real risks in fisheries. Does the stocking of hatchery-reared barramundi fry affect nodavirus prevalence in wild barramundi? Do Australian freshwater fishes have their own strains of nodaviruses? Are different primers required to pick up these strains of nodavirus? Little or no relevant information in these areas can be extrapolated from overseas publications, as there has been little or no research.

There are few reports on nodavirus prevalence (the percentage of healthy fish asymptomatically infected) in wild finfish. Different detection methods are used to report these prevalence estimates and they may not all be directly comparable. Mushiake *et al.* (1992) detected nodavirus antibodies in the serum of 65% of wild-caught striped jack (*Pseudocaranx dentex*) broodstock in a Japanese hatchery. Breuil *et al.* (2000) reported that 17% of wild European seabass were seropositive to nodavirus. Nodavirus was isolated in cell culture from the brain of one out of 440 (0.23%) winter flounder (*Pleuronectes americanus*) from Passamaquoddy Bay, Canada (Barker *et al.* 2002). Gomez *et al.* (2004) sampled and tested (by the nested RT-PCR) the brains from 111 wild marine finfish (19 different species) in Japan. Of those species where 10 or more individuals were tested, the nodavirus prevalence ranged from 100% in the Japanese parrot fish (*Oplegnathus fasciatus*) to 83% in the Japanese jack mackerel (*Trachurus japonicus*). Preliminary information from TAAHL produced during FRDC Project 99/205 has found a wide variation in nodavirus prevalence in captive broodstock barramundi populations (mostly originating from the wild) – from 5% to around 30% positive to the nodavirus nested RT-PCR.

Other than the results from FRDC Project 99/205, there were no published reports of VNN in fish reared in fresh water when this project began. Ikenaga *et al.* (2002) reported an experimental infection where the injection of nodavirus into the eye of freshwater angel fish (*Pterophyllum scalare*) resulted in immunohistochemistry detection of nodavirus in the retina and brain; confirming, as we did in our experiments, that nodavirus from a marine finfish can replicate in fresh water fish tissues. European seabass reared in freshwater developed VNN 8 months after transfer from a sea water hatchery (Athanassopoulou *et al.* 2002). Subsequently sturgeon (*Acipenser gueldestaedi*) on the same freshwater farm also developed VNN (Athanassopoulou *et al.* 2004). Chi *et al.* (2003) reported VNN in European eels (*Anguilla anguilla*) (30% mortality) and Chinese catfish (*Parasilurus asotus*) (100% mortality) on a freshwater farm. They describe how these farms in Taiwan reared multiple species including euryhaline species such as barramundi. This raised the possibility that the transferred seawater fish were the source of infection for freshwater fishes. Similarly Hedge *et al.* (2003) detected nodavirus infections in freshwater guppy (*Poicelia reticulate*) farmed in Singapore. Nucleotide sequence analysis determined that this nodavirus was identical to the nodavirus from greasy groupers, a marine finfish. While they reported no evidence of direct spread from the marine environment to the guppys, the possibility of a marine origin for the guppy nodavirus remained. These more recent reports all confirm the possibility that natural outbreaks of VNN and nodavirus infections can occur in farmed freshwater fishes. Also there is the suggestion that spread of nodavirus can occur between different species of fish in the fresh water ecosystems.

Detection tests for nodaviruses have undergone a continuous development in the past 5 to 10 years. The traditional tool of cell culture isolation systems had not been readily available in Australia (Frerichs *et al.* 1996; Lai *et al.* 2001; Chang *et al.* 2001; Lai *et al.* 2003). The barramundi cell line developed at TAAHL has now transformed at passage 36 and no longer expresses CPE after inoculation with the nodavirus (Moody, Horwood and McHardy 2004). 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) and real time PCR amplification have been published and some have been applied in Australia. For most of these detection technology areas further refinement can be proposed, as can the need for validation across a range of clinical material in Australia and a need to produce reagents locally. This has been recognised nationally. A nodavirus diagnostic test development project was funded under the Australian Government Federal Budget Initiative *Building a National Approach to Animal and Plant Heath*. This FRDC project 2001/626 ("Aquatic Animal Health Subprogram: development of diagnostic tools for the detection of nodavirus") made further refinements to the nested RT-PCR, produced polyclonal antibodies against a recombinant nodavirus coat protein and used these antibodies in an IHC and IFAT test. Attempts to develop new cell culture isolation systems were not successful (Moody, Horwood and McHardy 2004). The current project collaborated with FRDC project 2001/626 to apply the best practice refinements in the nested RT-PCR test and tissue sample selection. The national commitment to ensuring nodavirus detection technologies is available and applicable to Australian laboratories has continued with DAFF investment in 2006 in a consultancy to compare the ANZSDP for detection of VNN with commercial diagnostic PCR kits. A major ARC project titled "Biotechnology and epidemiology to control nodavirus in barramundi aquaculture" has begun at the University of Sydney where ELISA detection technologies will be developed and refined.

Strategies to control VNN have been based on somewhat limited epidemiological information. Current efforts to control the disease have focused on early detection of the virus in broodstock and subsequent elimination of the infected fish and their eggs and larvae. Essentially the approach has been to exclude the virus from larval cultures. There is evidence that the source of nodavirus infections of larvae is a broodstock carrier, that is to say vertical transmission, in striped jack (Arimoto *et al.* 1992) and Atlantic halibut (*Hippoglossus hippoglossus*) (Grotmol and Totland 2000). It has been assumed this is true for all fish species, although Mladineo (2003) found that 60% of adult European seabass had nodavirus in brain tissue, 40% in retina and 9% in liver but there was no nodavirus specific immuno-staining in gonad tissues. Munday, Kwang and Moody (2002) pointed out that nodavirus entry to a culture system may be from influent water containing fish shedding the virus. Nerland *et al.* (2007) report the detection of nodavirus in water during a VNN outbreak in Atlantic halibut larvae/fry using ultracentrifugation and RT-PCR. Studies on virus tissue distribution have detected nodavirus in the intestine, liver and kidney of diseased larvae (Grotmol *et al.* 1999; Chi, Lo and Lin 2001), in the kidney, liver and lower intestine of experimentally infected juvenile Atlantic halibut (Grove *et al.* 2003) and in the intestine and stomach of adult striped jack (Nguyen *et al.* 1997). It is not known if nodavirus is shed from the digestive tract or in urine, but contamination of water may occur by this route. In their report of the first record of VNN in North America, Curtis *et al.* (2001) concluded that the source of the nodavirus infection in white seabass (*Atractoscion nobilis*) fry was probably the water supply. Castric *et al.* (2001) found asymptomatic cultured sea bream (*Sparus aurata*) were infected with a nodavirus lethal to European seabass. They reported an infection was transmitted via water to healthy seabass after they were placed in a floating cage with sea bream infected with the nodavirus. Japanese researchers have more recently observed VNN in sevenband grouper often occurs a few weeks after nodavirus-free fish are transferred to sea cages (Gomez *et al.* 2004). Their research found that of the 111 wild marine fish sampled (brain tissue from 19 different species) 98% were nested RT-PCR positive for nodavirus. They considered this provided support for their suspicion that the VNN outbreaks in the grouper were caused by horizontal transmission from wild fishes. Initial reports found that live food organisms used to rear marine finfish did not carry nodavirus (Skliris and Richards 1998; Dalla Valle *et al.* 2000) but Chi *et al.* (2003) reported positive tests when a nested RT-PCR was used on *Artemia* nauplii, copepods and shrimp collected from marine finfish hatcheries. This suggests that water and live feeds are a possible pathway for horizontal infection in addition to vertical transmission.

Disinfection of eggs has been evaluated as a method to establish a hygiene barrier between the rearing water of the broodstock, including ovarian fluid, and the rearing water of the larvae by killing extracellular nodavirus. Only ozone appears to be an effective and practical egg disinfectant with 0.1mg 0_3/l for 2.5 minutes (Arimoto *et al.* 1996), 0.5mg 0_3/l for 5 minutes (Watanabe *et al.* 1998) and 4mg 0_3/l for 0.5 minutes (Grotmol and Totland 2000) recommended for different species. Disinfection of seawater used to hatch or rear larvae has also been integrated into control programs (Watanabe *et al.* 1998). Recent studies have revealed Atlantic halibut nodavirus is much more resistant to ultra-violet (*UV*) irradiation or ozonated seawater than previously described (Liltved *et al.* 2006). The results of that research suggest considerably higher Total Residual Oxidants concentrations and times of exposure are required to ensure seawater contains no viable nodavirus.

Vaccination may be a possible control strategy. Intra-muscular injection of recombinant nodavirus capsid protein and formalin inactivated nodavirus have been found to be protective in experimental infections and natural infections (Husgard *et al.* 2001; Tanaka *et al.* 2001; Yamashita *et al.* 2005). Further, Hegde *et al.* (2005) have shown that guppies bath immunised with formalin inactivated nodavirus or recombinant capsid protein produced a neutralising antibody against the nodavirus. Whether this immunity is protective or not requires further studies. The problems of economic vaccine delivery (injection would only be viable for broodstock), the effectiveness of achieving a protective immunity with early life-stage vaccination (if larvae are to be protected) and the effectiveness of vaccinating broodstock already infected all need to be determined. An effective vaccine would need to become widely available before vaccination can be shown to prevent VNN in larvae or fry (Bergh *et al.* 2001).

The method of screening broodstock so that nodavirus-infected (carriers) individuals can be removed from the breeding program has had close attention. ELISA tests to detect antibodies to nodavirus in serum can be used (Mushiake *et al.* 1992; Breuil and Romestand 1999; Breuil *et al.* 2000; Watanabe, Nishizawa and Yoshimizu 2000; Huang *et al.* 2001). 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, Nishizawa and Yoshimizu (2000) found that only barfin flounder broodstock with ELISA antibody titres >40 returned a positive RT-PCR test result. Thus ELISA tests are recommended for use in conjunction with RT-PCR detection tests (Breuil and Romestand 1999; Breuil *et al.* 2000; Watanabe, Nishizawa and Yoshimizu 2000). A more sensitive antigen capture ELISA using monoclonal antibodies against nodavirus have now been reported which may be useful in screening programs (Shieh and Chi 2005). Similar problems with sensitivity have also been reported for the molecular detection test, the one-step RT-PCR (Nishizawa, Muroga and Arimoto 1996). Five of seven tanks of larval striped jack had VNN even though broodstock tested negative to RT-PCR testing of spawning fluids.

As a consequence of detection test sensitivity the strategies to control VNN that have been published recommend repeated broodstock testing (Mori, Mushiake and Arimoto 1998; Watanabe *et al.* 1998). These protocols list testing of broodstock with ELISA on serum and RT-PCR on spawning fluids (both need to be negative) prior to spawning, disinfection of eggs and larval rearing water with ozone and testing of larvae every five days for sixty days using RT-PCR. Few commercial hatcheries in Australia would have the infrastructure or laboratory access that would make this approach easily achievable. While there are several marine finfish breeding programs in Australia that would be interested in nodavirus-free fry production, none as yet have successfully applied detection testing to ensure production of nodavirus-free fry.

The higher level of sensitivity of the two-step or nested RT-PCR, estimated at 100 times greater than the one-step RT-PCR (Thiery, Raymond and Castric 1999; Dalla Valle *et al.* 2000) and by 1,000 times (Moody, Horwood and McHardy 2004), may mean broodstock screening will result in all nodavirus-infected individuals being detected. When we ran a comparison of the RT-PCR (Nishizawa *et al.* 1994) and the nested RT-PCR (Thiery, Raymond and Castric 1999 – with some modifications) on barramundi spawning fluids, one set of thirteen samples were all negative to the one-step RT-PCR but eight were positive to the two-step nested RT-PCR (Anderson and Moody 2005). Of the 131 threadsail filefish (*Stephanolepis cirrhifer*) from a natural outbreak of VNN, 30 (23%) brain samples were RT-PCR positive but 116 or 89% were nested RT-PCR positive

(Gomez *et al.* 2004). Grotmol and Totland (2000) reported all Atlantic halibut gonad fluids were one-step RT-PCR negative but that the benefit of egg disinfection with ozone implies nodavirus was present in these fluids below the detection limit of the RT-PCR. Dalla Valle *et al.* (2000) emphasised the addition of the nested RT-PCR appears as an absolute requirement to ensure a sensitivity level adequate to screen for nodavirus-free broodstock. When seronegative adult European seabass were experimentally infected with nodavirus, and subsequently produced larvae with a vacuolating encephalopathy, one-step RT-PCR tests on ovary biopsies from the 7 fish were negative but nested RT-PCR tests were positive in 6 of the 7 (Breuil *et al.* 2002). Dalla Valle *et al.* (2000) using the nested RT-PCR also found that 72% of brain and 59% of blood samples were positive in the adult and juvenile European seabass while only 35% of the ovarian biopsies were PCR positive. Similarly in seabream, 39% of brain and 34% of blood samples were PCR positive. This raises the possibility of using the nested RT-PCR on blood samples to screen broodstock.

At this time there have been no publications describing the actual application of the nested RT-PCR in a fish breeding program to exclude nodavirus. Despite the higher level of sensitivity of the nested RT-PCR there are some further questions to be answered before an effective testing protocol can be recommended. Is one test on an individual enough to define its nodavirus status? Striped jack broodstock spawned through a breeding season tended to produce a higher incidence of VNN affected larval batches late in the season (Arimoto, Maruyuma and Furusawa 1994). A broodstock barramundi testing negative at the start of the breeding season may subsequently express active nodaviral replication due to the stress of spawning. Are all the progeny of a nested RT-PCR-negative broodstock always negative for VNN? What are the estimates of false-positive and false-negative test results using the nested RT-PCR? Can co-habitation of broodstock in the same tank result in transmission of infection? Can nodavirus-free fry/fingerlings become infected during grow-out? Through repeated, sequential testing of defined populations of captive barramundi (broodstock and their progeny) we would hope to answer these questions, even if only indirectly. If the assumption about broodstock being the primary source of nodavirus for larvae is true for most finfish species, the knowledge created in this project could well be the model to use for all marine finfish broodstock in breeding programs around Australia. Following the nucleotide sequence analysis of nodavirus isolates from different species and locations in Australia in FRDC project 2001/626, it was confirmed that the nested RT-PCR should detect most nodavirus strains from fishes in Australia (Moody, Horwood and McHardy 2004). Some minor changes to the primer sequence may be necessary to maximise the specificity of the nested RT-PCR for some species e.g., Australian bass.

3. Need

Nodavirus in wild barramundi populations:

* There is a need to address concerns about the effect of stocking hatchery-reared barramundi on the level of inapparent nodavirus infections (that is, the prevalence) in wild barramundi.
* The first step is to determine the prevalence of nodavirus in wild populations of barramundi (that is to say the natural level of inapparent nodavirus status – an infection without disease).
* The baseline nodavirus prevalence data will permit:
- Comparison of barramundi populations in areas where stocking has or has not occurred;
- Assessment of changes in prevalence of nodavirus in future years;
- Effective decisions about appropriate sources of replacement broodstock for breeding programs.

Nodavirus in freshwater fishes:

* There is a need to address concerns about the risk of possible lethal transmission of barramundi nodavirus to freshwater fishes.
* Recent investigations have shown a possible susceptibility of freshwater fishes to barramundi nodavirus and that nodavirus naturally occur in species other than barramundi in Australia, including the freshwater species, sleepy cod.
* There is a need to determine if there are nodaviruses in freshwater fishes as a risk analysis for translocation should include disease-status information in the receiving population.

Are the nodaviruses found in freshwater fishes related to barramundi nodavirus?

* If nodaviruses are detected in freshwater fish an analysis of relatedness (sequence analysis of PCR products) could indicate an association to previous stocking in that area of hatchery-reared barramundi. This information would support effective risk analysis for future translocation considerations.

A testing protocol for hatchery production of nodavirus-free fish fry:

* Broodstock screening protocols to identify nodavirus-free broodstock have been described from overseas but the detection tests used then are not as sensitive as the two-step or nested RT-PCR, and the protocols include a requirement for egg/water disinfection and repeat testing of larvae.
* There is a need to evaluate and validate the sensitivity of the two-step or nested RT-PCR to identify nodavirus-free broodstock and to determine if one or more tests are required to confirm the nodavirus-free status.
* There is a need to confirm in barramundi that larvae/fry become infected by nodavirus through the vertical transmission route (i.e. from their parent/s).
* There is a need to determine if fry can become infected via nodavirus-contaminated water once they are stocked into nursery systems.

* If the vertical infection route is the same for all fish species, the information on the testing protocol required to produce nodavirus-free barramundi fry will be a model testing protocol applicable to all fish species in breeding programs in Australia.

4. Objectives

1. To determine the natural level of nodavirus infection in wild barramundi.

2. To determine the presence or absence of nodavirus infections in freshwater fish species.

3. To describe the relatedness of any nodaviruses isolated from freshwater fishes.

4. To define the best testing protocol in barramundi hatchery production systems to ensure nodavirus-free fry production.

5. Nodavirus in wild barramundi populations

Under Objective One "determine the natural level of nodavirus infection in wild barramundi".

5.1 Methods

5.1.1. Sampling strategy

The primary strategy to measure the natural prevalence of nodavirus in wild barramundi in northern Queensland was to access adult barramundi via other activities so as to not kill this icon trophy fish just for this project. This also reduced project costs. The gonad tissue was selected as the target tissue as there was the implication that this was likely to be where the virus resided in the carrier state. Gonad tissue was also easier to access by non-professional staff than the other possible target tissue, the brain. A secondary objective was to compare the prevalence of nodavirus in at least two populations of wild adult barramundi, one population where there had been stocking of hatchery-reared barramundi fry and a population which were in a pristine fishery where fish stocking had never occurred. This was intended to evaluate the effect of hatchery-reared fingerlings (which could be assumed to have included fish with unapparent nodavirus infections at times) on virus prevalence in the wild population. Johnstone River on the east coast of northern Queensland is the only river that has been stocked with hatchery reared barramundi. The Gulf of Carpentaria barramundi populations are considered pristine (never exposed to hatchery-reared barramundi).

5.1.2. Sample collection

Gonad tissue from wild barramundi was collected by a variety of means through the life of the project. In the initial period project staff coordinated with DPI&F fishery biologists and fishery technicians. Fisheries research teams monitoring the population structure of barramundi in the Johnstone River would lethally sample some of the barramundi they caught during their fisheries operations. At the time of capture they collected their samples (length, weight, otoliths and gonad for histology) and removed gonad tissue for this project. Similarly, fisheries long term monitoring teams assessing the barramundi fishery resource in the Gulf of Carpentaria (Mitchell River) would collect some barramundi for population biology analyses and also removed gonad tissue for this project. The research and resource monitoring teams were provided with a sampling kit which included RNA*later*™ (QIAGEN) as an RNA stabiliser.

The following instructions were provided for their guidance:

- Gonad tissue will be the target tissue for the PCR detection test for barramundi nodavirus.
- Try to sample gonad tissue as fresh as practical (preservation of intact viral RNA will influence the accuracy of the nested RT-PCR).
- A commercial RNA stabilization reagent (RNA*later*™) is to be used to store the gonad samples. RNA*later*™ is non-toxic and will be provided in 4.5ml sample tubes. It can be held at room temperature until use. The gonad tissue should be placed in at least 10x the volume of RNA*later*™, and the tissue should be less than 0.5 cm thick, for the solution to penetrate and stabilise the viral RNA.

- Remove gonad tissue of about 0.5 x 0.5 x 0.5cm in size or a piece that is ≤ 0.5 cm thick and a size that comes up to the 0.5ml line on the sample tube (approx. 200mg of tissue).
- Some effort to avoid cross contamination between fish would be good (as the nested RT-PCR test is so sensitive it may be possible for gonad tissue containing nodavirus from one fish be transferred to the next gonad sample).
- Domestic bleach (a chlorine solution) is provided, dilute to ¼ strength in graduated sample pot to use for decontamination.
- Collection of gonad tissue samples.
  1. Immerse supplied instruments in ¼ strength bleach and then rinse in clean seawater.
  2. Take gonad sample at a site away from that sampled for histology and where the gonad surface has not been in contact with boat surfaces etc.
  3. The gonad tissue piece should trimmed down to about 0.5 x 0.5 x 0.5 cm in size but at least no thicker than 0.5 cm in one dimension (clean any surface used for trimming between fish with ¼ strength bleach and then rinse in clean water).
  4. Place in sample tube containing RNA*later*™ and ensure the tissue is covered by the solution.
  5. Immerse instruments in ¼ strength bleach until the next fish is to be sampled and then rinse in clean seawater before use.
  6. Take another gonad sample.
  7. Hold the samples at 2-8°C (refrigerate) overnight before placing in a freezer (at -20°C the RNA*later*™ will not freeze but crystals or a precipitate will form – this is OK) or holding in the refrigerator.
- Transport to Townsville (Tropical and Aquatic Animal Health Laboratory): Transport of samples to TAAHL in the sample box can be at room temperature but keeping the samples as cool as possible is desirable.

During the latter part of the project fisheries research priorities changed and the Gulf of Carpentaria barramundi resource monitoring utilised the fish caught by commercial fishers for their analyses. Gonad tissue for nested RT-PCR testing was collected by commercial fish processors in Innisfail and Cairns. Processors would identify those barramundi caught by commercial fishers in the Johnstone River, remove the entire viscera at processing, seal in a plastic bag and freeze at -20°C. Project staff would visit the processor when appropriate and collect the frozen barramundi viscera and return to the laboratory. The gonad tissue would be dissected and sampled in the laboratory and maintained frozen until processing for extraction. Professional fishers in the Gulf of Carpentaria would collect barramundi frames including viscera after filleting and freeze during their fishing operations in selected rivers, as requested by fisheries resource monitoring staff. Once sufficient frames were collected arrangements were made for freezer transport to the DPI&F Northern Fisheries Centre (NFC) in Cairns. Project staff would arrange to visit NFC when fisheries monitoring staff were to processing the group of barramundi frames to remove otoliths. Project staff would sample gonad tissue before the frames thawed out and then hold the samples on ice, immediately return to the laboratory for -20°C frozen storage until processed for extraction. Selected tissues from each batch of gonad tissue were evaluated for RNA integrity using the test outlined in section 5.1.5 below. Despite field collection by fishers or processors and storage at -20°C, the RNA quality in these samples was always sufficient for nodavirus nested RT-PCR testing (refer to 5.2).

Over the period of the project 64 wild barramundi gonad samples were collected from the Johnstone River and 220 gonad samples were collected from Gulf of Carpentaria rivers (94 from Mitchell River, 85 from Staaten River and 41 from Flinders River).

5.1.3. Nodavirus PCR detection test method

The nested RT-PCR test used in the project is based on the R3-F2 primers and RT-PCR cycling conditions described in Nishizawa *et al.* (1994) to produce a 426bp amplicon and the nested primers NR'3-NF'2 and nested PCR cycling conditions described in Thiery *et al.* (1999) to produce a 294bp amplicon as modified by Moody, Horwood and McHardy (2004).

Sample preparation

a. Remove gonad tissue onto a RNase-free surface (either chloroform treated in a fume cupboard or RNase Away treated solid surface).
b. Using a new sterile scalpel blade, divide tissue into portions of ~200 mg.
c. Homogenise one portion in two volumes of viral transport medium (VTM; medium 199 supplemented with 1000IU/ml benzylpenicillin, 1mg/ml streptomycin sulphate and 2µg/ml amphotericin B) in a stomacher bag using a hammer.
d. Transfer homogenate to a centrifuge tube (1.5 ml microfuge tube) and clarify by spinning at 10,000 rpm for 10 minutes at 4°C.
e. Transfer 200 µl supernatant to RNase-free 1.5 ml tube and proceed with RNA extraction.
f. RNA is extracted with the High Pure Viral Nucleic Acid Kit according to the manufacturer's instructions.

nested RT-PCR procedure

a. Aliquots of sample and known positive control RNA are heated to 90°C for 5 minutes and cDNA is transcribed in a 20µl reaction mix, containing 9.5µl RNA sample, 1x StrataScript™ buffer, 5U RNasin, 0.5mM dNTPs, 1.0µM R3 primer, 20U StrataScript™ RT and DEPC-treated deionised water, at 42°C for 60 minutes then 90°C for 5 minutes.

b. PCR amplification is carried out in a 25µl reaction mix, containing 2.5µl of the RT reaction, 0.2µM of each primer (R3 and F2), 2.5mM $MgCl_2$, 12.5µl of HotStarTaq™ Master Mix and deionised water in a thermal cycler programmed with the following cycles; 95°C for 15 minutes, 30 cycles of 95°C for 40 seconds, 50°C for 40 seconds, 72°C for 40 seconds and finally 72°C for 10 minutes.

c. The nested PCR amplification is carried out in a 25µl reaction mix, containing 0.5µl of the PCR reaction, 1.0µM NR'3 and NF'2 primers, 12.5µl of HotStarTaq™ Master Mix and deionised water, in a thermal cycler programmed with the following cycles; 95°C for 15 minutes, 25 cycles of 94°C for 40 seconds, 50°C for 40 seconds, 72°C for 40 seconds and finally 72°C for 10 minutes.

d. Reaction products are electrophoresed through a 2% agarose gel containing 10mg/ml ethidium bromide. Amplicons are visualised using a UV transilluminator. A positive reaction was recorded when an obvious nested PCR 294bp amplicon was produced.

5.1.4. Method for making synthetic RNA control material.

Due to the limited known nodavirus positive control tissue through the project, a synthetic RNA control material was usually used as the positive control in the nested RT-PCR tests.

a. Primary PCR amplicons from barramundi nodavirus were purified using QIAquick PCR purification spin columns (QIAGEN, USA) according to the manufacturer's instructions.
b. Purified amplicons were ligated into pGEM-T (Promega) with a 3:1 insert:vector ratio, according to the manufacturer's instructions. Plasmids were transformed into competent JM109 *E. coli*, cloned and selected for using blue/white screening with ampicillin. Isolated recombinant *E. coli* were picked and cloned further in LB medium with ampicillin. One ml stocks of each culture were removed and stored at -80°C. Recombinant plasmids were isolated from the remaining culture volumes using commercial miniprep kit (Promega).
c. Plasmids were linearised using the restriction enzyme *Xmn*I, purified with phenol:chloroform:isoamylalcohol and ethanol precipitation, and were resuspended in sterile nuclease-free water.
d. Single stranded RNA was transcribed from the DNA plasmid template using a commercial *in vitro* transcription system (Riboprobe®, Promega) containing a DNA dependent RNA polymerase according to the manufacturers instructions but omitting the probe labeling process. As the orientation of the A/T ligation was unknown, each plasmid was transcribed once in each direction, with SP6 or T7 RNA polymerase transcribing opposite strands of RNA. Plasmid DNA template was removed with a DNase 1 treatment and synthetic RNA was purified with phenol:isoamylalcohol followed by chloroform:isoamylalcohol. RNA was extracted from the purified solution with 7.5M ammonium acetate and ethanol precipitation.
e. Both strands of synthetic RNA were tested separately with the nodavirus RT-PCR to determine which strand represented the viral template. The correct promoter (SP6 or T7) for synthesizing template was noted for each of the stored recombinant cultures.
f. Synthetic RNA was stored in single use aliquots at -80°C.

5.1.5. Glyoxal RNA integrity test.

The collection of gonad samples in the Gulf of Carpentaria raised concerns about prior storage and transport conditions, and selected barramundi gonad tissue samples were tested to check the integrity of the RNA. The glyoxal denaturation agarose gel electrophoresis method was used for this purpose following the method described in Sambrook and Russell (2001).

5.2 Results and discussion

Selected gonad samples were assessed for RNA integrity. When the method of collection of samples changed in the second year of the project, that is when frozen filleted barramundi from the Gulf of Carpentaria were sampled at NFC and frozen abdominal organs were collected by fish processors, there were concerns there would be degradation

of the target RNA. The quality of RNA was assessed subjectively based on the amount of shearing present as determined by "smearing" in the glyoxal gels. Every sample assessed had an acceptable quality of RNA.

The mean prevalence of nodavirus in wild adult barramundi in northern Queensland was 23.9% (1SD = 24.9%) (Table One). There are few published studies from any species of adult fish describing the prevalence of nodavirus in asymptomatic carriers. It has been reported adult striped jack (wild and farmed) were 65% seropositive (anti-nodavirus antibodies as measured by ELISA; Mushiake *et al.* 1992), 17% of wild adult European seabass were seropositive (anti-nodavirus antibodies as measured by ELISA; Aspenhaug *et al.* 1999) and 0.23% of wild winter flounder had nodavirus isolated from brain and eye homogenates (SSN1 cell line; Baker *et al.* 2002). In the most comprehensive survey of wild fish to date, Gomez *et al.* (2004) using nested RT-PCR testing on brain samples reported a 92.8% prevalence for nodavirus (19 species). In those species where 10 or more fish were tested the prevalences ranged from 83 to 100%.

Table One: The location, number and nested RT-PCR test results on gonad tissue of wild adult barramundi sampled to estimate population prevalence.

Location		Number of individual fish sampled	Number of nested RT-PCR positive tests	Nodavirus prevalence (%)
Gulf of Carpentaria:	Mitchell River	94	1	1.1
	Staaten River	85	54	63.5
	Flinders River	41	2	4.9
	Total Gulf of Carpentaria	220	57	25.9
East Coast:	Johnstone River	64	11	17.2
Total for wild barramundi		**284**	**68**	**23.9**

While it is difficult to do cross species comparisons due to the different nodavirus detection methods and target tissues tested (a common theme in the nodavirus literature), the 23.9% the project measured falls in the mid-range of what has been reported from wild fishes. The only publication that matches the nested RT-PCR testing of gonad tissue used in this project was on adult captive European seabass and Shi drum (*Umbrina cirrosa*), with prevalences of 41.2% and 51.4% respectively (Dalla Valle *et al.* 2000). It could be suggested, particularly with the publication of the Gomez *et al.* (2004) study, the selection of gonad tissue was not ideal. However earlier literature (Munday and Naki 1997) suggested that, due to horizontal transmission of nodavirus gonad tissue was a likely site to find nodavirus in asymptomatic adults. The increased sensitivity of the nested RT-PCR (100 times that of the RT-PCR), as illustrated by Dalla Valle *et al.* (2000) and the preliminary studies reported by Moody, Horwood and McHardy (2004), also suggested gonad sampling was a valid option. Beuil *et al.* (2002) reported that in artificially infected seabass adults, 85.7% of gonad biopsies were nested RT-PCR positive (all test negative with one step RT-PCR and ELISA) while only 71.5% of the brain samples were positive

for nodavirus target. Ultimately the remote capture locations, utilising non-project staff for fish collections and the greater difficulty to remove the brain (and to a lesser extent retinal tissue) meant gonad sampling was a practical option for this aspect of the project.

The variation of prevalence between the three Gulf river's sampled is marked. As there were temporal differences in the sample collection between different rivers it is not possible to know if the differences are real and relate to the specific barramundi population in each river. There is no published literature on other species which examines variations in nodavirus prevalence on a geographic basis within the one species. The target of 150 individual samples were not collected from the Johnstone River (n=64) but the measured 17.2% could be expected to be a good estimate of the prevalence of nodavirus in that barramundi population.

Recognising that nested RT-PCR testing of brain tissue or even detection of anti-nodavirus antibodies in serum may be more sensitive test methods, these figures indicate surveys for inapparent nodavirus infections in wild barramundi need to assume, at this time, a 1% population prevalence as the Mitchell River prevalence was estimated at 1.1%.

The variation in nodavirus prevalence in the Gulf river's make it difficult to do a direct comparison between a pristine aquatic ecosystem where there has been no barramundi stocking with a river where stocking of hatchery-reared barramundi fingerlings has occurred. Despite this wide variation, superficially a comparison of the mean prevalence of nodavirus in Gulf barramundi and the prevalence in Johnstone River barramundi possibly indicates stocking has had no major effect. Again, there is no published literature where the effect of stocking of fish on the level of inapparent nodavirus infections in wild fish.

6. Nodavirus in wild freshwater fishes.

Under Objectives Two and Three "determine the presence or absence of nodavirus infections in freshwater fish species" and "describe the relatedness of any nodaviruses isolated from freshwater fishes".

6.1 Methods

6.1.1. Sampling strategy

There is no information on the prevalence of nodavirus in wild fresh water fishes in the literature. This component of the project targeted those east coast, northern Queensland rivers as areas where impacts of asymptomatic, hatchery-produced barramundi may have had an influence. Specifically, the Johnstone River had been stocked with hatchery-produced barramundi since the 1990s. The strategy was to collect 150 individual freshwater fish of four different species from at least two different rivers on the northern east coast of Queensland so that valid estimates of population prevalence could be stated. The fish collection was to be in conjunction with the DPI&F fisheries long-term monitoring team field collection activities. The species targeted would be bait fish, not the trophy fish which could not be collected in the target numbers. The target tissue was the heads (which include the retina and brain) which were to be combined into pools of no more than 5 individuals. Pooling was used to reduce costs and is consistent with OIE guidelines for virus surveillance and recommendations from Moody, Horwood and McHardy (2004).

6.1.2. Sample collection

The fisheries long-term freshwater monitoring teams utilise targeted electro-fishing to non-lethally collect freshwater fish to determine species, abundance and length (Jabreen *et al.* 2002). This freshwater monitoring targets three rivers in the northern east coast; the Daintree, Johnstone and Herbert River. In each river seven reaches are randomly selected then fixed for year after year sampling. Within each reach six replicated, randomly selected 3 minute 50 meter electro-fishing shots are used to stun fish. The fish are then collected and held in aerated tanks on the electro-fishing boat where they are anaesthetised for examination and measurement. Following recovery from the anaesthetic the fish are returned to the river.

Project staff would participate with the electro-fishing by the long-term monitoring team. Project staff would collect the target species from the electro-fishing boat and return them to an aerated tank on the side of the bank. The fish would be euthansed in benzocaine, their heads removed, individually placed in screw-top plastic tubes and placed on ice. The head tissue was targeted as it contains the brain and eye retinal tissue. Based on the records of abundance across the selected northern rivers the initial species targeted were the mouth almighty (*Glossamia aprion*), silver biddy (*Gerres filamentosus*), gudgeon (*Eleotris* spp.), carp gudgeon (*Hypseleotris compressus*) and rainbow fish (*Melanotaenia splendida*). With insufficient gudgeons being collected to achieve the target number of 150 individuals, from the second year of the project another two species, bassies (*Ambassies myopsis*) and Pacific blue-eye (*Pseudomugil signifier*) were also targeted. At the end of the project 150 individual fish or greater were collected of the species mouth almighty, silver biddy, carp gudgeon, rainbow fish and bassies (see Table Two).

6.1.3. Nodavirus PCR detection test method

The method described in 5.1.3 was followed with a variation in the method of sample preparation (below). In the event of a positive test on a pool of supernatant from five heads, individual supernatants would be retested.

Sample preparation

a. On arrival from the field transfer the samples to -20C for storage until processing for RNA extraction.
b. Homogenise each head in two volumes of viral transport medium (VTM; medium 199 supplemented with 1000IU/ml benzylpenicillin, 1mg/ml streptomycin sulphate and 2µg/ml amphotericin B) in a stomacher bag using a hammer.
c. Transfer homogenate to a centrifuge tube (1.5 ml microfuge tube) and clarify by spinning at 10,000 rpm for 10 minutes at 4°C.
d. Transfer aliquots from 5 individual supernatants to RNase-free 1.5 ml tube to form a pool and proceed with RNA extraction.
e. RNA is extracted with the High Pure Viral Nucleic Acid Kit according to the manufacturer's instructions.
f. Store individual supernatants in RNase-free 1.5ml tubes fitted with an O-ring at -80C for retesting as required.

6.1.4. Sequencing

Positive nested RT-PCR tests from four individual fish were selected from those samples testing positive in 2005.

a. PCR amplicons were purified using QIAquick PCR purification spin columns according to the manufacturer's instructions.
b. Amplicons were used as template material for nucleotide sequencing reaction using 10 ng template, 1 µl Applied Biosystems Big Dye v3.1 reaction mix and 3.2 pmol either primer NF'2 or NR'3, with two reactions in each direction. Reactions were cycled 25 times at 95^{o}C for 30 sec, 50^{o}C for 15 sec and 60^{o}C for 4 min.
c. Reactions were purified using Sephadex G50 columns and submitted to the Australian Genome Research Facility for capillary electrophoretic separation using Applied Biosystems equipment.
d. Resulting chromatograms were evaluated using Sequencher™ software (Gene Codes, Ann Arbor) and aligned to form contiguous nucleotide sequences for each amplicon from at least three reactions.

6.2. Results and discussion

6.2.1. Prevalence

There were no positive PCR test results on any of the fresh water fishes sampled during 2003 and 2004 (Table Two). In 2005, only fish from the Daintree River returned positive PCR tests. That being four of the *Glossamia aprion* (or 10% prevalence for that species) and five *Ambassies myopsis* (or 5% prevalence for that species). All other fresh water fishes collected during 2005 were test negative.

Table Two: The number, location, year of sampling and species of freshwater fish tested for nodavirus using the nested RT-PCR on head tissues.

Species	Date	Number of fish sampled: Daintree River	Johnstone River	Herbert River	Species total
Glossamia aprion (Mouth almighty)	2003	61	-	30	
	2004	-	-	27	
	2005	40	-	-	**157**
Gerres filamentosus (Silver biddy)	2003	40	55	26	
	2004	-	-	40	**161**
Eleotris spp. (Gudgeon)	2003	13	-	-	**13**
Hypseleotris compressus (Carp gudgeon)	2003	69	54	65	**188**
Melanotaenia splendida (Rainbow fish)	2003	-	4	52	
	2004	-	40	60	**156**
Ambassies myopsis (Bassies)	2004	-	37	-	
	2005	100	-	25	**162**
Pseudomugil signifier (Pacific blue-eye)	2005	52	50	1	**103**
River total		**375**	**240**	**326**	

6.2.2. Sequence analysis comparison

From the samples that had tested positive, four were chosen based upon those tests that had strongest amplification and representing examples from both species that tested positive.

5-45490 *Ambassies myopsis* (bassies) from Daintree River
5-45490 *Glossamia aprion* (mouth almighty) from Daintree River
5-45490 *Glossamia aprion* (mouth almighty) from Daintree River
5-45490 *Glossamia aprion* (mouth almighty) from Daintree River

These four amplicons were sequenced and consensus data formed for each amplicon.

Sample amplicons were aligned and found to be 100% identical.

Consensus sequence data (298 bases) for the PCR product was subjected to BLAST search of Genbank. The following matches were obtained (top ten shown only):

AY744705.1| Redspotted grouper nervous necrosis virus coat protein mRNA, Expect = 3e-161, Identities = 293/294 (99%)
AY140793.1| Barramundi nervous necrosis virus isolate B00GD coat protein gene, Expect = 6e-159, Identities = 292/294 (99%),

AF245003.1| Malabaricus nervous necrosis virus coat protein gene, complete, Expect = 6e-159, Identities = 292/294 (99%),
AF175516 *Dicentrarchus labrax* encephalitis virus RNA2 strain SG14 coat, Expect = 6e-159 Identities = 292/294 (99%),
AY140801.1| Grouper nervous necrosis virus isolate G9410YA coat protein gene, Expect = 2e-156, Identities = 291/294 (98%),
AF534998.3| *Epinephelus coioides* nervous necrosis virus coat protein gene, Expect = 2e-156, Identities = 291/294 (98%),
AY140800.1| Yellow-wax pompano nervous necrosis virus isolate YP99PD coat, Expect = 4e-154, Identities = 290/294 (98%),
AY140796.1| European eel nervous necrosis virus isolate EE98PH coat protein, Expect = 4e-154, Identities = 290/294 (98%),
AY140795.1| Cobia nervous necrosis virus isolate Co00CC1 coat protein gene, Expect = 4e-154, Identities = 290/294 (98%),
AY140794.1| Chinese catfish nervous necrosis virus isolate CC01YL1 coat protein, Expect = 4e-154, Identities = 290/294 (98%)

The consensus data was compared to the barramundi nodavirus sequence (the one we use as a control RNA) and was found to be 100% identical over the 298 bases.

Moody *et al.* (2007) found that the three sequences of nodavirus isolates from barramundi, estuary cod and barramundi cod in northern Queensland were 99.3-99.7% identical based on a 289bp sequence of the partial T4 region of the coat protein gene. The 100% identity of the four amplicons from the *Ambassies myopsis*, *Glossamia aprion* with the control nodavirus target (barramundi derived) would not have been expected if they were real isolates from the different species. Even the nodavirus strains isolated from the same farm of European seabass from one year to the next only had a 98% sequence identity (Ciulli *et al.* 2005). In all the well described nodavirus isolates from freshwater fishes or fish held in freshwater, there is a link to a marine source, either transfer to freshwater from marine hatchery systems or co-culture on a farm where marine fish were also farmed (Athanassopoulou *et al.* 2003; Chi *et al.* 2003; Hedge *et al.* 2003). The consensus in the literature is that betanodaviruses are endemic in marine finfish habitats (Munday *et al.* 2002). Even though these original samples were retested twice and repeatedly gave the same results, the positive PCR test from these nine freshwater fish from the Daintree River are false positives and this project does confirm northern Queensland freshwater finfish are nodavirus-free.

The absence of positive nodavirus tests from fish sampled from the Johnstone River does indicate that stocking of hatchery-reared barramundi has not resulted in transfer of barramundi nodavirus to other finfish species in the freshwater ecosystem.

7. Nodavirus free barramundi fry

Nodavirus in captive barramundi broodstock and their progeny.

Under Objective Four "define the best testing protocol in barramundi hatchery production systems to ensure nodavirus-free fry production".

7.1. Methods

7.1.1. Sampling strategy

The strategy was to apply the nested RT-PCR in a way that would fit into the normal operation of a commercial barramundi hatchery; to collect samples from broodstock and their progeny when the hatchery staff themselves would do so during the normal operations of the hatchery. The aim was to use the nodavirus detection technology in such a way that would be applicable to normal aquaculture operations in northern Queensland. As there have been some indications that broodstock fish will produce more nodavirus infected larvae at the end of the breeding season as compared to the start of the breeding season, a stress of spawning effect, it was proposed to sample the reproductive products of the broodstock at the start of the breeding season and at the end to see which was a more effective strategy to identify fish with inapparent nodavirus infections. The collection of reproductive products as the sample for testing was a good fit for standard operations in hatcheries. Barramundi broodstock are checked for gonad maturation to select those individuals most suitable for spawning induction using exogenous hormone. This is done by anaesthetising the fish, passing a catheter via the vent into the gonad and applying negative pressure to with draw a sample of ova or milt. This sample can be collected for RT-PCR testing. The progeny are easily available for sampling as one or two day old larvae, prior to stocking the larvae into larval rearing ponds or rearing tanks in the hatchery. The pond-reared larvae are readily available again for sampling when they are harvested prior to transfer to nursery tanks where they are weaned onto artificial diets. The two ages, that is the early larvae and the fry of 20-40 days of age, were selected as the practical times for sampling progeny. Due to test costs the larvae and fry would be pooled with arbitrary assignment of the level of pooling. This would allow an indirect assessment of the association of broodstock nodavirus test status with the nodavirus test status (and disease epizootics) of their progeny. No specific advice on how hatcheries should manage the broodstock or their progeny was provided. Hatcheries agreed to participate in this research on the basis that their individual nodavirus test results would not be made public.

There is limited information on the natural distribution of nodavirus in the organs of fish with inapparent nodavirus infections. An additional aim of opportunistically sampling one or more organs from barramundi broodstock that had died or were to be disposed off was included in the project strategy. RT-PCR and other testing for nodavirus would be performed on these organs.

The other tissue that can be non-lethally sampled in fish is the blood. Moody, Horwood and McHardy (2004) and others have reported on the results of nodavirus RT-PCR tests on blood. The project included a comparison of gonad material and peripheral blood as a target sample for screening with the nested RT-PCR as previous work had indicated blood was a more sensitive target tissue (Moody, Horwood and McHardy 2004). The

disadvantage of blood sampling is that few industry technicians or managers would have been trained to bleed fish.

7.1.2. Sample collection

At the start of the project three commercial and one government hatchery maintaining captive populations of barramundi broodstock participated with broodstock testing. Due changes in priorities, the government hatchery stopped holding barramundi broodstock at the end of the 2003/2004 summer. After that time the broodstock testing was restricted to the three commercial hatcheries.

7.1.2.1. Broodstock sample collection.

Project staff would visit the hatchery on the day arranged with the owner or hatchery manager. During the project it was found the hatcheries did not want their broodstock disturbed prior to the first spawning in the spring so as to ensure as many early fry could be produced by the hatchery as possible. As a consequence the early season sample was typically collected after the second or third full moon of the spring/summer. Project staff would assist the hatchery staff and would supply sterile catheters and sample containers but the hatchery staff would anaesthetise the fish and catheterise the gonad. The sample collected in the catheter would be expelled in a 1.5ml sterile vial with sealed screw cap and immediately placed on ice. A new catheter would be used for each fish. Only a proportion of the broodstock sampled in each tank would be in spawning condition with ova or milt withdrawn as a sample. The remaining fish had fluid from the reproductive tract taken as the sample for analysis (referred to as reproductive tract fluid or RTF in this report). All broodstock sampled would be identified by a numbered anchor tag or transponder. This number was recorded and the sample identified to that individual broodstock. Anchor tags could be lost occasionally, these would be replaced at the time of sample collection and the new number recorded. The gonad or RTF samples on ice would be returned to the laboratory the same day for processing.

During the last year of the project peripheral blood was collected at the same time as the gonad or RTF sample for four groups of broodstock. Following preliminary evaluation of several possible anticoagulants (unpublished data), the citrate-EDTA solution (0.45M NaCl, 0.1M glucose, 30mM tri-sodium citrate, 26mM citric acid and 2mM EDTA) of Balagi *et al.* (1989) was used at a 1:1 ratio of blood to collect an un-clotted blood sample. Using a 5ml syringe containing 1ml citrate-EDTA with an 18g 38mm hypodermic needle approximately 1ml of blood was withdrawn from the caudal vein accessed laterally in the caudal peduncle. The un-clotted blood was expelled into a sterile 5ml sample container, the broodstock identity was recorded, the un-clotted blood was immediately placed on ice and the sample returned to the laboratory the same day for processing.

When an adult barramundi was to be culled project staff or the hatchery technician would carry out an aseptic dissection, using chlorine disinfection of surfaces and instruments to minimise the chance of cross contamination between organs, collecting selected organs or tissues into individual, sterile 5ml sample containers. The sample containers would be held on ice until delivered to the laboratory or frozen at -20°C and delivered to the laboratory frozen.

7.1.2.3. Larvae and fry sample collection.

The supply of larval and fry samples depended entirely on hatchery staff collecting the required sample, packing in oxygenated water and dispatching by road to Townsville. The hatchery staff would identify the tank of origin and identity of the broodstock induced to produce the spawning. Project staff would collect the live larvae or fry at the freight depot and return to the laboratory for processing. Processing would begin immediately.

7.1.3. Nodavirus PCR detection test method

The method described in 5.1.3 was followed with variations in the method of sample preparation (below).

Sample preparation:

Broodstock gonad material and reproductive tract fluid

a. On the rare occasion that sample volume exceeds 200µl, split into 200µl aliquots.
b. Add 400µl binding buffer supplemented with RNA carrier (from Roche RNA extraction kit) and mix well, using a vortex mixer if really turbid.
c. Incubate at room temperature with shaking for 10 minutes.
d. Add sample (maximum 700µl) to QIAshredder spin column in a collection tube and spin at 14,000 g for three minutes. If there is more than one tube collected per sample, store all but one at -80°C.
e. RNA is extracted with the High Pure Viral Nucleic Acid kit according to the manufacturer's instructions.

Broodstock tissue.

Prepared as described in 5.1.3

Broodstock blood

a. Individual blood samples were lysed by addition of an equal volume of sterile deionised water and incubation at 4°C for 60 minutes.
b. Cellular debris was removed by centrifugation at 8,000 g for 10 minutes at 4°C.
c. Transfer 200 µl supernatant to RNase-free 1.5 ml tube and proceed with RNA extraction.
d. RNA is extracted with the High Pure Viral Nucleic Acid Kit according to the manufacturer's instructions.

Larvae

a. The live larvae would be concentrated into a small volume of the seawater they arrived in. An aliquot of this larval suspension would be placed into a plastic petri dish and individual larvae pipetted off into a 1.5ml sterile tube with a screw cap. Sitting the petri dish on crushed ice would slow their movement. Initially a range of pool sizes were trialled (eg., 30, 60 100 etc) resulting in a standard protocol of counting out in duplicate (if possible depending on the number of larvae supplied) pools of 150 and 300 larvae with any remaining larvae collected as an uncounted pool referred to as

>500. The samples would be held at -20°C until homogenisation, usually within 24 hours.
b. Individual pools are homogenised with an RNase-free autoclaved microfuge pestle.
c. Transfer homogenate to a centrifuge tube (1.5 ml microfuge tube) and clarify by spinning at 10,000 rpm for 10 minutes at 4°C.
d. Transfer 200 µl supernatant to RNase-free 1.5 ml tube and proceed with RNA extraction.
e. RNA is extracted with the High Pure Viral Nucleic Acid Kit according to the manufacturer's instructions.

Fry

a. The live fry would be euthanised in a solution of benzocaine (1000mg/l).
b. Individual heads were removed with a scalpel blade, the surfaces and scalpel disinfected with chlorine between heads. Pool into batches of 30 heads.
c. Homogenise one pool of 30 heads in two volumes of VTM in a stomacher bag using a hammer.
d. Transfer homogenate to a centrifuge tube (1.5 ml microfuge tube) and clarify by spinning at 10,000 rpm for 10 minutes at 4°C.
e. Transfer 200 µl supernatant to RNase-free 1.5 ml tube and proceed with RNA extraction.
f. RNA is extracted with the High Pure Viral Nucleic Acid Kit according to the manufacturer's instructions.

7.1.4 Histology and nodavirus immunohistochemical test

In addition to the dispatch of larvae and fry from the hatcheries for nodavirus PCR testing, five batches of fry were submitted for routine histopathology examinations. Live fry were euthanased in a 100mg/l solution of benzocaine then preserved in Bouin's solution. The fry would then be processed by routine methods for paraffin-embedding and sectioning at 4-5µm prior to staining with haematoxylin and eosin (H&E).

Paraffin sections were also processed for immunohistochemical testing using nodavirus recombinant coat protein sheep polyvalent antibody following the method of Moody, Horwood and McHardy (2004).

7.2. Results and discussion

7.2.1 Preliminary discussion on sampling strategy

The strategy to work with barramundi hatcheries to produce nodavirus-free fry was very much about the practical, on-farm application of a contemporary sensitive molecular test. As such the project design included no experimentation and limited control over variables. The sampling of the broodstock and their progeny followed as closely as possible to what would fit into the normal barramundi hatchery operation. Project staff had no specific control over the broodstock or larval husbandry. No attempt was made to take frequent sequential samples from the broodstock or their progeny. It is unlikely the hatchery managers/owners would have participated in this project if this was the case. The philosophy behind this strategy was that, if the nodavirus nested RT-PCR was to be used as an aid to produce nodavirus-free barramundi fry and to reduce, or eliminate, any of the

costs associated with viral nervous necrosis epizootics in barramundi larval rearing systems, it would only be adopted by industry if it complimented normal commercial husbandry practices and benefits were demonstrated in that context. This aspect of the project was not intended as a close examination of nodavirus biology and epidemiology in barramundi.

The four hatcheries that agreed to be involved in this project all produced barramundi for stocking, farm grow-out or sale under commercial conditions. Typically barramundi broodstock are managed as tank populations, not as individual fish, in these conditions. Individual fish can be moved between tank populations to balance the size and sex distribution in a tank. New, young males (noting barramundi are protandrous hermaphrodites) can be added to a tank as older males invert to females. However, in most instances each tank population is managed with as little change as possible, particularly if the tank has a history as a reliable producer of fertile spawnings. Also emphasised by hatcheries is husbandry with minimal interference involving the broodstock. Any handling could result in loss of spawning condition and failure to spawn at the next full moon. For a well run hatchery, the ideal is a natural spawning. A natural spawning demonstrates the fish are healthy, fully acclimatised to the artificial environment, are stress-free and can express natural maturation and mating behaviour. In this situation the male(s) and female(s) participating are unknown. More usually, the tank would be entered and individual fish checked by catheterisation of the gonad via the vent to sample reproductive material. This would allow identification of males actively producing milt and females with a stage of ova maturation that ensures viable embryos after natural fertilisation. To make sure the tank produce a viable spawning, two or three males and two or three females would be selected and injected with analogue luteinising hormone-releasing hormone (LHRH) to induce spawning. After the spawning the hatchery would not know which of these induced individuals actually produced the fertilised eggs. Repeated spawnings on the following night or two are not unusual. The mating behaviour and spawning may even stimulate non-induced fish in the tank to breed.

Fertilised eggs are harvested from the spawning tank and placed into separate hatching tanks. Hatching will occur in the first 24 hours and usually by the next day larvae are transferred to larval rearing ponds where they are left to feed on natural zooplankton (or, early in the season when water temperatures are low, into larval rearing tanks). Once stocked into a pond, larvae and fry are not readily available until they appear on sampling lift nets (15+ days of age) or when they are harvested. Getting hatchery staff to send one or two day old larvae was the easiest point in the early life cycle to sample. This was also a practical sample the hatcheries could submit if we could demonstrate screening for nodavirus using the nested RT-PCR at this point was effective. At rearing pond (or larval rearing tank) harvest (20-40 days of age) and transfer of fry to the nursery was the next point the progeny would be readily available for sampling. This was also the age after viral nervous necrosis epizootics would have been observed clinically. VNN in barramundi usually occurs between 9 and 21 days of age (Glazebrook *et al.* 1990; Munday *et al.* 2002).

Use of pooling is inevitable when testing larvae as they are so small. Ultimately pools of 150, 300 and greater than 500 were used. Such pooling is not unusual in an attempt to get sufficient target for the RT-PCR to detect. Pooling of 150 tiger prawn post larvae has been used to screen seed stock for White Spot Syndrome Virus in southeast Asia. The decision to pool fry into groups of 30 heads was quite arbitrary and was directly related to reducing

testing costs. In retrospect it may have been better to limit pools to 4 or 5 individual fry heads and test 150 individual fry from each spawning but in the context of developing a testing protocol we were sensitive to limiting testing costs. Without more clear evidence of economic benefit it is unlikely the hatcheries would pay thousands of dollars just for testing their fish.

The strategy used in this project was not meant to provide clear cut epidemiological knowledge. Due to the compromises inherent in this approach, the observational data must be interpreted acknowledging these weaknesses. Despite this we feel valuable information has been produced in the project and making clear cut decisions about the economic benefit of the use of nested RT-PCR alone for screening for nodavirus in barramundi is clearly questionable.

7.2.2. Summary of hatchery PCR prevalence

Table Three shows the number of hatchery broodstock tested through the project and the number of positive tests obtained using the nested RT-PCR on gonad material or reproductive tract fluid. The period at the start of each breeding season indicates sampling fish between October and December; the sampling at the end of each season was usually done between March and July. The broodstock tested in February shown in Figure One were replacement males not yet used for breeding. Through the project 952 individual nested RT-PCR tests were performed on gonad material or reproductive tract fluid (RTF) withdrawn from broodstock barramundi. Not one test was first step positive; all the positive tests refer to the amplification of nodavirus target RNA at the second or nested PCR step.

Table Three: Pooled nodavirus nested RT-PCR test results on gonad or reproductive tract fluid from individual captive barramundi broodstock in four hatcheries until the end of the 2003/04 summer then three hatcheries from the start of the 2004/05 summer.

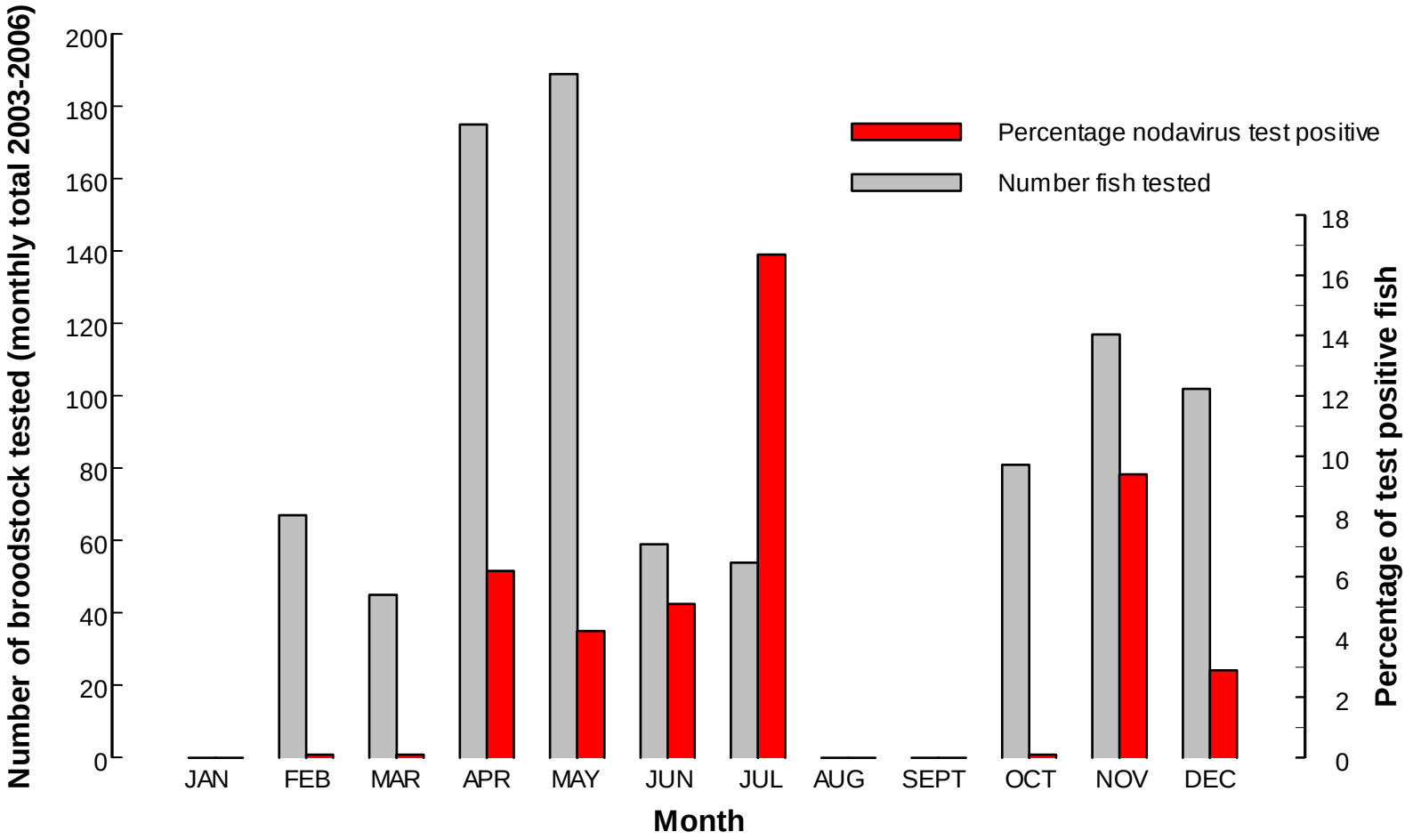
Time of sample collection		Number of individual fish tested	Number of nested RT-PCR positive tests	Nodavirus prevalence (%)
Summer of 2002/03	End	106	16	15.1
Summer of 2003/04	Start	119	0	0
	End	210	3	1.4
Summer of 2004/05	Start	137	11	8.0
	End	213	12	5.6
Summer of 2005/06	Start	91	3	3.3
	End	76	0	0

Project staff provided no guidance to the hatcheries on how they should manage those fish that tested positive. Test results for each sampling were provided to the hatchery as soon as completed by the laboratory. Staff emphasised the lack of definitive knowledge about the link between positive RT-PCR tests, the nodavirus status of their progeny and the

incidence of viral nervous necrosis in barramundi. Despite this hatchery owners/managers always avoided inducing those test positive fish and, in some instances, removed them from the breeding program. There was no pattern of test positive prevalence from year to year. Overall it appeared there was no increase in number of test positive broodstock from the start to the end of the breeding season. We might have expected this increase if the stress of spawning permitted activation of latent nodavirus infections. Mushiake *et al.* (1994) found some of the striped jack broodstock they were monitoring gonad material using RT-PCR at monthly intervals returned positive tests with the late spawnings. Arimoto *et al.* (1994) also found that as striped jack broodstock were repeatedly spawned through the season the incidence of VNN was higher in larvae produced late in the season. To better examine any possible breeding stress effect and seasonal influences the month by month test results are plotted in Figure One.

While the highest percentage of the test positive fish is in July, there is no real pattern showing an increasing proportion of test positive broodstock at the end of the breeding season. Due to the limited number of hatcheries tested on monthly basis (one to three), and the high variation in the number of fish testing positive (for example in April 0 of 43 and 11 of 133; July 0 of 35 and 9 of 19; November 0 of 42, 8 of 38 and 3 of 91) it was not possible to demonstrate any significant differences in test positive prevalence on a month to month basis.

Figure One: The distribution of nodavirus tests and test positive barramundi broodstock, in all hatcheries, by the month ('seasonality').



7.2.3. Repeat testing of broodstock

The published literature refers to the need for repeated testing (serology or one step RT-PCR methods) to identify those broodstock fish that were consistently negative (for example; Mori, Mushiake and Arimoto 1998; Watanabe *et al.* 1998; Breuil *et al.* 2002). Table Four shows the results of sequential testing of the barramundi broodstock. Individual anchor tags or transponders allowed individual fish to be identified from sampling visit to sampling visit, year to year. We found that some of the fish would lose their anchor tags between visits. The hatchery would retag with a new number at the time of sampling. Occasionally we could link the fish to its previous number i.e., only one fish in the tank population had no tag. More usually the fish was assigned a new identity. Thus a number of the fish will have been included in Table Four twice with one, two or three sequential tests. Also note that the time interval between each test and for different individuals can be different.

Of the 438 individual entries included in Table Four, 43 or 9.8% were test positive at one or more tests. This is a lower prevalence than the mean measured in wild populations and lower than the prevalence in Johnstone River barramundi. All the barramundi in the hatcheries would have come from the east coast region or descended from east coast broodstock. Of interest the 9.8% prevalence is similar to the 8.9% seropositve prevalence measured by Huang *et al.* (2001) using ELISA in farmed barramundi in Singapore. While some individual fish were consistently test negative, for example six fish had six sequential negative tests, there were 18 which first tested negative with the nested RT-PCR but subsequently tested positive. How do you interpret the seven fish that were test positive once then test negative on subsequent tests? These findings present information that suggests a one-off nested RT-PCR test on a gonad/RTF sample is not sufficient to identify broodstock barramundi with an inapparent nodavirus infection. This contrasts with Breuil *et al.* (2002) where they found only the nested RT-PCR method could detect nodavirus in all gonad samples (as compared to ELISA and one-step RT-PCR). We would have hoped for more consistent test results if the nested RT-PCR testing of gonad/RTF samples was to be recommended to screen out nodavirus carriers in a breeding program.

There is no published information on the use of sequential nested RT-PCR testing to identify adult fish with inapparent nodavirus infections.

Table Four: Sequential nested RT-PCR test results on gonad or reproductive tract fluid samples of individual captive barramundi broodstock (pooled results from all hatcheries sampled over three years). In the table "neg" = negative test result, "pos" = positive test result and "-" = not tested.

Grouping by first then subsequent test result	Nodavirus sequential test profile: nested RT-PCR test result of each sequential sample over time.*[1]						Number of individual barramundi with test profile*[2]
	1st	2nd	3rd	4th	5th	6th	
All negative	neg	-	-	-	-	-	174
	neg	neg	-	-	-	-	81
	neg	neg	neg	-	-	-	93
	neg	neg	neg	neg	-	-	18
	neg	neg	neg	neg	neg	-	23
	neg	neg	neg	neg	neg	neg	6
Positive	pos	-	-	-	-	-	16
Positive to negative	pos	neg	-	-	-	-	2
	pos	neg	neg	-	-	-	2
	pos	neg	neg	neg	-	-	1
	pos	neg	neg	neg	neg	-	2
	pos	neg	neg	pos	-	-	2
Negative to positive	neg	pos	-	-	-	-	6
	neg	neg	pos		-	-	4
	neg	neg	neg	pos	-	-	1
	neg	neg	neg	neg	neg	pos	1
	neg	pos	neg	-	-	-	5
	neg	neg	pos	neg	-	-	1

*[1] Note that the time interval between each test and for different individual barramundi can be different.

*[2] As some barramundi broodstock had lost identification anchor tags through the period of the project, they were retagged with a new number and would be included twice in this table as a fish with only one, two or three sequential test results.

7.2.4. Tissue distribution of nodavirus in adult barramundi

Table Five shows the results of opportunistic testing of tissues and organs from 14 adult barramundi. No consistent tissue distribution of nodavirus was found. None of the fish that had a positive test on gonad/RTF material (catheter sample) also had test positive gonad tissue (necropsy sample). In fact the only nodavirus positive gonad tissue was a fish (40277) that actually had a primary negative test. The results for fish A660, where selected preserved tissues were processed for nodavirus IHCT, also present inconsistent information on the presence of nodavirus nuclear material (nested RT-PCR) and antigen (IHCT) in various tissues. These results may indicate changes in nodavirus tissue distribution over time, noting the primary test on gonad/RTF samples was done 88 days before the fish was euthanased and tissues were sampled for the secondary testing.

Alternatively the results from fish A660 may indicate differing test sensitivities of the two test platforms used but those tissues tested with both nested RT-PCR and IHCT (gonad and brain tissue) in the secondary testing were in agreement.

There are few publications that describe the tissue distribution of nodavirus in asymptomatic juvenile or adult fishes. Much of the tissue distribution data comes from larvae/fry with clinical viral nervous necrosis. In clinical disease it is clear the retina, brain and spinal cord are the target tissues for nodavirus replication (reviewed by Munday *et al.* 2002). Eight month Atlantic halibut artificially infected with nodavirus did not show any clinical disease. RT-PCR testing of organs 41 days post-infection found all of the head tissue homogenates, kidneys, livers and posterior intestine tissues were test positive. Gill tissues and hearts were negative (Grove *et al.* 2003). Four adult striped jack who tested positive for nodavirus on gonad material were reported to have nodavirus positive (IFAT confirmed with RT-PCR) eye, liver, intestine and stomach, with the brain, spinal cord, gill, spleen, heart and skin test negative (Nguyen *et al.* 1997). In their analysis of juvenile and adult fish Dalla Valle *et al.* (2000) found 42.4% brains, 59.5% blood samples and 41.2% gonad material samples from European seabass were nested RT-PCR positive. The gilt head seabream they tested had 27.8% of the brains and 34.1% of the bloods test positive. Unfortunately none of the reported results were aligned to individual fish.

Clearly a more systematic study of nodavirus distribution in asymptomatic adult barramundi needs to be performed using the most sensitive detection test available.

Table Five: Tissue distribution of nodavirus in adult barramundi. In the table "neg" = negative test result, "pos" = positive test result and "-" = not tested.

			Secondary tissue nested RT-PCR test result							
Fish ID	**Primary** Gonad/RTF* nRT-PCR test result	Time between sampling (days)	Gonad or RTF*	Gonad tissue	Retina tissue	Brain tissue	Caudal vein blood	Heart blood	Intestine tissue	Caudal kidney tissue
A638	pos	0	-	neg	-	-	-	-	-	-
40277	neg	95	-	pos	-	-	-	-	-	-
K58039	pos	94	-	-	neg	neg	-	-	-	-
03054	pos	94	-	-	neg	neg	-	-	-	-
A660	pos	88	-	neg	-	neg	pos	neg	-	-
	A660: IHCT‡ test result		-	neg	pos	neg	-	-	neg	neg
602	pos	52	neg	neg	neg	-	neg	-	neg	neg
202	pos	52	neg	neg	neg	-	pos	-	pos	neg
D52	pos	52	neg	neg	neg	-	neg	-	neg	neg
305	pos	52	neg	neg	neg	-	neg	-	neg	neg
D09	pos	52	neg	-	-	-	neg	-	-	-
54D	pos	52	neg	-	-	-	neg	-	-	-
27B	pos	52	neg	-	-	-	neg	-	-	-
371	pos	52	-	-	-	-	neg	-	-	-
439	pos	52	-	-	-	-	neg	-	-	-

* Gonad material or reproductive tract fluid obtained via catheterization of the vent.

‡ Immuno-histochemistry test on histological tissue sections from fish A660.

7.2.5. The use of blood samples for nodavirus PCR screening of barramundi broodstock

Blood was studied as an alternative non-lethal sample for nodavirus testing using the nested RT-PCR. Blood can be taken from an anaesthetised broodstock fish at the time of checking gonad maturation. Dalla Valle *et al.* (2000) and Moody Horwood and McHardy (2004) both found the percentage of individual fish with nodavirus positive blood was higher than the gonad material samples on nested RT-PCR testing. Table Six gives the results of four sets of paired gonad/RTF and blood samples from individual broodstock. Both samples were collected at the same time from normal broodstock tank populations. These results confirm the earlier research in that 7.2% of the fish had nodavirus positive blood against 3.3% that returned positive tests on gonad/RTF material. Unfortunately not one fish had both positive blood and positive gonad/RTF material. This lack of correlation suggests we cannot yet recommend the best target tissue for screening broodstock using the nested RT-PCR. We did not do any repeated blood sampling nor correlate the blood test result with nodavirus status in progeny due to the limited number of larval/fry batch results available. There is no published information examining these alternatives and further studies are required.

Table Six: Comparison of blood (collected in anticoagulant from the caudal vein) and gonad or reproductive tract fluid samples for the detection of nodavirus using the nested RT-PCR test.

	Number of fish			
	Brood stock population			
	A	B	C	D
Number of paired samples from individual fish	49	50	51	31
Sample type and nested RT-PCR test result				
Blood and Gonad/RTF* Negative	46	49	44	23
Blood Positive – Gonad/RTF Negative	1	0	7	5
Gonad/RTF Positive – Blood Negative	2	1	0	3
Blood and Gonad/RTF Positive	0	0	0	0

* Gonad material or reproductive tract fluid

7.2.6. Does cohabitation of broodstock result in the spread of nodavirus infection?

One of the questions asked during project planning was, does an inapparent nodavirus infection in broodstock barramundi spread to other broodstock held in the same tank? As the hatcheries aimed to maintain as stable a tank population as possible many of the same broodstock could spend years living together. Generally the published literature does not support adult to adult transmission of nodavirus infection although there is at least one report of experimental nodavirus transmission to adult European seabass that resulted in nodavirus infected larvae (Breuil *et al.* 2002). In this study seronegative adults were injected with 0.4ml of $9x10^8$ CFU/ml nodavirus and showed no clinical disease. More commonly the literature describes a reduction in susceptibility to infection and reduced incidence of, or no, clinical disease with increasing age of the fish, this occurs as early as 4

to 6 weeks in barramundi (Anderson and Moody 2005; Aranguren *et al.* 2002; Grove *et al.* 2003). There are no reports of successful bath challenge (the route expected to be closer to a natural infection) of adult fish resulting in a patent nodavirus infection.

The illustrations in Figure Two show individual tanks in the four hatcheries (A, B, C and D) sampled through the project. Those tanks where the populations were relatively stable are shown. In hatchery B there were significant tank transfers but the movements of the majority of the fish could be followed and are shown with arrows. While each of the tank populations were relatively stable some individuals were removed due to positive nodavirus tests, ill health, no breeding activity or to make up the number of males in another tank. Fish could be added as replacement males.

The graphs are an attempt to show any gross change in the number of test positive broodstock over time. Is there a spread of nodavirus infection through a tank population over time? The bar length is the number of fish tested at each tank sampling. The black part of the bar is the number of test positive fish. Note that each tank population need not be sampled on the same day as other tanks sampled in the same period of the season. Also the interval between each sampling period was not the same.

As the graphs are just observational data and each tank population was not exactly the same only comments on trends can be given. Test positive fish could be recorded at any time during the project, including the last sampling. Over all there did not appear to be a strong trend for increasing numbers of nodavirus infected broodstock. Our feeling is that the project test results do not support the suggestion that nodavirus infections are readily transmitted from one adult barramundi to another sharing the same tank.

Figure Two: Sequential test results of selected barramundi broodstock tanks from 2003/04 to 2005/06 to illustrate the changing occurrence of nodavirus test positive barramundi broodstock in each tank over time. a) Hatchery A.

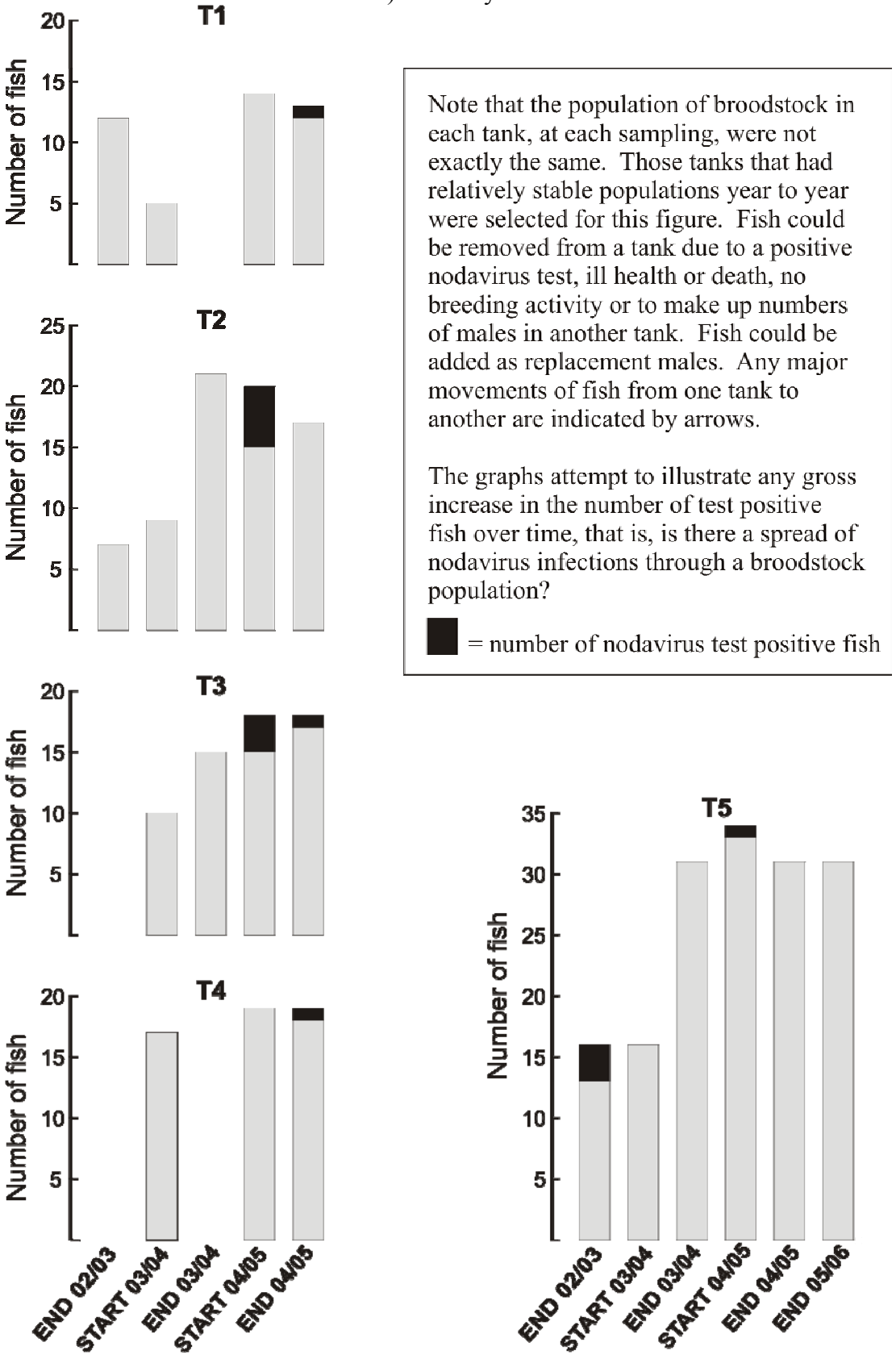


Figure Two: b) Hatchery B.

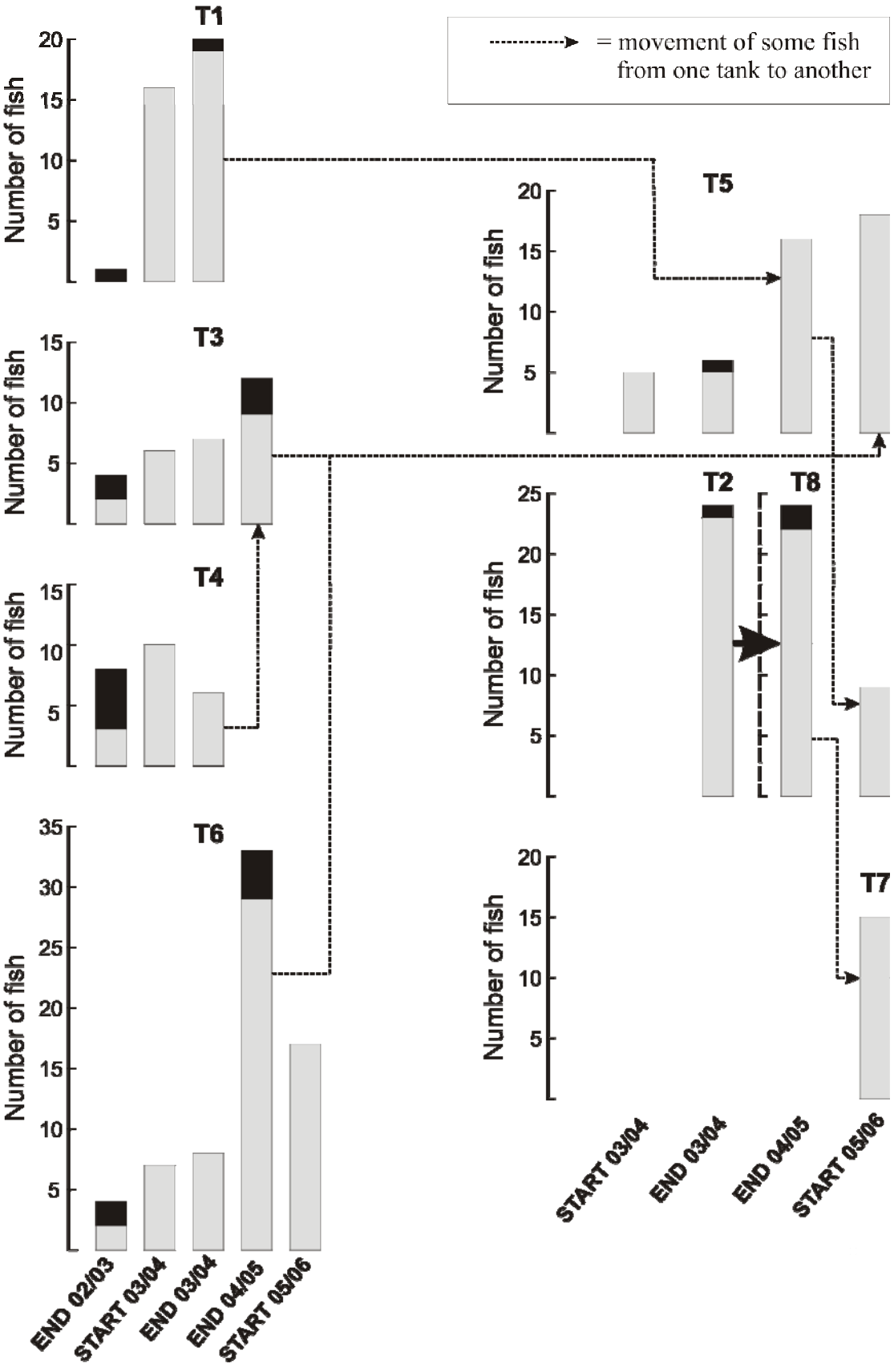
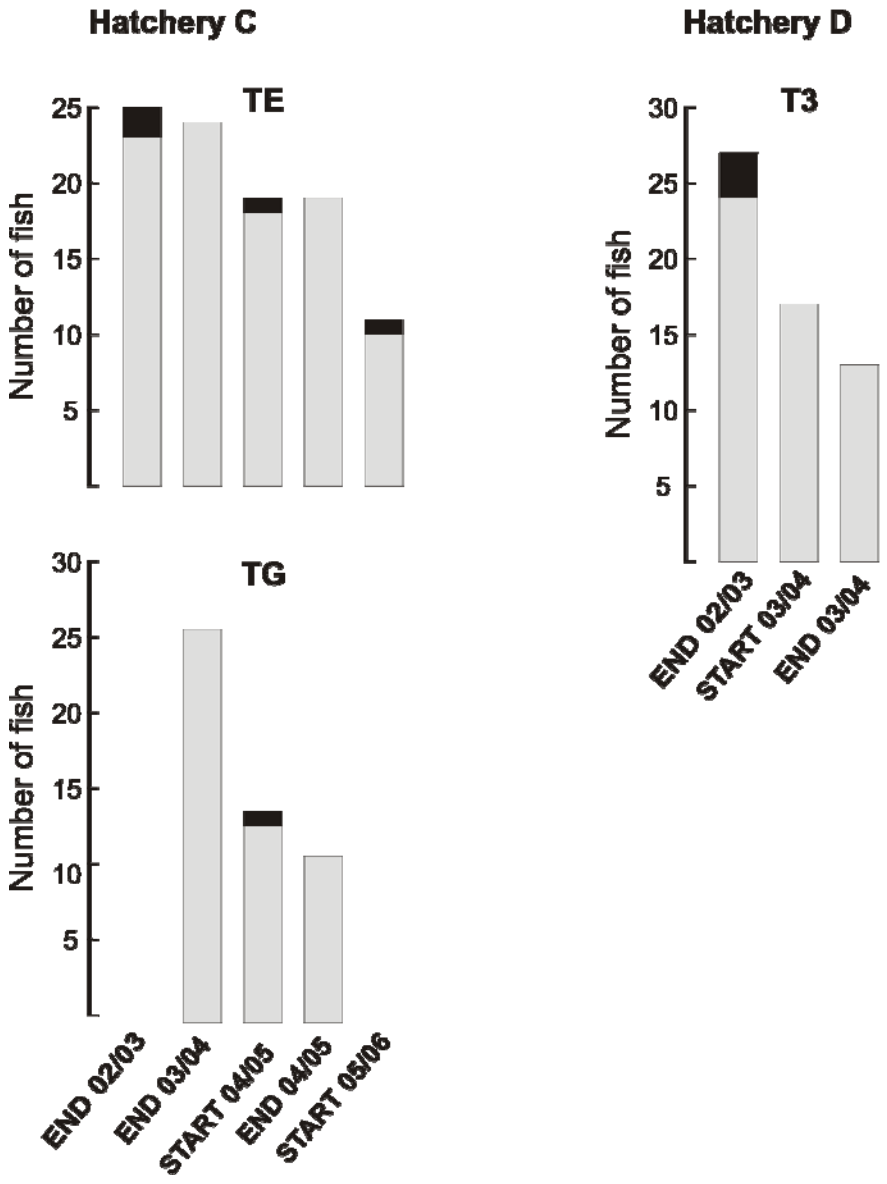


Figure Two: c) Hatcheries C and D.



7.2.7. Nodavirus in the larvae and fry progeny

The hatcheries sent 42 batches of barramundi larvae or fry to the laboratory for testing during the project; either as requested in the project sampling protocol or known batches for routine health testing. Unfortunately due to the demands of hatchery production and work pressures on the hatchery managers/technicians we did not receive samples from every batch produced during the period of the project. We recognise that better compliance with the sampling protocol could have been achieved if project staff repeatedly visited each hatchery at appropriate times. This would have been logistically difficult and not consistent with the aim of following practices that would fit normal hatchery operational capacity. The aim was to analyse each batch of progeny of identified broodstock as 1 or 2 day old larvae and again as fry when the batch was transferred to the nursery system. As can be seen from Table Eight this was not often achieved. When the larval and fry progeny of a single spawning were submitted the results are presented on a

single row of Table Eight. Full details on number of pools tested, pool size(s), tests used (if not the nested RT-PCR) and test result (positive or negative) are all provided in Table Eight. A summary of the full results is given in Table Seven to illustrate the trend of the results from the progeny testing. This summary table over-simplifies some of the complexities we found in this part of the project. Some care is needed to not over interpret these summary results.

Table Seven: Summary of nodavirus testing on submitted batches of barramundi progeny. In the table "neg" = negative test result, "pos" = positive test result and "-" = not tested.

Broodstock nodavirus status		Larvae nodavirus status	Rearing system	Fry nodavirus status	No. of batches with test profile
Tank*	Induced fish				
pos	neg	neg	n/a	-	3
		pos	n/a	-	2
		neg	pond	neg	1
		neg	pond	pos	1
		neg	tank	neg	3
		-	pond	neg	1
		-	tank	neg	3
		-	tank	pos	1
neg	neg	neg	n/a	-	6
		pos	n/a	-	4
		neg	pond	neg	1
		pos	pond	pos	1
		-	pond	neg	4
		-	pond	pos	1
		-	tank	neg	5
		-	tank	pos	3

* A positive tank result refers to the presence of one or more test positive broodstock in the tank where spawning of that batch occurred.

In the results tables the nodavirus test status of the broodstock refers to the test result from the sampling prior to the spawning of that batch of eggs. This sample may have been obtained some months prior to the spawning. From the information provided by the hatcheries all the broodstock selected for spawning induction were test negative prior to spawning and were negative on the next test of the sampled gonad/RTF material. The specific test status of the spawning broodstock in batch #1 and #36 was unknown as the tanks had a natural spawning (induction using exogenous hormone not done) – we have assumed a negative test status. In these tanks at least one of the broodstock population at the time of spawning was a known test positive fish. The batch #5 spawning involved a wild caught male of unknown test status – we have assumed a negative test status. The tank test status is positive when one or more of the fish in that tank tested positive at the sampling prior to the spawning. Note these test positive broodstock did not contribute eggs or milt to the fertilised eggs produced in the tank. Their theoretical influence may have been that they released nodavirus in faeces or urine, contaminating the water making them a possible source of nodavirus for the fertilised eggs. The number of test positive

broodstock of the total number of broodstock in the tank is given in the 'Tank' column. The hatcheries informed project staff that the test positive broodstock in the tanks that produced the batches #1, 2, 4, 5, 6, 7, 29, 30 and 31 were removed prior to spawning. The result tables still refer to the tank test status as positive for these batches. The theoretical possibility that the tank environment had been contaminated by nodavirus was still present.

After the initial larval sample analyses, batch #1 and 2, we attempted to evaluate various pool sizes, and in duplicate, in an attempt to identify the number of larvae needed to provide sufficient nodavirus RNA target for the nested RT-PCR to give a consistent result. As can be seen in batches #20, 21, 22, 24 and 30 there were inconsistent test results. Most usually replicates did not give the same result. In some larval batches, #20, 21, 22 and 24, a positive test would occur in the smaller pool but not exclusively so (for example batch #22). A positive test on any of the pools was considered a positive for that batch in the summary table (Table Seven). An explanation for this result may relate to very low levels of nodavirus and the limits of detection of the nested RT-PCR. A similar variability was reported by M. Crane (pers. comm.) when the same imported prawn tissue sample was tested with the White Spot Syndrome Virus nested PCR. Positive and negative test results were returned when the same sample was repeatedly retested. The extent of pooling could influence detection efficiency as the target RNA is diluted. Breuil *et al.* (2002) used pooling of 30 larvae, sampled every two days post hatch, to test for the early detection of nodavirus in European seabass. Histological lesions of VNN were detected in the brain at 14-22 days of age. ELISA detection of nodavirus antigen in larvae was usually at the same time except for the progeny of one spawner which was at 11 days post hatching. These workers did not provide specific details but did indicate that fertilised eggs were both RT-PCR and nested RT-PCR test positive (number or mass of eggs not stated). Does this imply pooling of 30 seabass larvae is acceptable? In an early study using the one-step RT-PCR Nishizawa, Muroga and Arimoto (1996) sampled and pooled approximately 20 larvae each day. From gonad material-nodavirus RT-PCR positive females the larval pools tested positive for the first time at 3, 5 or 7 days of age. This suggests that the levels of nodavirus in/on younger larvae were too low to be detected. Even though the project used the nested RT-PCR, 1,000x more sensitive than the one-step RT-PCR (Moody, Horwood and McHardy 2004), was nodavirus present in some of the larval pools but not detected due to their age? Does the 150 pool or greater result in too much target dilution? In the project batch #24 larvae and fry were test positive but batch #33 were test negative as larvae and test positive as fry. All the other paired batches (larvae to fry) were test negative.

The effect of pooling may have been a factor in the fry test results. Five of the fry pools were test positive but batch #25 illustrates an issue with test sensitivity when testing pooled specimens as the fry were test positive for other methods but negative for nested RT-PCR. These 21 day old fry were submitted for a standard health test prior to interstate translocation. This involves a histological examination of the central nervous system. 2.1% of the fry had VNN lesions and were IHCT positive but the pool of 30 fry heads was nested RT-PCR negative. By examining the effect of pooling on barramundi fry involved in an experimental nodavirus transmission trial Moody, Horwood and McHardy (2004) recommended that no more than 4 heads from clinically normal fry be pooled to be sure one asymptomatic, infected fry can be detected. The findings in this project indicate a systematic study is required to identify the acceptable level of pooling to detect inapparent nodavirus infections in barramundi larvae, fry and fingerlings.

None of the broodstock used to produce the batches of fry tested in this project were test positive. This did not result in nodavirus-free fry. The presence of test positive broodstock in the tank where the batch originated did not predict the subsequent test status. Table Seven is divided into two parts based on broodstock tank nodavirus test status. In the lower part of the table where all the progeny originated from tanks where all the broodstock were test negative (test negative tank group) 36.0% of the batches (larvae and/or fry) were test positive while only 26.7% of the batches from the test positive tank group were positive. Another factor to consider was the possibility of nodavirus-free larvae becoming infected during pond larval rearing. This rearing method involves filling earthen ponds with seawater that has been filtered down to a level to exclude wild fish eggs and larvae. Otherwise the seawater is raw and birds and other animals have access to the pond. There did not appear any consistent result where pond reared fry became test positive as compared with tank reared fry (Table Seven). The numbers of batches tested in this study does not allow a statistical analyses of the different effects on each batch i.e. broodstock tank test status or rearing method.

The results of broodstock screening and larval and fry testing have not revealed consistent results which would lead to a specific recommendation for an effective testing protocol to ensure production of nodavirus-free fry.

Table Eight: a) The nodavirus status of submitted batches of progeny. In the table "neg" = negative test result, "pos" = positive test result and "-" = not tested.

Batch Number	Batch ID	Nodavirus test status **prior to spawning** Tank*[2]	Induced fish(es)	**Larvae** nodavirus *nested* RT-PCR test Age (days)	Pool size*[3]	Result	Pond or Tank rearing	**Fry** nodavirus *nested* RT-PCR test *[1] Age (days)	Pool size*[4]	Result
1-7/03	815	pos (2/16)	Natural spawning	1	30 >500	neg pos		-	-	-
2-8/03	021/990	neg	neg	1	30 60 100 150 300 >500	neg neg neg neg neg neg	Pond	30	30	neg
3-9/03	832	pos (2/16)	neg	-	-	-	Tank	35	30 x 5	all neg
4-9/03	232/870BS 1	pos (3/8)	neg	1	100 x 2 150 x 2	neg; neg neg; neg	Tank	22	30	neg
5-9/03	232BS2	pos (2/4)	neg F; wild M*[5]	1	150 x 2 300 x 2	neg; neg neg; neg	Tank	22	30	neg
6-9/03	232BS3	pos (2/4)	neg	1	150 x 2 300 x 2	neg; neg neg; neg	Tank	22	30	neg
7-10/03	513	pos (2/25)	neg	1	100 x 2 150 x 2 300 x 2 >500 x 2	neg; neg neg; neg neg; neg neg; neg		-	-	-

Table Eight: b) The nodavirus status of submitted batches of progeny. In the table "neg" = negative test result, "pos" = positive test result and "-" = not tested.

Batch Number	Batch ID	Nodavirus test status **prior to spawning** Tank*[2]	Nodavirus test status **prior to spawning** Induced fish(es)	**Larvae** nodavirus *nested* RT-PCR test Age (days)	Pool size*[3]	Result	Pond or Tank rearing	**Fry** nodavirus *nested* RT-PCR test *[1] Age (days)	Pool size*[4]	Result
8-10/03	931	neg	neg	1	100 x 2 150 x 2 300 x 2 >500 x 2	neg; neg neg; neg neg; neg neg; neg		-	-	-
9-11/03	242	neg	neg	1	100 x 3 150 x 2 300 x 2 >500 x 2	neg; neg neg; neg neg; neg neg; neg		-	-	-
10-11/03	250	neg	neg	-	-	-	Pond	22	30	neg
11-1/04	136	neg	neg	-	-	-	Pond	22	30	neg
12-2/04	066	neg*[6]	neg	-	-	-	Tank	33	24/155	IHCT*[7] pos
13-2/04	236	neg	neg	-	-	-	Pond	21	30	neg
14-2/04	395	neg*[6]	neg	-	-	-	Tank	40	30	pos
15-2/04	654	neg*[6]	neg	11	>500	pos		-	-	-
16-2/04	695	neg*[6]	neg	11	>500	neg		-	-	-
17-9/04	540	neg	neg	-	-	-	Tank	27	30	neg

Table Eight: c) The nodavirus status of submitted batches of progeny. In the table "neg" = negative test result, "pos" = positive test result and "-" = not tested.

Batch Number	Batch ID	Nodavirus test status **prior to spawning** Tank*[2]	Nodavirus test status **prior to spawning** Induced fish(es)	**Larvae** nodavirus *nested* RT-PCR test Age (days)	Pool size*[3]	Result	Pond or Tank rearing	**Fry** nodavirus *nested* RT-PCR test *[1] Age (days)	Pool size*[4]	Result
18-9/04	164	neg	neg	1	150 x 2 300 x 2 >500 x 2	neg; neg neg; neg neg; neg		-	-	-
19-9/04	785	neg	neg	-	-	-	Pond	28	30	neg
20-11/04	913	neg*[6]	neg	3	150 x 2 300 x 2 >500	neg; pos neg; neg neg		-	-	-
21-11/04	952	neg*[6]	neg	4	150 x 2 300 x 2 >500 x 2	neg; pos neg; neg neg; neg		-	-	-
22-11/04	000	neg*[6]	neg	1	150 x 2 300 x 2 >500 x 2	neg; pos neg; neg pos; pos		-	-	-
23-11/04	135	neg	neg	-	-	-	Tank	22	150	histo*[8] neg
24-11/04	143/394	neg*[6]	neg	1	150 x 2 300 x 2 >500 x 2	neg; pos neg; pos neg; neg	Pond	41	30	pos
25-11/04	344/472	neg*[6]	neg	-	-	-	Pond	21	3/144 2/96 30	histo pos IHCT pos PCR neg

Table Eight: d) The nodavirus status of submitted batches of progeny. In the table neg = negative test result and pos = positive test result.

Batch Number	Batch ID	Nodavirus test status **prior to spawning** Tank*[2]	Nodavirus test status **prior to spawning** Induced fish(es)	**Larvae** nodavirus *nested* RT-PCR test Age (days)	**Larvae** nodavirus *nested* RT-PCR test Pool size*[3]	**Larvae** nodavirus *nested* RT-PCR test Result	Pond or Tank rearing	**Fry** nodavirus *nested* RT-PCR test *[1] Age (days)	**Fry** nodavirus *nested* RT-PCR test *[1] Pool size*[4]	**Fry** nodavirus *nested* RT-PCR test *[1] Result
26-11/04	506	neg*[6]	neg	-	-	-	Tank	27	30	pos
27-11/04	611	neg*[6]	neg	-	-	-	Tank	28	150	histo neg
28-11/04	626	neg*[6]	neg	-	-	-	Tank	30	10 x 3	all neg
29-12/04	483	pos (1/20)	neg	-	-	-	Tank	20	30	neg
30-12/04	742	pos (1/34)	neg	2	150 x 2 300 x 2 >500 x 2	neg; pos neg; pos neg; pos		-	-	-
31-12/04	796	pos (1/20)	neg	-	-	-	Tank	27	30	neg
32-1/05	160	pos (1/18)	neg	2	150 x 2 300 x 2 >500	neg; neg neg; neg neg		-	-	-
33-1/05	322/186	pos (1/34)	neg	1	150 x 2 300 x 2 >500 x 2	neg; neg neg; neg neg; neg	Pond	28	30	pos
34-2/05	325	pos (1/18)	neg	-	-	-	Tank	42	20/100 2/2	histo pos IHCT pos
35-2/05	372	pos (1/34)	neg	1	45 150	neg neg		-	-	-

Table Eight: e) The nodavirus status of submitted batches of progeny. In the table "neg" = negative test result, "pos" = positive test result and "-" = not tested.

Batch Number	Batch ID	Nodavirus test status **prior to spawning** Tank*[2]	Nodavirus test status **prior to spawning** Induced fish(es)	**Larvae** nodavirus *nested* RT-PCR test Age (days)	Pool size*[3]	Result	Pond or Tank rearing	**Fry** nodavirus *nested* RT-PCR test *[1] Age (days)	Pool size*[4]	Result
36-2/05	461/685	pos (1/34)	natural spawning	1	150 300 >500	neg neg neg	Pond	17	30	neg
37-4/05	750	pos (1/18)	neg	-	-	-	Tank	42	30	neg
38-6/05	242	neg	neg	-	-	-	Tank	35	30	neg
39-9/05	646	neg	neg	2	150 x 2 300 x 2 >500 x2	neg; neg neg; neg neg; neg		-	-	-
40-9/05	135	neg	neg	1	150 x 2 300 x 2 >500 x 2	neg; neg neg; neg neg; neg		-	-	-
41-12/05	010	pos (3/31)	neg	-	-	-	Tank	42	30	pos
42-12/05	346	pos (3/31)	neg	-	-	-	Tank	22	30	neg

Table Eight: f) Footnotes: The nodavirus status of submitted batches of progeny.

*[1] The *nested* RT-PCR test was used to detect nodavirus nucleic acid in most batches of fry tested. The results of histological examination (**histo**) and immunohistochemical test (**IHCT),** using nodavirus recombinant coat protein sheep polyvalent antibody, of fry are also included for batch #s 12, 23, 25, 27 and 34. A positive histology test is the presence of a vacuolating degeneration of brain neuronal cells and basophilic intracytoplasmic inclusions with the number of fry with lesions over the total number examined given in the 'pool size' column. A positive IHCT is the specific immuno-staining of neuronal cells in the brain with the number of fry with positive brain cell staining over the total number of fry tested given in the 'pool size' column.

*[2] The tank result refers to the result of the last *nested* RT-PCR testing of gonad/reproductive tract fluid obtained by catheterization via the vent of the broodstock in the tank where spawning of that batch occurred. This testing could have been several months prior to the spawning event. A positive result refers to the presence of one or more test positive broodstock with the number of broodstock testing positive over the number of broodstock tested given in brackets under the test status. Information provided by the hatcheries indicated the test positive broodstock in the tanks that produced batch #s 1, 3, 4, 5, 6, 7, 29, 30 and 31 had been removed from the tank prior to the induction and spawning event. Broodstock that had tested nodavirus positive in the proceeding sampling were never induced to spawn in any batch listed here. The parents of larvae produced from natural spawning events were unknown.

*[3] Live larvae were chilled on ice, transferred with pipettes and counted into individual pools of a specific number of larva or a great number of larvae (indicated as >500). In some batches these pools of larvae were duplicated; indicated as 'x 2'.

*[4] Live fry were euthanased, processed to remove the entire head and these individual heads were combined into a single pool of, usually, 30 heads for PCR testing. In some batches multiple pools of fry heads were tested; indicated as 'x 5'. When histological ('histo') or immuno-histochemical tests ('IHCT') results as reported for a batch, this column gives a number of fry that test positive over the total number of fry examined/tested.

*[5] Batch #5 involved the induction of one test negative female broodstock and mating by one of five untested, wild-caught male broodstock.

*[6] At the next tank sampling and gonad/reproductive tract fluid PCR testing, this tank included one or more test positive broodstock but not the specific broodstock used to produce this batch of larvae. **Note** that the fry and larvae of batch #s 12, 14, 15 and 16 all came from the same broodstock tank; the fry and larvae of batch #s 20, 21 and 25 all came from the same broodstock tank; and the fry and larvae of batch #s 24, 26, 27 and 28 all came from the same broodstock tank.

*[7] IHCT is an immunohistochemical test using nodavirus recombinant coat protein polyvalent sheep antibody.

*[8] histo is the histological examination of fry in midline, longitudinal sections stained with H&E.

8. Benefits and adoption

This project applied a contemporary molecular detection test, the nested RT-PCR, for nodavirus in two areas. The first was to determine the level of inapparent nodavirus infections in wild barramundi and freshwater fishes in north Queensland rivers. The estimates of population prevalence will benefit aquatic animal health managers and professionals when planning surveillance projects for nodavirus in wild finfish. The estimate of population prevalence is one parameter used to accurately calculate sample sizes when designing surveillance projects. A reduction in sample size will reduce the costs of surveillance for barramundi. The prevalence estimates in wild barramundi will provide baseline data for comparison of the natural level of nodavirus in future surveillance in barramundi at the locations sampled and others, and in other wild marine finfish populations. The project compared the natural distribution of nodavirus in pristine river systems and those stocked with hatchery-reared barramundi fry. The nodavirus prevalence in barramundi in the Johnstone River indicated it is unlikely the stocking of hatchery-reared barramundi fry (which could be assumed to have included fish with inapparent nodavirus infections at times) had resulted in increased levels of nodavirus. No nodavirus was detected in the freshwater bait fish sampled from the Johnstone River. This knowledge supports fisheries management decisions to permit limited open water stocking of barramundi in regard to nodavirus risks. The baseline prevalence estimates will allow evaluation of future stocking of hatchery-reared fishes. The knowledge also benefits barramundi hatcheries showing the background level of nodavirus in Johnstone River barramundi stocks is unaffected. The collection of replacement broodstock from this population still requires hatcheries consider the nodavirus status of the wild barramundi as a proportion of the fish are going to be naturally asymptomatic nodavirus carriers, even those barramundi from pristine river systems. The project outputs indicate that nodavirus is not transmitted from clinically normal barramundi in rivers and in hatchery broodstock populations to other non-infected fish. This knowledge implies a benefit through the reduction of nodavirus risk to wild fish populations and benefit in the management of captive broodstock populations.

The objective of the application of the nested RT-PCR in barramundi hatcheries was to be the production of nodavirus free barramundi fry. This would have benefited the commercial hatcheries through reducing direct nodavirus disease costs, by avoiding problems with supply continuity of fingerlings for on-growing and to reduce compliance costs associated with interstate fry sales. There would also have been the benefit of eliminating any risk of nodavirus to freshwater fishes in those regions outside of the natural range of barramundi by ensuring translocated barramundi were virus free. The project outputs have lead to the realisation that these benefits will not be delivered using the current knowledge of nodavirus biology and epidemiology, and nested RT-PCR testing alone. While not expected, the commercial barramundi hatcheries have benefited from this knowledge by avoiding test costs that are not efficacious for the purpose intended. The costs of the routine use of the relatively expensive nested RT-PCR for screening gonad and reproductive tract samples, and 1-3 day larvae, are not yet justifiable as the project could not demonstrate a profile of required samples in a testing protocol that would ensure nodavirus free fry at this time. As a consequence commercial barramundi hatcheries have not adopted routine screening of broodstock or their progeny using the nested RT-PCR.

Information on the natural distribution of nodavirus in wild barramundi and the absence of nodavirus in freshwater fishes has been presented to several fish stocking committee

workshops. Project objectives and preliminary outcomes were presented at an Australian Barramundi Farmers Association conference. Several letters to collaborating barramundi hatcheries have directly informed them of the test results of their broodstock and summaries of overall results. Presentations of project results, implications and requirements for future research have been given at FRDC Aquatic Animal Health Subprogram scientific conferences.

9. Further Development

Since this project began the scientific literature on detection tests for nodavirus have shown their use in a range of applications. The range of different test platforms used on different tissues makes it difficult to know the best method to screen asymptomatic fish with inapparent nodavirus infections. Serology and antibody detection with ELISA is reported for selection of virus free broodstock although the specificity is moderate and only a minority of fish are repeatedly seronegative. This may not be ideal when restricted numbers of broodstock are available for breeding. Neither is blood sampling a common skill in commercial hatchery technicians. Screening of larvae and fry is not possible using blood samples. From the results of this project is seems that the information on nodavirus biology and the epidemiology of infections in marine finfish is not directly applicable to the euryhaline, protandrous hermaphrodite barramundi. There is a need to undertake a systematic study of the tissue distribution of nodavirus in asymptomatic advanced juveniles and adult barramundi using the full range of detection tests; cell culture isolation, antigen capture ELISAs, antibody detection ELISA, immunohistochemistry, *in situ* hybridization, nested RT-PCR and real-time PCR. The study of tissue distribution in adult barramundi would need to include investigation of changes over the annual cycle, that is does the spawning condition of broodstock affect the distribution of the virus? The effect of pooling of brain, retina, blood or other tissues on the detection of inapparent nodavirus infections is required. This research needs to be done in Australia on barramundi living in their natural range. While the focus can be on captive barramundi some consideration and study of wild barramundi would be essential. Unfortunately this type of research would require lethal sampling. The aim of tissue distribution studies would be to identify the best tissue for surveillance samples and to determine the sensitivity of testing non-lethal samples from living broodstock.

Further sequential testing of the progeny of barramundi broodstock of known nodavirus test status is required to demonstrate vertical nodavirus transmission as well as the actual circumstances that lead to effective transmission of the infection resulting in disease or to persistent infection. Daily testing of fertilised eggs, larvae and fry, again with the range of detection test platforms, will give an accurate knowledge of the earliest age where nodavirus can be detected in clinically normal fish. An evaluation of the effect of pooling and tissue selection needs to be completed for larvae and fry. An investigation of nodavirus in the hatchery and extensive larval rearing pond environment would add to the understanding of the virus epidemiology. As most barramundi fry are produced in semi-open earthen ponds we need to understand if infections are possible via the intake water or wild fish in the pond environment. The nodavirus strain present in broodstock, eggs, larvae or fry will need to be sequenced to confirm vertical or horizontal transmission, although sequencing still may not definitively indicate the source of the infection. To complete the understanding of the pathogenesis of VNN and the fate of nodavirus in surviving fish long term transmission studies which use bath infection models of larval barramundi are required. This will provide information on the expected prevalence of inapparent infections in survivors and indicate the sensitivity of the test that is required to detect a carrier status. Proof of concept in regard to a testing protocol, detection test platform(s) and hatchery husbandry would be required to demonstrate a consistent production of nodavirus free barramundi fry. Until industry is convinced a particular protocol will guarantee they can produce nodavirus-free fry it is unlikely they will spend money on testing or modifications to their production methods.

More extensive surveillance of wild barramundi is needed to clearly establish estimates of nodavirus prevalence in different river systems. Prevalence data over time and from region to region will provide better evidence for implementation of appropriate translocation protocols by fisheries managers. The relatedness of different isolates from different marine finfish would be required to thoroughly assess potential translocation risks. Many of the further research needs that apply to barramundi can also be considered for other species, particularly other euryhaline species in our opinion. Even the application of protocols used overseas on marine finfish need to be demonstrated as applicable in Australia. For example the correct dose of ozone for egg disinfection is species specific. Different species have eggs of different morphology and thus different sensitivity to ozone toxicity. The detection components of a test platform need to be shown to be sufficiently specific and sensitive, and reliable, for the nodavirus in the particular fish species being used in a breeding program.

10. Planned Outcomes

The project application stated the "major outcome will be a reduced effect, perceived or real, of nodavirus on freshwater fishes and in barramundi breeding programs" as seen through a range of fisheries management decisions, well designed surveillance programs for tropical finfish viruses and the production of nodavirus free fry from hatcheries.

The project outputs of nodavirus prevalence estimates for barramundi from Gulf of Carpentaria rivers and Johnstone River contributes to knowledge that will enhance the design of nodavirus surveillance by allowing calculation of more specific sampling level targets. The project has created the first prevalence data set of virus carrier status in wild barramundi in Australia. This will remain base line data for future comparisons. The absence of nodavirus in Johnstone River freshwater fishes, and the nodavirus prevalence in asymptomatic Johnstone River barramundi, has shown transmission of nodavirus from hatchery reared barramundi fry is unlikely to be a significant issue. The output contributes knowledge that supports controlled stocking of hatchery reared barramundi with minimal nodavirus risk to freshwater fishes.

The project output of a recommended testing protocol to use in barramundi hatcheries to produce nodavirus free fry was not achieved. The ability to reduce the effect of nodavirus in barramundi breeding programs requires further studies. The use of the nested RT-PCR on the samples collected in this project was shown not to provide sufficient information to predict a route of vertical transmission of nodavirus in barramundi. While the outcome of reduced nodavirus effects in this context were not met, the knowledge created in the project provides good information which will inform future research directions. There remains a need for confirmation that alternative or multiple nodavirus detection methods, and alternative sampling profiles, can lead to nodavirus free barramundi fry production.

11. Conclusion

The translocation of any live fish, particularly if the fish are to be released into open water systems or culture systems where water is discharged directly to nature waters, carries with it the risk of also transferring a disease or pathogen to the new aquatic environment. The betanodavirus of barramundi has been a virus which has faced particular scrutiny by fisheries managers in barramundi translocations due to the potential risk to freshwater fishes in the receiving waters, particularly those aquatic environments outside the natural range of barramundi. Nodavirus infections are a significant issue in marine aquaculture causing disease and deaths in intensively reared larvae and fry. Viral nervous necrosis (VNN), the disease caused by nodavirus, has been seen in barramundi larval rearing systems since 1990. The barramundi farming industry wants to remove the risk of VNN from their commercial hatcheries. The main economic loss is through the costs of fingerling supply interruptions to meet farm marketing schedules. Costs associated with interstate market access for barramundi fingerling sales can also be problematic. The implementation of the nested RT-PCR for nodavirus meant that the project had a very sensitive detection test to address several of the issues of concern. These were the prevalence of inapparent nodavirus infections in barramundi and freshwater fishes in northern Queensland; the effect of stocking hatchery-reared barramundi on nodavirus prevalence in northern Queensland; and the recommended testing protocol using the nested RT-PCR to produce nodavirus-free barramundi fry from hatcheries.

The first objective of the project was to establish the natural level of inapparent nodavirus infection in wild barramundi. By sampling barramundi from the Gulf of Carpentaria (Mitchell, Staaten and Flinders Rivers) and the Johnstone River (Queensland north-east coast) the project achieved the objective to measure the effect of stocking hatchery reared barramundi fry. Two hundred and eighty-four (284) gonad samples from wild barramundi, 220 caught in Gulf rivers and 64 from the Johnstone River, were tested with the nodavirus nested RT-PCR. The aim was to sample 150 from the Johnstone River, this was not achieved but the prevalence estimate is still acceptable. The overall mean prevalence of positive nodavirus tests was 23.9% (n=284; gonad tissue). Specifically there was a 17.2% prevalence in Johnstone River barramundi, and 1.1% in Mitchell River barramundi, 63.5% in Staaten River barramundi and 4.9% in Flinders River barramundi making a mean prevalence in Gulf river barramundi of 25.9%. The overall prevalence of 23.9% falls within the mid-range of the reported prevalences in fish overseas. The reason for the wide range of nodavirus prevalence between the three Gulf rivers may reflect an actual population difference or, some how, a temporal difference as the samples were collected in different years. These results indicate that the stocking of hatchery-reared barramundi has most likely had no effect on the natural level of nodavirus in that wild barramundi population.

There is no information on the presence or absence of nodavirus in natural populations of freshwater fishes. There is a concern that translocation of barramundi nodavirus could result in transmission of nodavirus to wild freshwater fishes when hatchery reared barramundi fry are stocked into rivers. The project tested 941 freshwater bait fish, five species with more than 150 individuals tested and from three northern east coast rivers including the Johnstone River where stocking of hatchery-reared barramundi fry had occurred meeting the planned objectives. The project also aimed to determine the relatedness of any nodavirus target detected at nested RT-PCR testing. Nine positive tests were returned from the Daintree River samples collected in 2005, all other tests through

the project were negative. Sequence analysis determined these were false positive tests, which may be attributed to sample or laboratory contamination. As no nodavirus was likely detected in the freshwater fishes from the Johnstone River, the stocking of hatchery-reared barramundi fry did not result in transmission of nodavirus to the five species sampled from the Johnstone River. We conclude it is unlikely nodavirus is a natural infection of freshwater fishes in north Queensland.

The final aim of the project was to define the best testing protocol in a barramundi hatchery production system to ensure nodavirus-free fry are produced. Published literature reports that efforts to control the effects of nodavirus have been to exclude the virus from larval cultures. Vertical transmission is prevented by early detection of nodavirus in broodstock and subsequent elimination of the infected fish. Ozone disinfection of eggs is also reported to eliminate the virus in larvae although there is now some question about the Total Residual Oxidants dose required to effectively inactivate nodavirus (Liltved *et al.* 2006). Elimination of viable nodavirus in larval and fry rearing water has been recommended by some scientists (e.g., Watanabe *et al.* 1998).

The project worked directly with commercial barramundi hatcheries (and one government hatchery until the end of the 2003/2004 summer). The nested RT-PCR detection test for nodavirus was used to test the reproductive products or reproductive tract fluid of all the captive broodstock, early in the breeding season and at the end. The hatcheries sent live samples of larvae, after hatching, and fry, when they were harvested, from the larval rearing tanks or ponds. The age of the barramundi progeny when tested was selected as it was at this point when the sampling could be accomplished in normal barramundi hatchery operations. No specific advice on how hatcheries should manage the broodstock or their progeny was provided. This part of the project was not intended as a close investigation of nodavirus biology and epidemiology in barramundi. The aim was to apply a sensitive molecular detection test to samples collected in a manner that was likely to be applicable and acceptable to a commercial barramundi hatchery. The aim was that interpretation of the test results would indicate the best testing protocol for nodavirus free barramundi fry production.

No trend for increased test prevalence was seen from the start of the breeding season to the end. Of the 438 individual fish tested 9.8% were test positive at one or more tests. While many of the broodstock were consistently test negative, the sequential testing of individual broodstock revealed that 18 fish were test negative on the first test then test positive at a subsequent test. Transmission of nodavirus from broodstock to broodstock in the same tank did not occur. When paired blood samples and gonad samples were tested there was no correlation with no fish testing positive on both blood and gonad samples. Neither the test status of the spawned broodstock nor the test status of the broodstock tank population predicted the test status of either the larvae or fry. Neither was there any correlation between the test status of the progeny as larvae and as fry. Tank or pond rearing did not appear to have any effect on the fry nodavirus test status. Over all the nodavirus nested RT-PCR test results have not revealed consistent results which could be the basis of recommendations for an effective testing protocol to nodavirus free fry production. More research is required on the biology of nodavirus infections in barramundi, particularly the fate of the virus in asymptomatic adults. A detailed study on the pathogenesis of nodavirus infections would provide information to support the timing and tissues to target in screening programs.

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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

IG Anderson, Principal Veterinary Pathologist (Fish Disease)
HJ Oakey, Senior Molecular Biologist
AP Fisk, Senior Technician (Fish Disease)
N Levy, Project Technician
N Nolan, Temporary Project technician
J Munro, Temporary Project technician

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