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Surveillance for nervous necrosis virus-specific antibodies in barramundi *Lates calcarifer* in Australian hatcheries

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ABSTRACT: We conducted single point-in-time and repeated cross-sectional studies of the prevalence of antibodies against nervous necrosis virus (NNV) in populations of adult barramundi *Lates calcarifer* in Australia. Serum samples collected between 2002 and 2012 were analyzed with indirect ELISA (n = 468). Most of the samples were sourced from broodstock with unknown exposure history, and these were compared with reference populations with confirmed history of exposure to NNV. Non-lethally collected gonad fluid samples from economically valuable barramundi broodstock (n = 164) were tested for the presence of NNV using RT-quantitative PCR at the time of blood sampling to compare infectivity with serostatus, but no virus was detected. NNV-specific immunoreactivity in broodstock was significantly lower than that for immunized and persistently infected populations. Seroprevalence increased over time in broodstock sampled longitudinally, probably reflecting repeated exposure to NNV in a region where the virus was endemic. The seroprevalence for the broodstock was 23.8% over the entire sample period while a cross-sectional survey conducted in 2012 found a seroprevalence of 34.5% with no significant difference between populations based on the geographic region or the history of occurrence of viral nervous necrosis (VNN) disease in the progeny in the respective hatcheries. Although serological surveillance was useful for studying the history of exposure of barramundi to NNV, the lack of association between serostatus in broodstock and the subsequent occurrence of VNN disease in their progeny indicates that ELISA tests for anti-NNV antibodies are not suitable for the purpose of preventing vertical transmission of NNV in barramundi.

KEY WORDS: Nervous necrosis virus · NNV · Nodavirus · Barramundi · Surveillance · Serology · Vertical transmission

INTRODUCTION

Viral nervous necrosis (VNN) is an economically important disease caused by nervous necrosis virus (NNV), family *Nodaviridae* (Nakai et al. 2009). One of the earliest reports of the disease was in farmed barramundi or Asian sea bass larvae in Australia (Glazebrook et al. 1990). Since then, efforts have been made to prevent and control VNN in the barramundi industry, mainly by intensifying biosecurity measures, including the implementation of ozonation of eggs and use of indoor recirculation systems for

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larval rearing (Schipp et al. 2007). However, although the incidence of outbreaks has decreased, they still occur sporadically.

The virus is thought to be transmitted both horizontally and vertically. The notion of vertical transmission of NNV as a pathway of infection for larvae has been supported by cumulative evidence: the disease occurs in populations of very young fish (Breuil et al. 1991); NNV antigens and nucleic acid have been detected in gonadal fluid of broodstock of several species (Arimoto et al. 1992, Mushiake et al. 1992, Azad et al. 2006, Kuo et al. 2012), and high NNV antibody titers in barfin flounder (*Verasper moseri*) broodstock were concurrently associated with the occurrence of VNN in the progeny of individual broodstock (Watanabe et al. 2000). This has led to the idea that testing and exclusion of test-positive broodstock was a key measure for the control of VNN. Molecular tests for the screening of gonadal fluid were initially recommended by Mushiake et al. (1994), but the measure was found to be ineffective in a number of cases (Nishizawa et al. 1996, Mori et al. 1998, Watanabe et al. 2000), including in the Australian context, where Anderson & Oakey (2007) found no relationship between RT-PCR results in the gonadal fluids and the occurrence of VNN in larvae of barramundi.

The evidence with regard to the effectiveness of serological screening of broodstock has been conflicting. After unsuccessful attempts to control vertical transmission of NNV in barfin flounder by screening broodstock with PCR, Watanabe et al. (2000) found an agreement between high antibody titers measured by ELISA (48% of the fish), the detection of the virus by PCR of gonadal fluids, and the occurrence of the disease. Therefore, they suggested screening broodstock with both ELISA and PCR for control of VNN in larvae. Similarly, Breuil et al. (2000) found that 72% of the sea bass identified as seronegative were also negative by PCR and virus isolation (VI) in matched gonadal fluids. These authors suggested that ELISA could be a valuable method for the screening of broodstock. However, test results for NNV infection of seropositive fish and the incidence of disease in the surveyed populations were not reported. In contrast, Mushiake et al. (1992) discouraged the use of antibody ELISA for screening. These authors found that the prevalence of antibodies to NNV in farmed striped jack *Pseudocaranx dentex* did not match with the presence of the viral antigens in gonadal fluid. Hence, it is still unclear whether serological screening of broodstock can be useful for VNN control or whether it is only useful for some species.

Although persistent infection with NNV can occur in several species (Johansen et al. 2002, 2003, López-Muñoz et al. 2012), it is unknown how long NNV infections can persist or how the relationship between persistent infection and serostatus manifests over time. We expect NNV serological surveillance of barramundi broodstock to offer some insight into the occurrence of NNV exposure in hatcheries, given that serological tests can accurately indicate previous exposure at the population level due to a cumulative experience effect, more so in the case of chronic infections (Graham et al. 2007, Jaramillo et al. 2017).

To advise the barramundi industry in Australia and elsewhere on effective strategies to control VNN, we evaluated the utility of ELISA as a screening method for identifying NNV exposure among barramundi broodstock. We conducted a serosurvey for NNV in barramundi hatcheries using a validated indirect ELISA and analyzed the statistical relationship between the serostatus of broodstock and the occurrence of NNV infection in larvae. Our specific objectives were to evaluate NNV seroprevalence in barramundi broodstock held in hatcheries, analyze longitudinal changes in NNV seroprevalence over time, compare NNV seroprevalence between hatcheries on the basis of the occurrence of VNN in larvae, and evaluate the relationship between the NNV serostatus of individual broodstock and the occurrence of VNN in their progeny.

MATERIALS AND METHODS

Case definition

For the purpose of this study, NNV infection was defined by the detection of NNV by real-time reverse transcriptase quantitative PCR (RT-qPCR) in the nervous tissue of fish and confirmed by virus isolaton in SSN-1 cell culture. An infection was considered to be subclinical in the absence of clinical signs of VNN, namely above baseline mortality and evidence of neurological dysfunction including abnormal swimming or behavior.

Serum samples

Reference samples from fish with known history of exposure to NNV were sourced from barramundi in aquaculture facilities (Table 1). A panel of 319 serum samples was collected purposively from one hatchery between 2002 and 2012 as part of a longitudinal

Table 1. Panel of barramundi sera used for the survey for anti-nervous necrosis virus (NNV) antibodies. Tissue samples are non-lethal samples analyzed by RT-qPCR in this study. NT: Northern Territory; QLD: Queensland; NSW: New South Wales; GF: gonadal fluid; N/A: not available

Populations	Exposure	History	Location	Serum samples (n)	Tissue sample type (n)
Surveyed broodstock	Unknown	Captive and wild caught broodstock in hatcheries	NT	220[a]	GF (68)
		Captive and wild caught broodstock in hatcheries	QLD	99[a]	GF (96)
Reference	Naturally exposed	Persistently infected cohorts with subclinical infection	NT	96	N/A
	Immunized	Captive young adults with certification of freedom from NNV infection	QLD/NSW	16	N/A
		Apparently healthy adults	NT	33	N/A
	Not exposed	Captive young adults with certification of freedom from NNV infection	NSW	4	N/A
Total				468	164

[a]Details are in Table 2

Table 2. Time of sampling and sample sizes for the surveyed population: barramundi broodstock with unknown history of exposure to nervous necrosis virus (NNV). Data are number of serum samples (fish). –: no samples collected

Region	Hatchery	2002	2003	2007	2008	2010	2011	2012	Total
NT	A	2	36	9	20	42	51	60	220
QLD	B	–	–	–	–	–	–	7	7
	C	–	–	–	–	–	–	79	79
	D	–	–	–	–	–	–	13	13
Total		2	36	9	20	42	51	159	319

study, and from multiple farms in 2012 as part of a cross-sectional survey (Table 2). Where possible, fish were tested using tissues collected at necropsy or non-lethal samples from gonadal fluids to evaluate the presence of NNV by PCR. Blood samples were collected from the caudal vein and dispensed into tubes with no additives. After allowing the blood to clot, tubes were centrifuged at 2000 × *g* for 20 min and serum was recovered and stored at −20°C.

Surveyed populations

Samples were obtained from individually tagged barramundi broodstock with unknown infection status and history of exposure to NNV (Table 2). Four hatcheries were surveyed, 1 in the Northern Territory (NT) and 3 in Queensland (QLD). The latter 3 sources enabled analysis of differences in seroprevalence between regions and hatcheries. The hatchery in NT was sampled repeatedly, but hatchery management prevented a long-term longitudinal census so several fish were sampled just once. Occurrence of NNV infection in larvae and juvenile fish produced at each hatchery was recorded to enable statistical comparison of serological status in broodstock and the detection of NNV by qPCR in larvae-juveniles (see Section 'RT-qPCR'). One hatchery in QLD had a history of freedom from VNN; all others had a history of occurrence of VNN outbreaks in larvae. Due to the high commercial value of the fish, RT-PCR was not conducted on nervous tissue of any broodstock other than those sampled in 2008 in NT as part of the molecular longitudinal study of Hick (2010) and on one of the studied broodstock that died of unknown causes with non-specific clinical signs. The RT-qPCR and virus isolation results were negative for brain, retina and a comprehensive range of tissue samples from these fish (n = 21).

Reference populations

Naturally exposed. Populations of fish were considered to have been exposed to NNV infection (clinical or subclinical) if a high proportion of the cohort tested positive for NNV by RT-PCR or virus isolation on brain and retinal tissue. Reference samples were

obtained from 3 cohorts with a history of NNV infection after they had been transferred to grow-out farms from Hatchery A (n = 96) (Hick et al. 2011a). Some of these fish were euthanized at the time of blood collection to enable matched ELISA testing of blood and RT-qPCR testing of nervous tissue. All fish were collected from farms with open water systems and were apparently healthy, although clinical signs of VNN had occurred in some sub-populations prior to 40 d of age. The aetiology for all cases was confirmed during the peak of the infection on randomly selected individuals by virus isolation and the apparent prevalence using RT-qPCR was 100%.

Not-exposed. Five juvenile barramundi (78–141 g) were collected from a freshwater recirculation system at a commercial farm in NSW in 2010. The population was certified free of VNN and NNV infection at 21 d post-hatch (dph) based on histopathology of 150 fish and RT-PCR tests of 60 individuals, according to the biosecurity regulations for interstate fish movements that applied at the time.

Immunized. The juvenile barramundi that were not previously exposed to NNV were immunized with recombinant NNV capsid protein or purified virus antigens, according to the schedule described in Jaramillo et al. (2016b). Serial samples were collected from 5 barramundi over a period of 11 wk (total 16 collected, as some fish died during the trial). An additional 33 samples were sourced from barramundi broodstock that were immunized with a heat-inactivated clarified tissue homogenate derived from larvae infected with NNV in 2003.

Indirect ELISA

The indirect sandwich ELISA was conducted according to the validated protocol described by Jaramillo et al. (2016b). Briefly, sheep anti-NNV primary antibodies were used to capture semi-purified NNV. After adding the fish serum diluted 1:150, rabbit anti-Australian bass antibodies were used as detection antibodies and detected with a donkey anti-rabbit horseradish peroxidase (HRP) conjugate. The diagnostic sensitivity and specificity of this assay were estimated to be 81.8 and 86.7%, respectively (Jaramillo et al. 2016a). Serum samples from the survey were diluted 1:150 and tested in 4 wells per sample: duplicate wells with NNV antigen and duplicate wells without antigen for the calculation of the optical density ratio (ODR). The optical density (OD) values were used to assess the reactivity of the antibodies towards the antigen while ODR indicated the specificity of the reaction. ODR was calculated by dividing the mean value of the duplicates of the sample tested in the presence of the virus (testing wells) by the mean value of the duplicates tested in the absence of the virus (background noise).

For standardization purposes, the same positive control sera from immunized fish were tested on each plate. Results for each plate were considered valid only when the ODR for the positive control was 8 or above; otherwise the plate was repeated. The mean OD for duplicate test samples was normalized across plates according to the variation detected in the controls. The positive–negative cut-off point was established at ODR ≥2.4 according to the distribution of results for reference positive and negative samples.

RT-qPCR

Larval cohorts at Hatchery A

Longitudinal sampling was conducted as part of a NNV active surveillance program on cohorts produced at Hatchery A since 2007 to allow early detection of NNV during latent periods. The sampling was intended as an intensive survey on random samples from several developmental stages including fertilized eggs before and after ozonation, 1, 5, 10 and 15 dph larvae and 19–21 dph juveniles. The samples were to be stored at −20°C for retrospective studies as appropriate. Tissue processing and qPCR testing were conducted as described in Hick & Whittington (2010). Upon the detection of NNV by RT-qPCR, confirmatory testing by virus isolation in SSN-1 cells was carried out as described in Hick et al. (2011b).

Gonadal fluids

Based on prior studies reporting the detection of NNV in gonadal fluids (Mushiake et al. 1994, Valero et al. 2015), non-lethal sampling for PCR detection of NNV was conducted on gonadal fluid when it was available, to complement the serological evaluation for NNV exposure. Gonadal fluid samples (eggs and sperm) were collected by cannulation at the time of blood collection for sero-surveillance from resident broodstock at Hatchery A (NT) in June 2011 (n = 68) and resident broodstock at hatcheries in QLD in 2012 (n = 96). For the preparation of the samples, approximately 100 mg was homogenized 1:10 in Leibovitz-15 medium (L-15) (Gibco™), and nucleic acid extraction was performed as indicated for nervous tissue

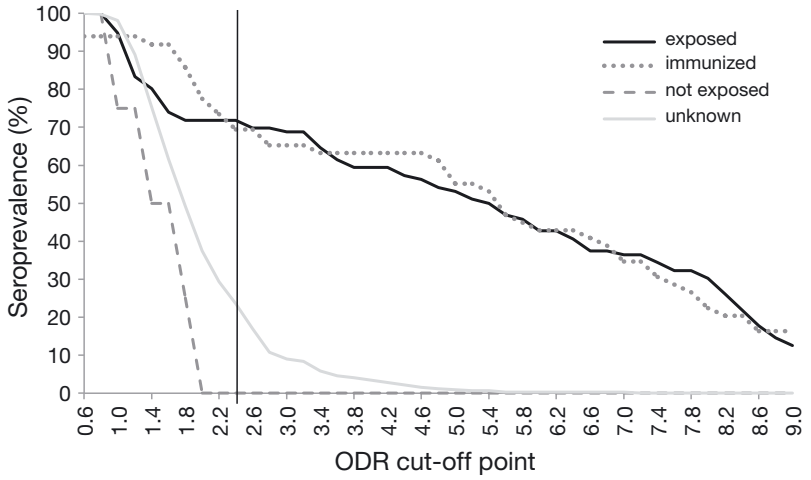

Fig. 1. Effect of the cut-off point on the seroprevalence of nervous necrosis virus (NNV) antibodies in barramundi populations with different exposure status. ODR: optical density ratio between testing wells and blank wells for each sample. Vertical line represents the cut-off point used for final analysis: ODR ≥ 2.4

(Hick & Whittington 2010). All samples with an amplification curve crossing the threshold before the 45 cycles were considered positive.

Statistical analysis

Data analysis was carried out using the statistical software Genstat (14th edn, 2011, VSN International). Analysis of continuous variables was conducted using the non-parametric, Mann-Whitney *U*-test to avoid invalid assumptions of normality of the data. Confidence intervals for prevalence estimates were calculated using the Epitools platform (confidence intervals for a sample proportion; Sergeant 2004). Pairwise correlation (Pearson's), chi-squared and logistic regression analyses were calculated using Stata (StataCorp, 2013, Stata Statistical Software: Release 13).

RESULTS

Serosurvey

Compared to the reference samples from unexposed and immunized barramundi, the population with unknown history of exposure had an intermediate seroprevalence. This conclusion was true for a range of possible cut-off points (Fig. 1).

At the defined cut-off point (ODR ≥2.4), the overall period seroprevalence of broodstock was 23.8%, 95% confidence interval ($CI_{95\%}$) = 13.9–37.6%, estimated based on linearized variance estimation using each sampling event (farm and date) as the primary sampling unit. The seroprevalence in broodstock was significantly lower than the seroprevalence in exposed and immunized populations ($p < 0.001$). The overall seroprevalence found in the cross-sectional analysis of broodstock in 2012 was 34.5% ($CI_{95\%}$ = 27.5–42.4%) with no significant difference in prevalence based on their location or the history of occurrence of the disease in the hatcheries at which they were kept (Table 3).

The seroprevalence increased over time in the resident broodstock at Hatchery A that were sampled on multiple occasions; it was the highest in 2012 (47%) and the lowest in 2008 (4.7%)—2002 not included. Seroconversion was observed in 34% (n = 10) of the individually identified broodstock tested between 2010 and 2011 and in 31% (n = 8) of the individuals tested between 2011 and 2012.

The reactivity of the serum of barramundi broodstock with unknown history of exposure to NNV was generally low, and non-specific reactivity was common. The pairwise correlation between OD and ODR was moderate (0.5) in contrast with that of the exposed populations in which it was high (0.88). Thus, the specificity of the antibodies from barramundi with unknown exposure was lower than that of the

Table 3. Seroprevalence in barramundi broodstock resident at hatcheries. Samples were collected at a single time point in 2012 and compared on the basis of their location and the occurrence of the disease in the hatchery. VNN: viral nervous necrosis

Factor	No. of fish per factor	Seroprevalence % (95% CI)	p-value[a]	Odds ratio (95%CI)
Region				
NT	60	47 (34.9–59.0)	0.09	0.86
QLD	99	27 (18.3–35.7)		(0.28–2.66)
Occurrence of VNN at hatchery				
Yes	80	41 (31.1–52.2)	0.79	2.62
No	79	28 (19.2–36.8)		(0.84–8.14)
Overall				
	159	34.5 (27.5–42.4)		

[a]Multifactorial logistic regression analysis with ELISA outcome as the dependent variable and region and occurrence of VNN at the hatchery as predictors

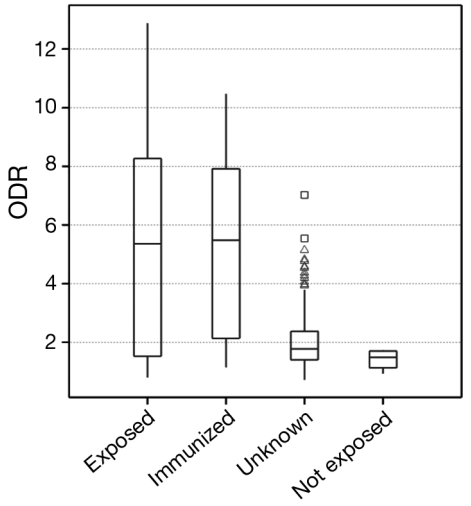

Fig. 2. Boxplot of optical density ratio (ODR) results by indirect ELISA on sera from barramundi populations with different nervous necrosis virus (NNV) exposure status: Exposed, Immunized, Unknown and Not exposed. ODR is the ratio between testing wells and blank wells for each sample. For each box: the middle horizontal line is the median; the top and bottom boxes: 1st and 3rd quartiles, respectively; whiskers: minimum and maximum values; symbols: outliers

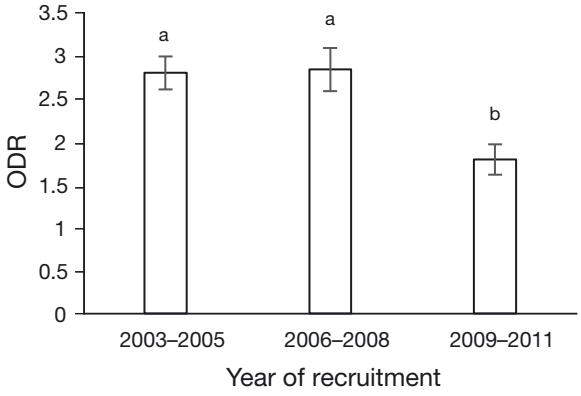

Fig. 4. Cross-sectional analysis (mean ± SE) of optical density ratio (ODR) results of barramundi with unknown exposure to nervous necrosis virus (NNV) at Hatchery A in 2012, based on the year of recruitment. ODR is the ratio between testing wells and blank wells for each sample. Letters above bars indicate statistical differences among groups with different letters

barramundi with a recent history of exposure to NNV antigen. The mean OD for all samples was 0.67 ($CI_{95\%}$ = 0.63–0.71%) and the ODR was 1.98 ($CI_{95\%}$ = 1.88–2.07%) so that the antibody response of the barramundi with unknown exposure was significantly lower than that of populations with history of exposure (exposed and immunized fish) ($p < 0.001$) (Fig. 2).

The mean NNV-antibody response of the barramundi broodstock that were sampled at Hatchery A in a longitudinal study increased over time (Fig. 3). Between 2007 and 2012, the mean OD ranged from 0.65 to 0.9 while the ODR increased substantially from 1.5 to 2.5, indicating that both the reactivity and the specificity of the antibodies towards NNV antigens increased overtime.

The cause of the increase in NNV-specific reactivity of sera in the ELISA was explored by analyzing the time each fish had been resident at Hatchery A. Cross-sectional analysis of results in 2012 indicated that a higher ODR was obtained for fish that had been resident at the hatchery for longer periods of time (Fig. 4). Fish recruited from 2003 to 2005 and from 2006 to 2008 had significantly higher test results than fish recruited from 2009 to 2011 ($p = 0.005$). The age of the fish was unknown, so that the association between ODR and age could not be assessed.

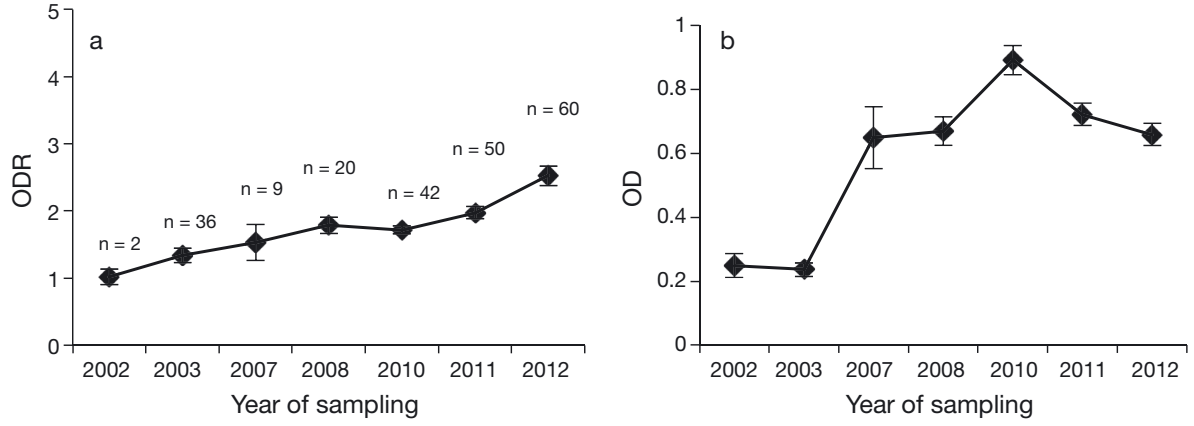

Fig. 3. Antibody levels (mean ± SE) for a population of barramundi at Hatchery A that had an unknown history of exposure to nervous necrosis virus (NNV). ELISA results are given in (a) optical density ratio (ODR) and (b) optical density (OD). ODR is the ratio between testing wells and blank wells for each sample

Table 4. Cohorts (given as mm/yy) with detection of nervous necrosis virus (NNV) by RT-qPCR at Hatchery A during the longitudinal study conducted from 2007. All detections were confirmed by virus isolation. Age: age at first detection; dph: days post-hatch. Mortality: High = above routine mortality; Low = within hatchery routine mortality

Cohort	Positive/tested	Pool size	Age (dph)	Clinical signs	Mortality	Observations
09/09	8/8	$4 \times 5 + 4 \times 10$	5	Yes	High	
10/09	7/7	10	34	Yes	High	
04/11	5/5	1	64	No	Low	Fish stressed due to transport and poor water quality conditions

Serostatus in broodstock and the occurrence of NNV infection in offspring

At Hatchery A, where ongoing surveillance was conducted on the broodstock and their respective cohorts of larvae, 3 batches out of 17 produced were infected with NNV during the observation period (2009–2012) (Table 4). Cohorts 09/09 (September 2009), 10/09 (October 2009) and 04/11 (April 2011) had tested positive by RT-qPCR and virus isolation, with a high prevalence of infection in each, cohorts 09/09 and 10/09 experienced clinical VNN while cohort 04/11 had a subclinical presentation of the infection. The occurrence of the disease in larvae was not associated with the serological status of the broodstock ($p = 0.29$, χ^2). For cohorts 09/09, 10/09 and 04/11, 2 out of the 9 spawners were NNV seropositive. The 14 batches that were not infected with NNV were produced from 50 broodstock, 9 of which were seropositive (ODR of up to 7.02) (Table 5).

RT-qPCR on blood and gonadal fluids

The virus could not be detected by RT-qPCR in any gonadal fluid samples (n = 164) that gave rise to affected and unaffected batches of fish. However, not all broodstock involved in the production of larvae could be sampled and tested with RT-qPCR.

DISCUSSION

Serological testing of fish populations for antibodies to NNV was a useful tool for understanding the immune response and epidemiology of VNN without the need for euthanasia of the fish. All populations surveyed in NT and QLD had a seroprevalence above 20%, suggesting widespread exposure to NNV. We observed that the serological status of the populations was dynamic over periods of years, possibly reflecting the pattern of ongoing exposure to the virus. There was evidence of an increase in both the reactivity and the specificity of the antibodies during the observation period, suggesting higher immunoaffinity and repetitive exposure to the viral antigens. This finding is similar to observations made by Lorenzen & Dixon (1991), who found that lymphocystis seroprevalence was higher in flounder *Platichthys flesus* of older age sampled from wild populations with healthy appearance. This was interpreted as a sign of frequent exposure of the population to lymphocystis virus.

As the newly introduced broodstock had the lowest NNV-specific immunoreactivity during the observation period and the fish were kept in tanks with continuous inflow of sand-filtered seawater, we believe that the fish were repeatedly exposed to the virus via the incoming water. It is possible that the exposure to the viral antigens occurred under one or more of the following circumstances: (1) The fish are exposed to sufficient quantities of the virus to trigger an immune response but not enough to cause infection/disease. (2) The fish are exposed to viral antigens but the virus itself is not infective. (3) The fish are not permissive to the virus at the age when they are exposed; regardless of the quantities of the virus, the infection did not become established.

Based on the results of this survey, we consider that the increased seroprevalence and seroconversion in adult barramundi are evidence of the virus being endemic in the surveyed populations at NT and QLD in Australia. While such data from fish populations are rare due to lack of published investigations, Whittington et al. (1999) found evidence of seroconversion in trout held in epizootic haematopoietic necrosis virus (EHNV) endemic farms.

This study indicates that broodstock selection based on screening tests for anti-NNV antibodies are not useful for the prevention of VNN outbreaks in barramundi larvae and juveniles. We could not identify a clear association between the serostatus of individual broodstock and the occurrence of VNN in their brood. We also found no association between the seropreva-

Table 5. Spawning cohorts and serology results for the individual broodstock fish that were involved with larval production in each cohort. These broodstocks were from spawning cohorts involved with the production of larvae that were considered infected or free of nervous necrosis virus (NNV) based on detection by RT-qPCR (Table 4) during the observation period at Hatchery A. Age: age at first detection; OD: optical density; ODR: optical density ratio; N/A: not available; N/D: not done

Cohort	Spawning date	Age (d)	Broodstock ID	Closest serum sampling date	OD	ODR	Call[c]
NNV-infected cohorts							
09/09	Sep 2009	5	0658C4DD	Apr 2012	0.42	2.32	–
			065DB5E7	Aug 2010	0.75	1.97	–
			065DC568	Jun 2011	0.78	2.1	–
10/09	Oct 2009	34	01C90BC8[a]	Apr 2012	0.87	2.99	+
			0658C2EA	Jun 2011	0.49	1.83	–
			065D865E	Apr 2012	0.43	2.5	–
04/11	Apr 2011	61	065D81C7	Jun 2011	0.40	2.20	–
			065DB5E7	Jun 2011	0.96	1.84	–
			065DB601	Jun 2011	1.02	2.65	+
NNV-free cohorts							
01/08	Jan 2008	N/A	065ECE78	Aug 2010	0.63	1.84	–
			065EC762[a]	N/D	N/D	N/D	N/D
			065ED4C5[a]	Apr 2012	0.27	1.84	–
03/08	Mar 2008	N/A	065D7D06[a]	Apr 2012	0.80	4.28	+
			065ED4C5[a]	Apr 2012	0.27	1.84	–
			065D99CF[a]	Jun 2011	0.42	2.29	–
04/08	Apr 2008	N/A	065EC8B1[a]	Jun 2011	0.53	1.28	–
			065DA7FO	Aug 2010	1.00	1.22	–
			065D8B78	Aug 2010	0.51	1.52	–
09/08	Sep 2008	N/A	01C8F901	Jun 2011	0.65	2.23	–
			065DC568	Jun 2011	0.78	2.11	–
			0658D8B7[a]	Apr 2012	0.88	4.07	+
11/08	Nov 2008	N/A	065DB5E7	Aug 2010	0.76	1.97	–
			0658B7CE[a]	Jun 2011	1.07	1.37	–
			065EBA11[a]	Jun 2011	0.43	1.28	–
02/09	Feb 2009	N/A	065DB5E7	Aug 2010	0.76	1.97	–
			065EBA11[a]	Aug 2010	0.43	1.28	–
			065EBE3A[a]	Jun 2011	0.53	1.22	–
04/09	Apr 2009	N/A	065DB5E7	Aug 2010	0.76	1.97	–
			0658E6CB	Aug 2010	0.75	2.25	–
			065DB991	Aug 2010	0.43	1.59	–
			065DB601	Aug 2010	1.00	1.95	–
02/10	N/A	N/A	065DB5E7	Aug 2010	0.76	1.97	–
			01FBA90A	Aug 2010	0.46	1.62	–
			0658BF3B[a]	Aug 2010	0.80	2.03	–
			0658E6CB	Aug 2010	0.75	2.25	–
			065DB601	Aug 2010	1.00	1.95	–
			065D7C90[a]	Aug 2010	0.62	2.11	–
			0658D85B	Aug 2010	0.68	2.18	–
			0658B9D7	Aug 2010	1.33	1.94	–
05/10	May 2010	N/A	01FAB749	Aug 2010	0.82	2.44	+
			0658B9D7	Aug 2010	1.33	1.94	–
			065DB991	Aug 2010	0.43	1.59	–
08/10	Aug 2010	N/A	01C90BC8	Apr 2012	0.87	3.00	+
			0658C4DD	Apr 2012	0.42	2.33	–
			065EAF45[a]	Apr 2012	0.43	1.55	–
			065DC697	Apr 2012	0.68	2.38	–
			065DC568	Jun 2011	0.78	2.11	–
01/11	Jan 2011	N/A	065DAD56[a]	Jun 2011	0.80	1.87	–
			0658BF3B[a]	Jun 2011	0.80	7.02	+
			065DB601	Jun 2011	1.02	2.65	+
09/11	Sep 2011	N/A	01C8F901	Jun 2011	0.65	2.23	–
			06E21B5B[a]	Aug 2011	0.45	1.75	–
			06E21DC9	Apr 2012	0.29	1.29	–
11/11	Nov 2011	N/A	01FA6E0E[a,b]	Jun 2011	0.58	2.80	+
			06E24B9E[a]	Jun 2011	0.61	1.28	–
			06E221BF	Jun 2011	0.80	1.11	–
			065D8B78[a]	Apr 2012	0.66	1.90	–
03/12	Mar 2012	N/A	01FA6E0E[a,b]	Apr 2012	0.51	2.81	+
			065D81C7	Jun 2011	0.40	2.20	–
			065D7C90[a]	Apr 2012	1.09	4.20	+

[a]Gonadal fluid tested by RT-qPCR
[b]Post-mortem examination of this fish was conducted in November 2012 soon after being euthanized due to unrelated poor health. Retina and brain samples tested negative to NNV by RT-qPCR.
[c]–: negative; +: positive

lence in resident broodstock populations at hatcheries and the history of occurrence of the disease in larvae. However, as the time of recruitment/year of sampling was an important predictor for serostatus in broodstock, our findings are limited by the lack of longitudinal data on hatcheries from QLD.

While Anderson & Oakey (2007) found negligible value in screening gonad fluids using nested RT-PCR, because of a lack of correlation between positive results and occurrence of VNN, we could not detect any NNV genome in gonadal fluids using a more sensitive and specific RT-qPCR. We concluded that currently available methods for the screening of broodstock, namely serology and nucleic acid detection tests in gonadal fluid, are ineffective for the prediction and control of VNN in barramundi larvae. Fin clips have been proposed for the non-lethal screening of NNV (Kuo et al. 2011). However, the test has not been validated and rather than suggesting the quest for more sophisticated tests, we question the paradigm of persistently infected barramundi broodstock acting as source of infection for the larvae.

The evidence presented here is consistent with a longitudinal study at the same hatchery in which it was conclusively demonstrated that outbreaks of VNN were caused by horizontal transmission of NNV from an environmental source (Hick et al. 2011a). This suggested that management interventions to prevent vertical transmission should be balanced with those to prevent horizontal transmission of NNV in barramundi hatcheries. Increasing seroprevalence in barramundi contributes further evidence that NNV is endemic in these barramundi farming regions in Australia; therefore, exposure to the virus is likely and can result in disease outbreaks when susceptible young fish are exposed.

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