



Differential expression profiling of orange-spotted grouper larvae, *Epinephelus coioides* (Hamilton), that survived a betanodavirus outbreak

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Abstract

Nervous necrosis virus (NNV), a piscine nodavirus, has caused serious viral nervous necrosis and viral encephalopathy and retinopathy in hatchery-reared larvae and juveniles of a wide range of marine teleost species worldwide in the last two decades. Although the mortality of NNV-infected larvae is nearly 100%, there are still some larvae that survive this catastrophe. To comprehensively understand the variations of these survivors at the molecular level, we collected orange-spotted grouper larvae that survived an NNV outbreak in an indoor hatchery in southern Taiwan to study differential gene expression. Healthy larvae with high, medium and low levels of detected NNV were compared with morbid larvae using a 9600-clone-containing grouper larva cDNA microarray, and differential gene expression was further confirmed by a quantitative real-time polymerase chain reaction. Significant variation exists in healthy larvae. The following genes were upregulated: adenylate kinase 1-2, myosin binding protein H-like, myosin light chain 2, myosin light chain 3, tropomyosin, fast/white muscle troponin T embryonic isoform, and parvalbumin 1 and 2 genes. The following genes were downregulated: apolipoprotein A-I, trypsinogen, pyruvate kinase and astacin-like metalloprotease. Moreover, immunoglobulin M heavy chain gene transcription was significantly higher in healthy larvae that had high virus levels, indicating that

humoral immunity might protect organisms from viral infection. These results suggest that some non-immune-related genes may have played important roles in survival during the larval metamorphosis stage, after betanodavirus infection.

Keywords: betanodavirus, DNA microarray, larvae, orange-spotted grouper, quantitative real-time polymerase chain reaction.

Introduction

Piscine nodaviruses can infect more than 30 species of marine fish during the seedling period and the culture process; it is one of the major viral pathogens that have seriously damaged the aquaculture industry worldwide. The nearly 100% mortality caused by the virus results in significant economic losses. Typical clinical signs include a change in body colour, loss of appetite and an abnormal spiral swimming behaviour (Munday, Kwang & Moody 2002). The causative agent is a nodavirus that belongs to the genus *Betanodavirus*, family Nodaviridae. It contains two single-stranded positive-sense RNA molecules (RNA1 and RNA2) that encode two main proteins – RNA-dependent RNA polymerase and a coat protein (Nagai & Nishizawa 1999).

After a virus crosses anatomical barriers and penetrates host cells, many events are triggered by host–virus interactions. To survive a host's defence mechanisms, viruses have evolved processes to prevent or delay attacks by host cells and further utilize the host's machinery to produce the material for viral replication and increase viral progeny (Tortorella,

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Gewurz, Furman, Schust & Ploegh 2000). In contrast, to survive a virus attack, the innate immune system of the host employs several strategies for antiviral defence, such as interferon (IFN)-induced Mx responses, inflammatory reactions and phagocytosis (Lieschke & Trede 2009). Recently, three forms of Mx genes were identified in the orange-spotted grouper, *Epinephelus coioides* (Hamilton), and were demonstrated to be Mx proteins that can inhibit nervous necrosis virus (NNV) propagation (Lin, John, Lin & Chang 2006). Several immune responses were also reported against betanodavirus infections, including interferon regulatory factor 1, interleukin (IL)-1 β , IL-8, IL-10, tumour necrosis factor (TNF)- α , transforming growth factor (TGF)- β , cyclooxygenase (COX)-2, viperin and immunoglobulin M (IgM) (Poisa-Beiro, Dios, Ahmed, Vasta, Martinez-Lopez, Estepa, Alonso-Gutierrez, Figueras & Novoa 2009; Montes, Figueras & Novoa 2010; Rise, Hall, Rise, Hori, Browne, Gamperl, Hubert, Kimball, Bowman & Johnson 2010; Scapigliati, Buonocore, Randelli, Casani, Meloni, Zarletti, Tiberi, Pietretti, Boschi, Machado, Martin-Antonio, Jimenez-Canizano, Bovo, Borghesan, Lorenzen, Einer-Jensen, Adams, Thompson, Alonso, Bejar, Cano, Borrego & Alvarez 2010). Moreover, it was found that some non-immune-related proteins participate in the host–virus interaction, such as mda-5, galectin, ATP-dependent RNA helicase DHX58, sarsin and heat shock protein 90AB (Dios, Poisa-Beiro, Figueras & Novoa 2007; Poisa-Beiro *et al.* 2009; Chen, Kuo, Wang, Shie, Wang, Huang, Tsai, Chen, Chen & Chen 2010; Rise *et al.* 2010). To investigate molecular variation in hosts, differential gene expression profiles in specific tissues were also obtained after nodavirus infection from various cultured fish, including sea bream, *Sparus aurata* L.; turbot, *Scophthalmus maximus* (L.); Atlantic halibut, *Hippoglossus hippoglossus* (L.); sea bass, *Dicentrarchus labrax* (L.); and Atlantic cod, *Gadus morhua* L. (Dios *et al.* 2007; Park, Osborne, Montes, Dios, Nerland, Novoa, Figueras, Brown & Johnson 2009; Patel, Malde, Lanzén, Olsen & Nerland 2009; Poisa-Beiro *et al.* 2009; Rise *et al.* 2010).

Most previous research used juvenile or adult stages of fish as test specimens (Rise *et al.* 2010; Scapigliati *et al.* 2010) and used experimental situations to investigate related immune responses to either a direct virus challenge (Poisa-Beiro *et al.* 2009; Montes *et al.* 2010) or challenge after vaccination using different antigens (e.g. inactivated virus, recombinant proteins showing good

antigenicity). Although mature fish have well-established immune systems and are easy to handle, they may be inappropriate models for virus infection, given that NNV primarily infects larvae. Together, the combination of the different stage of life of the organism and artificial manipulations might not reflect the real situations in which infection occurs. We were curious about the extremely small number of larvae that survive NNV infection and fortunately obtained NNV-infected, metamorphosis-stage orange-spotted grouper samples from an indoor hatchery in southern Taiwan, including both morbid and surviving larvae. In an attempt to determine what responses in the host act against viral infection, differential gene expression profiles of grouper larvae were examined using a whole-larvae cDNA microarray and results were further validated using a quantitative real-time polymerase chain reaction (qPCR) method.

Materials and methods

Clinical samples and RNA preparation

Clinical grouper larvae samples (body length 1.8–2.4 cm; body weight 0.07–0.18 g) were kindly provided by an indoor hatchery in Pingtung, Taiwan. Ten larvae of each were randomly collected from larvae with normal and abnormal swimming patterns at 35 days after hatching. Total RNA of a whole larva was isolated immediately using a TRIzol[®] reagent extraction (Invitrogen), followed by DNase I (Qiagen) digestion and clean-up using an RNeasy[®] Mini kit (Qiagen). RNA quality control using an RNA 6000 Nano Chip kit (Agilent) was performed before further experiments. For viral titre comparison, total RNA of 1 mL GB-3 cell cultured 10⁷ TCID₅₀ mL⁻¹ yellow grouper NNV (YGNNV) (Lai, Murali, Chiu, Ju, Lin, Chen, Guo, Fang & Chang 2001) was isolated and prepared as above.

Viral detection by semi-quantitative reverse transcription polymerase chain reaction

Reverse transcription was performed with 1 μ g of total RNA and a random hexamer in a final 25- μ L reaction using MMLV reverse transcriptase (Promega). The reaction proceeded at 37 °C for 1 h, followed by 72 °C for 10 min. The NNV coat protein (NNVCP) was determined by reverse transcription polymerase chain reaction (RT-PCR)

using specific primers (forward primer CP1 5'-AATGGTACGCAAAGGTGA-3' and reverse primer CP4 5'-TTCTGCTTTCCCACCAT-3') and EconoTaq PLUS Green 2x Master Mix (Lucigen) with an aliquot (0.1 µL) of cDNA in a final 10-µL reaction. Amplification conditions were an initial denaturation at 95 °C for 3 min, followed by 27 amplification cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a final elongation at 72 °C for 10 min. β -Actin was used as an internal control using the specific primers: forward primer actinF (5'-CACACCTTCTACAATGAGCTG-3') and reverse primer actinR (5'-CGGTCAGGATCTTCATGAGG-3'). NNVCP standard was generated from a 10-fold serial dilution of cDNA that was synthesized from total RNA of 10^7 TCID₅₀ mL⁻¹ YGNNV supernatant. NNVCP amplicons were sequenced and compared with published nodavirus sequences in the NCBI database using the BLASTX program.

cDNA library construction and cDNA chip preparation

The construction of an orange-spotted grouper larvae cDNA library was performed as previously described (Cheng, John, Wu, Lee, Lin, Lin & Chang 2006). Briefly, total RNAs from 1-, 2-, 3- and 4-week-old NNV-free grouper larvae (whole weight 0.5 g) were extracted by TRIzol[®] reagent (Invitrogen), followed by FastTrack[®] 2.0 mRNA Isolation Kit (Invitrogen) to purify messenger RNAs. Messenger RNAs from whole larvae of different ages were pooled equally to accomplish cDNA synthesis and library construction using a ZAP-cDNA[®] Synthesis Kit (Stratagene) and Gigapack[®] III Gold Packaging Extract (Stratagene), according to the manufacturer's protocols. A total of 9600 clones were randomly selected and amplified from the constructed library. Plasmid DNA was extracted with a Mini Plus[™] Plasmid DNA Extraction System (Viogene), and the purified plasmid DNA was re-dissolved in 50% DMSO and spotted onto GAPS[™] II-coated slides (Corning).

Microarray analysis

A microarray analysis was carried out as previously described (Yeh, Chen, Wu, Chen, Yuan, Pan, Chang, Chuang & Chang 2008). Briefly, total RNA was extracted from grouper larvae with different health status as described above. On the basis of viral detection results, total RNAs from

each whole larva were classified and pooled into four subgroups isometrically: morbid larvae, healthy larvae with high viral content (hvh), medium viral content (hvm) and low viral content (hvl). The cDNA of each subgroup was synthesized from 10 µg of total RNA with an anchored oligo(dT)₂₀ primer and was indirectly coupled with Alexa Fluor[®] dyes using a SuperScript[™] Plus Indirect cDNA Labelling System (Invitrogen), according to the manufacturer's protocol. The cDNAs of each health subgroup (hvh, hvm and hvl) were labelled with Alexa Fluor[®] 647, and the cDNA of the morbid subgroup was labelled with Alexa Fluor[®] 555. Meanwhile, the cDNA array was prehybridized at 42 °C for 1 h with blocking buffer, which included 1× bovine serum albumin, 4× saline-sodium citrate buffer (SSC) and 0.5% sodium dodecyl sulphate (SDS). After washing with sterilized water twice and isopropanol once, the chip was dried by a short centrifugation. This prehybridized array was hybridized with a mixture that included equal volumes of Alexa Fluor[®] 647 and 555 dye-labelled cDNA at 42 °C for 16 h. After hybridization, the glass slide was washed at room temperature with shaking; the washing solutions were as follows: 2× SSC with 0.1% SDS, 1× SSC with 0.1% SDS, 0.5× SSC and 0.1× SSC. The glass slide was dried by a short centrifugation and directly scanned using a high-resolution DNA microarray scanner (Agilent), and the corresponding images and GPR files were produced automatically. To obtain the appropriate experimental data, two biological replicates were performed for each comparison. After Lowess normalization and baseline transformation to the median of all samples, the obtained microarray data, which were filtered by present flag value, were further filtered by twofold expression and the entity lists were created by GeneSpring GX 10 (Agilent).

Sequencing and homology search

DNA sequencing was performed by an ABI 3730xl DNA Analyzer (Applied Biosystems) and edited by removing vector sequences using DNASTAR software. The resulting nucleotide sequences were used as query sequences to search the translated nucleotide database at NCBI using the BLASTX program.

Quantitative real-time polymerase chain reaction

The cDNAs of each sample were prepared and pooled isometrically into four subgroups as de-

scribed above. Ten microlitres of the reaction contained 3.33 μL of 50-fold-diluted cDNA of each subgroup, 0.33 μL of a gene-specific forward/reverse oligo primer mixture and 5 μL of Maxima™ SYBR Green/ROX qPCR Master Mix (2 $\times$) (Fermentas) and was adjusted with sterilized water. The reaction was performed using a LightCycler® 480 Real-Time PCR System (Roche). Amplification conditions were an initial denaturation at 95 °C for 10 min, followed by 45 amplification cycles of 95 °C for 15 s and 60 °C for 1 min, with a final melting curve step. The delta-delta Ct method was performed to calculate the relative quantity values of the experimental genes. Each experiment was performed in triplicate, and the mean of each gene expression was calculated from at least three experiments. Gene-specific oligo primers used in this study were designed by Primer Express® software version 3.0 (Applied Biosystems) (Table 1). 18S ribosomal RNA was used as an internal control for the qPCR.

Statistical analysis

The qPCR data were compared using the paired samples *t*-test by PASW Statistics 18.0 software (IBM), and the level accepted for statistical significance was $P < 0.05$.

Results and discussion

Clinical fish samples

Nervous necrosis virus-infected, orange-spotted grouper metamorphosis-stage larvae from the hatchery in southern Taiwan in June 2009 started to exhibit the abnormal swimming behaviour that was similar to the spiral swimming pattern caused by NNV infection 25 days after hatching. Morbid larvae died within 2–3 days, and this epidemic spread to the entire population within a week. The morbidity was nearly 100% and mortality was more than 99% at 35 days after hatching. These surviving late-metamorphosis larvae were in a stable condition at 40 days post-hatching.

Nodavirus detection

Ten larvae of each were randomly collected from normal and abnormally swimming larvae at 35 days post-hatching. After the RNA quality control test, one of the normal swimming samples was

eliminated. Using an NNV coat protein (NNVCP)-specific primer set, clear predicted bands were amplified in ten morbid larvae by 27 cycles of semi-quantitative RT-PCR. However, amplicon yields showed significant variation in normal larvae. Similar bright bands to those of the abnormal samples appeared in two samples (more than 10^5 TCID₅₀ mL⁻¹ NNV), minor bands appeared in another four larvae (about 10^3 – 10^4 TCID₅₀ mL⁻¹ NNV) and extremely weak bands appeared in another three larvae ($< 10^3$ TCID₅₀ mL⁻¹ NNV), in the agarose gel analysis (Fig. 1). With reference to the NNVCP standard, all abnormally swimming larvae were classified into one subgroup (morbid), and normal swimming larvae were classified into three subgroups: healthy but with a high (hvh), medium (hvm) and low virus content (hvl). Amplicons were sequenced and analysed using the NCBI database, and results indicated that the disease-causing virus belonged to the RGNNV genotype (data not shown).

Survey of variant gene expression using a cDNA microarray

Although the main target region of nodaviruses is the central nervous system (CNS), we tried to determine how the whole body resists the viral infection. In this experiment, a transcriptomic analysis was performed using a whole grouper larvae cDNA microarray to explore variations in gene expression between morbid larvae and three groups of healthy grouper larva containing different levels of virus. This microarray, containing 9600 clones from an orange-spotted grouper larvae cDNA library, was hybridized with dual-colour dye-labelled probes from samples of morbid larvae and healthy subgroups containing different levels of virus. After normalization, a statistical analysis of the twofold expression threshold was calculated: 79 upregulated (expression higher in healthy larvae) and 351 downregulated clones (expression higher in diseased larvae) were found. After sequencing and searching in the NCBI database, 45 genes and 19 unknown sequences (considered novel identifications) were found and accession numbers were obtained. Among these 430 clones, 14 genes and three unknown sequences were upregulated in larvae surviving the NNV outbreak (Table 2), whereas 31 genes and 16 unknown sequences were downregulated (Table 3). These viral-repressed and viral-induced genes might contribute to host defence after nodavirus infection or transform the

Table 1 Specific gene designed quantitative PCR primers used in this study

Primer	Abbreviation	Sequence
18S ribosomal RNA F	18S	5'-CGCTCTGAGGGTAACTGATGG-3'
18S ribosomal RNA R		5'-GTCCTTTGGGAGCAGCCATG-3'
Adenylate kinase 1-2 F	ADK1-2	5'-CGAGAAGAAGATCGGCAAACC-3'
Adenylate kinase 1-2 R		5'-ACGGCCGCTGGTCTCA-3'
Cytochrome c oxidase subunit 7C F	CxIV7C	5'-GGCTTTGCATTCCCTTCAT-3'
Cytochrome c oxidase subunit 7C R		5'-TATGCTCCAGCTGACGACCTT-3'
Glyceraldehyde 3-phosphate dehydrogenase F	GAPDH	5'-CGACCCCTCACTCCTCCATCTT-3'
Glyceraldehyde 3-phosphate dehydrogenase R		5'-GCTGTAGCCGAACGCTTGTGTC-3'
Nucleoside diphosphate kinase B F	NDKB	5'-CCGCAACCAAGCCTTCTATC-3'
Nucleoside diphosphate kinase B R		5'-AACGCTTAATGATCTCGCCG-3'
Histone H2B F	H2B	5'-AAAGAAAGGCGCCACAAAGA-3'
Histone H2B R		5'-GCTTCAGCACCTTGACACGTAGA-3'
Myosin binding protein H-like F	Mybp-H	5'-TGGAGACGACCCCCAAGTACAG-3'
Myosin binding protein H-like R		5'-CAGGGCCTTGACAGGAGTACA-3'
Myosin light chain 2 F	MLC2	5'-CATGTTTGAGCAGAGCCAGATT-3'
Myosin light chain 2 R		5'-AGCACGTCCCTCAGGTCATC-3'
Myosin light chain 3 F	MLC3	5'-TCCAGCTCCTTCTCTCCAAAAT-3'
Myosin light chain 3 R		5'-ACCTGGCTGTACCGACTCT-3'
Tropomyosin F	TM	5'-GCCCCGAAGCTGGTCATCATT-3'
Tropomyosin R		5'-TTTTCAACTTTCCTCAAGCTCAGA-3'
Troponin I F	TnI	5'-AGGCCGCCCTCATGGA-3'
Troponin I R		5'-TTGTCGGCCTTCTGCACTTT-3'
Fast/white muscle troponin T embryonic isoform F	TnT	5'-AGAAGTTTCGACCACATGGAGAGA-3'
Fast/white muscle troponin T embryonic isoform R		5'-GCTCCCTTCTTGCTGAATTTACTG-3'
Tributyltin binding protein type 2-like F	TBTbp2	5'-TTAGCCCTAGAGGCCGATACC-3'
Tributyltin binding protein type 2-like R		5'-GCCTGTTTCTTGATTGTATG-3'
Parvalbumin 1 F	PV1	5'-GGAGACAAACCTAAATCAAAAATGG-3'
Parvalbumin 1 R		5'-GCTTGAAAGAGTCGGCAGCTT-3'
Parvalbumin 2 F	PV2	5'-CCTTCGCTGGTGTCTCAAT-3'
Parvalbumin 2 R		5'-GCCGCATGCCTTGAAGAA-3'
Astacin like metalloprotease F	Metallo	5'-AGCCGTGGCTGCAAGTG-3'
Astacin like metalloprotease R		5'-CGGGTGATGATGGCCTTCT-3'
Pyruvate kinase F	PK	5'-GAGCCGTGAGACTGCCAAG-3'
Pyruvate kinase R		5'-CATCTGCCTGTGGAACATGG-3'
Apolipoprotein A-I F	ApoA-I	5'-GCCCCACCGTCAAAATCTTA-3'
Apolipoprotein A-I R		5'-CAGCGTGCCTGGTGAATA-3'
Trypsinogen 1a F	Tryp	5'-GAGTCACCGAGGGCAAGAG-3'
Trypsinogen 1a R		5'-GCTTGATCAGCATGATGTCATTG-3'
Eclatsa001 F	Ec001	5'-ACTTCTCCAGCCGCTCTTT-3'
Eclatsa001 R		5'-ACACCCCTCCACAGCCTTCAG-3'
Mx-I F	Mx1	5'-AAGTGGTGCGGGAAGATAAGC-3'
Mx-I R		5'-AGAGGCAATGTCCAGGGTGAT-3'
Mx-II F	Mx2	5'-GGAGGAACCAAGCTGTCAAGAGA-3'
Mx-II R		5'-GTGGACTCTTTTCTTGCTTAATGG-3'
Mx-III F	Mx3	5'-TCGACCTCATCCACCGTATTC-3'
Mx-III R		5'-TGTCACAATGCCACTTCCTCTT-3'
Complement component 3 F	C3	5'-CCGCACTGCAGGGATTCTT-3'
Complement component 3 R		5'-TGCTTCTGTGGGCCAGTACTC-3'
Cluster of differentiation 8 α F	CD8a	5'-AGGAGCCGGTGAAGACGTAA-3'
Cluster of differentiation 8 α R		5'-GAGAACTCGAAACCAGATGACCAT-3'
Cluster of differentiation 8 β F	CD8b	5'-CTAACATCACCTGTGACTACGTCTACTG-3'
Cluster of differentiation 8 β R		5'-TGCTCCTCTGCTTATTTTGAAC-3'
Interleukin-1 β F	IL1b	5'-CCCAGATGACATGCAACGTT-3'
Interleukin-1 β R		5'-GCCACGTGCTTCATTGTCACT-3'
Interleukin-8 F	IL8	5'-CCTGACAGAGAGCAACCCATT-3'
Interleukin-8 R		5'-CTTCTTACCCAGGGAGCTT-3'
Tumour necrosis factor α F	TNF α	5'-CTCAGGGCGGCTTCACT-3'
Tumour necrosis factor α R		5'-TCGCTGCAGGAGACTCTGAAC-3'
Transforming growth factor β F	TGF β	5'-CTTCCCGCTTCATCACCA-3'
Transforming growth factor β R		5'-CTCCGCTATCCTCATTTCC-3'
Cyclooxygenase-2 F	COX2	5'-AGAGGCAGCACAAGCTCAGACT-3'
Cyclooxygenase-2 R		5'-TCGACACCCACTTCCTTGACT-3'
Immunoglobulin heavy chain V _H -I F	IgMhc	5'-ATACGTCGCTTTACTTCCCATCA-3'
Immunoglobulin heavy chain V _H -I R		5'-TTCCCTAAGTGGCTCTATCCAGTCTT-3'



Figure 1 Reverse transcription polymerase chain reaction amplification of nervous necrosis virus coat protein (NNVCP) gene. (a) Naturally infected grouper larvae. Lanes 1–10, morbid larvae samples; lanes 11–19, healthy larvae samples with different viral contents (lanes 11–12, high; lanes 13–16, medium; lanes 17–19, low). β -Actin was used as an internal control. (b) Tenfold serial dilution of yellow grouper NNV for viral titre comparison. Lane 1–6, 10^1 – 10^6 TCID₅₀ mL⁻¹.

Table 2 Twofold relative upregulated grouper genes after microarray analysis

Genes	Accession no.	Repeats		
		Hvh	Hvm	Hvl
Energy				
Adenylate kinase 1-2	HQ207653			4
Cytochrome c oxidase subunit 7C, mitochondrial precursor	GU982569			1
Glyceraldehyde 3-phosphate dehydrogenase	GU982531		1	1
Nucleoside diphosphate kinase B	GU982527			1
Structure				
Histone H2B	GU982558		2	
Muscle contraction				
Myosin binding protein H-like	GU982537	2	2	3
Myosin light chain 2	HQ207654	1	2	5
Myosin light chain 3	HQ207658		4	1
Tropomyosin	HQ207648			12
Troponin I	GU982534			2
Fast/white muscle troponin T embryonic isoform	HQ207651		3	17
Lipid metabolism				
Tributyltin binding protein type 2-like protein	HQ207659		1	1
Nervous system				
Parvalbumin 1	HQ207649			3
Parvalbumin 2	GU933125			17
Others				
EclaEST0002	HO663750			1
EclaEST0003	HO663751		1	
EclaEST0004	HO663752			1

host into an environment fit for viral survival, replication and progeny production.

qPCR validation

To validate the cDNA microarray data, a gene-specific qPCR was performed. On the basis of a

quantitative analysis, the trend of 14 upregulated genes was negatively correlated with the viral content of larvae (Fig. 2a). Among these 14 genes, the transcriptomic expression levels of only three genes were at least twofold higher than those of diseased larvae at all three virus-containing levels, including H-like myosin binding protein (Mybp-H), myosin light chain 2 (MLC2) and the embryonic isoform of fast/white muscle troponin T (TnT) (Table 4). Five genes showed around twofold higher significant induction in the hvl-larvae than diseased larvae; these genes were adenylate kinase 1-2 (ADK1-2), myosin light chain 3 (MLC3), tropomyosin (TM) and two types of parvalbumin (PV1 and PV2). ADK is a phosphotransferase enzyme that participates in adenine nucleotide interconversion; MLC2, MLC3, Mybp-H, TM, TnT and PV are plentiful in skeletal muscle cells, implying involvement in muscle contraction (Cantino, Brown, Chew, Luther & Squire 2000; Galinska-Rakoczy, Engel, Xu, Jung, Craig, Tobacman & Lehman 2008). As mentioned above, betanodavirus-infected larvae displayed clinical signs that include unnatural swimming patterns, such as swirling and intermittent burst swimming, which might have been due to functional deficiencies in some movement- or nerve-related genes. With screening at greater than twofold expression and confirmation with qPCR, the results of upregulated genes in survivors, including energy- and muscle contraction-related functions, support the hypothesis that diseased larvae have motor deficits. PV was the major protein among upregulated genes, accounting for 25.3% (20, two types) of the total 79 clones. PV has a helix-loop-helix structure that can specifically bind calcium ions, and its distribution includes skeletal muscles, brain and retinas (Endo, Kobayashi, Kobayashi & Onaya 1986). In addition to contributing to acceleration of muscle movements, PV is also implicated in the CNS. PV-expressing GABAergic interneurons pre-

Table 3 Twofold relative downregulated grouper genes after microarray analysis

Genes	Accession no.	Repeats		
		Hvh	Hvm	Hvl
Proteolysis				
Alpha-2-macroglobulin	GU982539	3	5	5
Astacin like metalloprotease	GU982555	22	23	23
Chymotrypsinogen	GU982573			1
Chymotrypsinogen 2	HQ207652	2	2	2
Chitinase	GU982589	1		
Elastase 1 precursor	HQ207655			1
Serine/cysteine proteinase inhibitor	GU982595			1
Trypsinogen	GU982529			18
Trypsinogen 1a	HQ207647			44
Energy				
Fructose-bisphosphate aldolase A	GU982528	8		
Lactate dehydrogenase-A	GU982565	2		
Putative malate dehydrogenase	GU982594			1
Pyruvate kinase	GU982526	30	20	29
Structure				
Collagen, type X, alpha 1	GU982557	1		
Fetuin B	GU982581	2	2	2
Fibrinogen alpha chain	GU982586			1
Fibrinogen beta chain precursor	GU982579			3
Ictacalcin	GU982543	4	2	
Lily-type lectin	GU982544	4	4	3
Muscle-specific beta 1 integrin binding protein 2	HQ207660	1		
Lipid metabolism				
14 kDa apolipoprotein	HQ207650		5	13
Apolipoprotein A-I	HQ207646		3	89
Apolipoprotein A-IV1	HQ207656	1		
Similar to apolipoprotein B	GU997624	9	6	8
Liver-type fatty acid-binding protein	GU982566	1		
Immune related				
Complement C1q-like adipose specific protein	GU982535	1		
Nucleotide hydrolysis				
Similar to inosine-uridine preferring nucleoside hydrolase	GU982593	1		
Others				
Prostate stem cell antigen precursor-like	GU982584			1
Proto-oncogene protein c-Fos	GU982590		1	
Similar to sperm plasma glycoprotein 120	GU982532	7	8	10
Seminal plasma glycoprotein 120	GU982591	1	1	1
EclaEST0005	HO663753	1	1	1
EclaEST0008	HO663756	1	1	
EclaEST0010	HO663758	1	1	
EclaEST0011	HO663759		1	
EclaEST0012	HO663760		1	1
EclaEST0013	HO663761	1	1	1
EclaEST0015	HO663763	1		1
EclaEST0016	HO663764	1		1
EclaEST0018	HO663766		1	1
EclaEST0020	HO663768			1
Eclatsa001	HP429415		25	21
Eclatsa002	HP429416	3	3	3
Eclatsa003	HP429417	1	2	2
Eclatsa004	HP429418	1	2	1
Eclatsa005	HP429419	1	2	2
Eclatsa006	HP429420			3

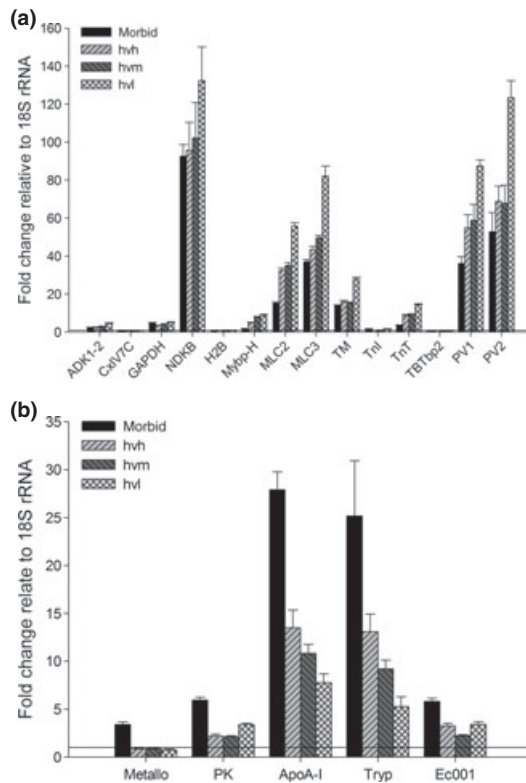


Figure 2 Quantitative polymerase chain reaction (PCR) validation of grouper genes selected from a twofold microarray analysis. Fourteen upregulated genes (a) and five downregulated genes (b) were chosen for validation using qPCR. 18S ribosomal RNA was used as an internal control, and its expression level is indicated in each panel by a continuous line. ADK1-2, adenylate kinase 1-2; CxIV7C, cytochrome *c* oxidase subunit 7C; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NDKB, nucleoside diphosphate kinase B; H2B, histone H2B; Mybp-H, H-like myosin binding protein; MLC2, myosin light chain 2; MLC3, myosin light chain 3; TM, tropomyosin; TnI, troponin I; TnT, fast/white muscle troponin T (embryonic isoform); TBTbp2, tributyltin binding protein type 2-like; PV1, parvalbumin 1; PV2, parvalbumin 2; Metallo, astacin-like metalloprotease; PK, pyruvate kinase; ApoA-I, apolipoprotein A-I; Tryp, trypsinogen 1a; Ec001, Eclatsa 001.

dominantly exist in chandelier and basket cells in the cortex and are subdivided into several subtypes of pyramidal cells in the hippocampus that contribute to network oscillations (Klausberger, Marton, O'Neill, Huck, Dalezios, Fuentealba, Suen, Papp, Kaneko, Watanabe, Csicsvari & Somogyi 2005). PV is also regarded as a good marker for amacrine cells, which are interneurons in the retina, and might participate in the maturation of amacrine cells in retinal differentiation, as a result of variations in PV expression during the growth period (Bejarano-Escobar, Blasco, Degrip,

Martín-Partido & Francisco-Morcillo 2009). After viral infection, vacuolated lesions and propagated viruses can be detected in the CNS including brain, spinal cord and retina (Lai *et al.* 2001), which are also areas in which PV is distributed. qPCR results indicate that PV expression was negatively correlated with viral content, implying PV's potential anti-NNV role.

Four downregulated genes and one novel sequence were positively correlated with the viral content of larvae (Fig. 2b). The two largest proportions of downregulated genes were apolipoprotein A-I (ApoA-I) and trypsinogen (two types), accounting for 25.4% (89) and 17.7% (62) of the total 351 clones, respectively. ApoA-I is the major protein component of high-density lipoprotein in plasma and is implicated in the efflux of cellular cholesterol from tissues to the liver (Eisenberg 1984). A previous report indicated that overexpression of human ApoA-I resulted in the inhibition of lipopolysaccharide-induced serum cytokines: IL-1 β , IL-6 and TNF- α (Li, Dong & Wu 2008). Trypsinogen is the precursor of the pancreatic enzyme, trypsin, a serine protease found in the digestive system and a useful enzyme used in tissue culture. Abnormal trypsinogen activation might cause cell apoptosis and in turn result in pancreatitis (Ji, Gaiser, Chen, Ernst & Logsdon 2009). After nodavirus infection, the virus might induce expression of ApoA-I and trypsinogen to interfere with host self-defence systems, to create a suitable environment for virus propagation. With quantitative validation, significant induction of pyruvate kinase (PK) and astacin-like metalloprotease was also seen in diseased larvae. PK is an enzyme involved in the final step of making pyruvate in glycolysis. Astacins are a family of zinc-dependent metalloproteases, which are involved in morphogenesis, digestion, extracellular matrix formation and fish embryonic hatching (Semenova & Rudenskaya 2009). Furthermore, 46 clones (13.1%) belonged to a novel sequence (Eclatsa 001) that had significantly higher induction compared with all healthy larvae. Further research is required to elucidate the real roles of these downregulated genes in host–virus interactions within betanodavirus-infected larvae.

Detection of expression of immune-related genes

Although there were no typical immune response-related genes detected by the microarray data, we

Table 4 Related gene expression level in grouper larvae of different health status vs. morbid larvae

Subgroup	Upregulated genes														
	ADK1-2	CxIV7C	GAPDH	NDKB	H2B	Mybp-H	MLC2	MLC3	TM	TnI	TnT	TBTbp2	PV1	PV2	
Hvh	1.06*	1.15	0.77	1.03	1.07	2.86*	2.14*	1.17*	1.14*	0.47*	2.38*	1.99*	1.52*	1.30*	
Hvm	1.28*	1.42*	0.92	1.10	1.07	4.60*	2.31*	1.34*	1.11*	0.58*	2.46*	1.95*	1.63*	1.28	
Hvl	1.95*	1.55*	1.12	1.43	0.93	5.08*	3.68*	2.22*	1.99*	1.02	3.95*	1.62*	2.42*	2.34*	
Subgroup	Downregulated genes														
	Metallo	PK	ApoA-I	Tryp	Ec001										
Hvh	0.26*	0.37*	0.48*	0.52	0.56*										
Hvm	0.26*	0.36*	0.39*	0.37	0.39*										
Hvl	0.22*	0.57*	0.28*	0.21*	0.59*										
Subgroup	Immune-related genes														
	Mx1	Mx2	Mx3	C3	CD8a	CD8b	IL1b	IL8	TNFa	TGFb	COX2	IgMhc			
Hvh	0.46*	0.55*	0.38*	0.39*	0.68*	0.69*	0.27*	0.21*	0.51	0.91	0.38*	1.56*			
Hvm	0.21*	0.25*	0.28*	0.29*	1.05	1.18	0.24*	0.18*	0.52	0.79	0.46*	0.36*			
Hvl	0.27	0.25*	0.21*	0.24*	1.04	1.10	0.39*	0.32*	0.78	0.80	0.40*	0.33*			

Full gene names as in the legends of Figs 2 & 3.

*Significant difference ($P < 0.05$) between individual healthy subgroups and the morbid subgroup.

selected 12 immune-related genes that have been reported in response to nodavirus treatment and evaluated whether their expression levels varied with different infection status using real-time qPCR, including Mx-I, Mx-II, Mx-III, complement component 3 (C3), cluster of differentiation (CD) 8 α and β subunits, IL-1 β , IL-8, TNF- α , TGF- β , COX-2 and IgM heavy chain (IgMhc) (Lin *et al.* 2006; Overturf & Lapatra 2006; Poisa-Beiro *et al.* 2009; Scapigliati *et al.* 2010). Significant variations were observed between healthy and morbid organisms in ten genes, but not TNF- α and TGF- β . However, expression levels of all genes were lower than 18S ribosomal RNA, except IgMhc in hvh-larvae (Fig. 3). IFN-inducible Mx proteins can be detected soon after stimulation with either poly I:C or virus and showed non-specific antiviral properties (Leong, Trobridge, Kim, Johnson & Simon 1998). Lin *et al.* (2006) demonstrated the propagation inhibition of three types of Mx proteins against a betanodavirus in grouper cell culture. The expected higher expression of Mx proteins was not observed in the surviving larvae in this study but was observed in morbid larvae, and Mx expression showed a positive correlation with the viral content within organisms, as did C3 expression. C3 is a humoral immune gene that plays a central role in complement activation, which contributes to innate and acquired immunity, and significant C3 induction was also detected after virus challenge in teleosts (Overturf & Lapatra 2006). C3 had the second highest expression in all grouper larvae, and the higher level was also seen in morbid larvae.

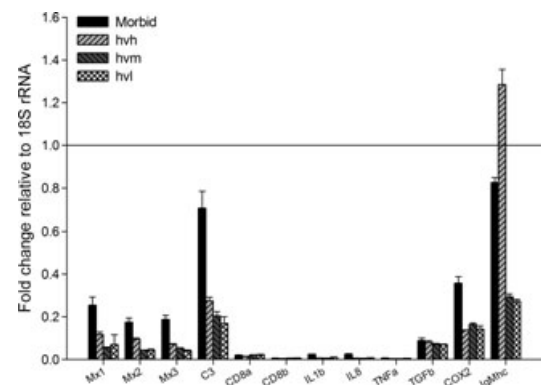


Figure 3 Quantitative polymerase chain reaction (PCR) detection of immune-related grouper genes. Expression levels of immune-related genes in larvae with different viral levels were evaluated by qPCR. 18S ribosomal RNA was used as an internal control, and its expression level is indicated by a continuous line. Mx1, Mx-I; Mx2, Mx-II; Mx3, Mx-III; C3, complement component 3; CD8a, CD8 α ; CD8b, CD8 β ; IL1b, interleukin-1 β ; IL8, interleukin-8; TNFa, tumour necrosis factor α ; TGFb, transforming growth factor β ; COX2, cyclooxygenase-2; IgMhc, immunoglobulin M heavy chain.

Several cytokines were also investigated, and significant induction of IL-1 β , IL-8 and COX-2 was seen in diseased larvae. These results reveal that the expression of immune-related molecules was elevated after viral invasion and was positively correlated with viral content. However, the enhancement of immune gene expression does not guarantee that infected larvae would survive virus attack. Immunoglobulins have critical roles in humoral immunity and the prevalent form is an IgM tetramer in the serum of teleosts, rather than

the pentameric form in mammals (Solem & Stenvik 2006). Among these immune-related genes, IgMhc showed the highest expression in all larvae. The higher expression in hvh-larvae than in morbid organisms suggested that IgM activation was implicated in defence against viral infection during the early growth stage. This IgM-mediated protection is supported by an early developmental stage thymus-independent immune study, which responded to viral antigen stimulation 4 weeks after grouper larva hatching (Chang, Chiu & John 2008). A similar magnitude was not seen in the other two healthy subgroups, perhaps because of the lower virus content. Induced IgM expression also occurred in diseased larvae; however, it could not prevent them from dying. These details require further study.

In this study, we examined surviving and diseased grouper larvae after an epidemic of NNV by performing a microarray analysis containing 9600 orange-spotted grouper larvae cDNA clones, and 45 genes plus 19 sequences were selected and confirmed by real-time qPCR. Several candidate genes that were not immune related but were potentially related to the clinical signs of betanodavirus infection were found in surviving larvae. Immune-related gene expression profiles were also evaluated using a quantitative analysis.

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