Vol. 114: 89–98, 2015
doi: 10.3354/dao02853

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published May 21

RNAi-based inhibition of infectious myonecrosis virus replication in Pacific white shrimp *Litopenaeus vannamei*

Rubens Galdino Feijó[1], Rodrigo Maggioni[2], Pedro Carlos Cunha Martins[2], Keuly Ladislau de Abreu[2], João Mafaldo Oliveira-Neto[2], Cristhiane Guertler[3], Emily Bruna Justino[3], Luciane Maria Perazzolo[3], Luis Fernando Marins[1,*]

[1]Laboratório de Biologia Molecular, Instituto de Ciências Biológicas (ICB), Universidade Federal de Rio Grande (FURG), Av. Itália, Km 8, CEP 96203-900, Rio Grande, RS, Brazil

[2]Laboratório de Biologia Molecular, Centro de Diagnóstico de Enfermidades de Organismos Aquáticos (CEDECAM), Instituto de Ciências do Mar (LABOMAR), Universidade Federal do Ceará (UFC), Av. Abolição, 3207, Meireles, CEP 60165-081, Fortaleza, CE, Brazil

[3]Laboratório de Imunologia Aplicada à Aquicultura, Departamento de Biologia Celular, Embriologia e Genética (BEG), Universidade Federal de Santa Catarina (UFSC), CP 476, CEP 88040-900, Florianópolis, SC, Brazil

ABSTRACT: Disease in Pacific white shrimp *Litopenaeus vannamei* caused by the infectious myonecrosis virus (IMNV) causes significant socioeconomic impacts in infection-prone shrimp aquaculture regions. The use of synthetic dsRNA to activate an RNA interference (RNAi) response is being explored as a means of disease prophylaxis in farmed shrimp. Here, survival was tracked in *L. vannamei* injected with long synthetic dsRNAs targeted to IMNV open reading frame (ORF) 1a, ORF1b, and ORF2 genome regions prior to injection challenge with IMNV, and real-time RT-PCR was used to track the progress of IMNV infection and mRNA expression levels of the host genes *sid1*, *dicer2,* and *argonaute2*. Injection of dsRNAs targeting the ORF1a and ORF1b genes but not the ORF2 gene strongly inhibited IMNV replication over a 3 wk period following IMNV challenge, and resulted in 90 and 83 % shrimp survival, respectively. Host gene mRNA expression data indicated that the Sid1 protein, which forms a transmembrane channel involved in cellular import/export of dsRNA, increased in abundance most significantly in shrimp groups that were most highly protected by virus-specific dsRNA injection. Subclinical IMNV infections present in the experimental *L. vannamei* used increased markedly in the 2 d between injection of any of the 4 virus-specific or non-specific dsRNAs tested and IMNV challenge. While handling and injection stress are implicated in increasing IMNV replication levels, the underlying molecular factors that may have been involved remain to be elucidated.

KEY WORDS: IMNV · *Litopenaeus vannamei* · RNAi therapy · Gene expression · RT-qPCR

INTRODUCTION

Infectious diseases, especially those of viral etiology, cause significant economic losses in shrimp aquaculture worldwide and remain a critical challenge faced by the industry. Infectious myonecrosis (IMN) disease was first observed in 2002 in Pacific white shrimp *Litopenaeus vannamei* being farmed in the state of Piauí in northeast Brazil (Lightner et al. 2004) and subsequently in *L. vannamei* farmed in Indonesia (Senapin et al. 2007, 2011). IMN in *L. vannamei* is characterized by necrosis of striated muscle fibers in the abdomen, appendices, and cephalothorax, as well as lesions in gill and lymphoid organ

*Corresponding author: dqmluf@furg.br

tissues (Lightner et al. 2004). Lesions emerge as muscle opacity mainly in the distal abdomen and tail regions. Muscle areas with a milky appearance are typical in the early stages of disease. In advanced-stage disease, muscle liquefaction can occur, and tissue decomposition in affected regions can lead to a red discoloration of the abdomen similar to that of boiled shrimp (Nunes et al. 2004).

IMN is caused by the infectious myonecrosis virus (IMNV), a non-enveloped icosahedral virus of ~40 nm in diameter comprised of 4 proteins (24, 42, 106, and 149 kDa). The IMNV genome comprises a single long dsRNA (7560 bp) that encodes 2 open reading frames (ORFs) that overlap by 199 nucleotides (nt). The ORFs can be translated as a single long polypeptide via use of a −1 ribosomal frameshift element. ORF1 (136–4953 nt region) encodes the major capsid protein (MCP), and its N-terminal region encodes a dsRNA-binding motif and 2 '2A-like' cleavage motifs. ORF2 (5241–7451 nt region) encodes an RNA-dependent RNA polymerase (RdRp; Nibert 2007).

The RNA interference (RNAi) process in plants and animals utilizes dsRNA-specific RNAse III endonucleases such as Drosha and Dicer that cleave either endogenous or exogenous long dsRNA molecules into small interfering RNAs (siRNAs, 20–28 bp; Meister & Tuschl 2004). These siRNAs guide the degradation of mRNAs in a sequence-specific manner within the RNA-induced silencing complex (RISC; Meister & Tuschl 2004). The RNAi mechanism exists in shrimp, where it can be mobilized as a component of the antiviral defense response. Synthetic dsRNAs targeted to various viral and host gene sequences have thus been investigated for their ability to activate prophylactic RNAi responses against viruses including the Taura syndrome virus (TSV, Robalino et al. 2004), white spot syndrome virus (WSSV, Robalino et al. 2005), yellow head virus (YHV, Yodmuang et al. 2006, Tirasophon et al. 2007), and IMNV (Loy et al. 2013). To activate the RNAi mechanism in shrimp effectively, long dsRNA molecules have been delivered by injection. Transmembrane channels comprised of Sid1 protein appear to be responsible for internalizing dsRNA into the cell cytoplasm (Labreuche et al. 2010). Once internalized, dsRNA is cleaved by the Dicer2 enzyme, allowing siRNAs to interact with the Trbp-1 and Argonaute2 enzymes to facilitate RISC-mediated destruction of the target viral RNA (Chen et al. 2011).

Despite being a subject of substantial investigation, RNAi technology is still being refined for use in applications that benefit shrimp aquaculture productivity and sustainability. Here, RNAi responses to injected long dsRNAs targeted to IMNV ORF1a, ORF1b, and ORF2 genome regions were determined in *L. vannamei* challenged 2 d later by muscle injection of an IMNV inoculum. The effects of dsRNA injection on mRNA expression levels of the *sid1*, *dicer2,* and *argonaute2* genes, which encode proteins involved in the shrimp RNAi response, were also determined.

MATERIALS AND METHODS

dsRNA production

Long dsRNAs targeted to 3 IMNV genome regions (ORF1a, 197–789 nt; ORF1b, 4239–4839 nt; and ORF2, 5939–6536 nt, GenBank AY570982) and to a zebrafish *Danio rerio* immunoglobulin gene region (IGSF4D, 4568–5246 nt, GenBank AL954312) were synthesized from DNA products amplified by PCR using primers containing a 5′-terminal bacteriophage T7 promoter sequence (Table 1).

PCR-amplified DNA was precipitated using 0.3 M sodium acetate and 2 vol ethanol, resuspended, and further purified using a Wizard SV Gel and PCR Clean-Up System kit (Promega) according to the manufacturer's instructions. dsRNA was transcribed using a T7 RiboMAX Express Large Scale RNA Production System kit (Promega) according to the manufacturer's instructions and the following incubation protocol: 42°C for 4 h, 70°C for 10 min followed by a gradual (0.5°C per 20 s) reduction of the reaction temperature to 20°C. dsRNA was adjusted to 0.5 M NaCl with 3 M NaCl and incubated with DNase I (to remove dsDNA/ssDNA) and RNase A (to remove ssRNA) at 37°C for 30 min. dsRNA was then purified through a Wizard SV Minicolumn (Promega) employing a denaturing solution (4 M guanidine thiocyanate in 0.01 M Tris-HCl, pH 7.5) to remove remaining proteins, free nucleotides, and degraded nucleic acids, and stored at −80°C until used. Before intramuscular injection into the third abdominal segment of the shrimp, dsRNA was quantified using the Qubit™ fluorometric quantitation assay (Invitrogen) and diluted in 100 µl TE buffer (10 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.0) to accommodate injection of 2 µg dsRNA g^{-1} shrimp body weight.

IMNV inoculum preparation

IMNV inoculum was prepared essentially as described previously (Prior et al. 2003) using *Litopenaeus*

Table 1. Sequences of primers used in PCR tests to amplify DNA templates for dsRNA synthesis and to quantify mRNA expression levels of various *Litopenaeus vannamei* genes. Asterisk (*) indicates the T7 promoter (TAA TAC GAC TCA CTA TA) sequence at the 5′-end of a primer. IMNV: infectious myonecrosis virus; ORF: open reading frame

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon (bp)	GenBank acc. no.
IMNV ORF1a	*GGGCCGGAGCTGACCACA TTCCAAGC	*GGGAGGGCCTGCTGTTGTG GTACCA	593	AY570982
IMNV ORF1b	*GGGGCCATGGCCAATGCC AGAAGGAA	*GGTGCCACATATTGCTGGGA ACGGG	600	AY570982
IMNV ORF2	*GGCTGAGTGACGCTTTGAC GAAAAACCA	*GGGTGTCCAACCGCGTACTC CTGTC	598	AY570982
Zebrafish IGSF4D	*GGGGTTTGTCTGTCCCTCGTGGT	*GGGAGGTGCAGGTTTTCCTTTT	678	AL954312
sid1 RT-qPCR	GAAGCGATTGGCAGTCTATGAAC	TGGAAGCCTATCTCTGCAACTTG	62	HM234688
dicer2 RT-qPCR	AGGAAATGCAATGTCGTGGTT	ACGAGCCCTCCCCCTAGATT	77	HQ541163
argonaute2 RT-qPCR	GATGGCATGAAGTCTGCAGTTG	TGCGCACGACCATCACTAAG	61	HM234690
ef1α RT-qPCR	CCACCCTGGCCAGATTCA	GCGAACTTGCAGGCAATGT	75	DQ858921
β-*actin* RT-qPCR	CATCAAGGAGAAACTGTGCT	GATGGAGTTGTAGGTGGTCT	114	AF300705

vannamei (~9 g) collected from IMN-affected farms in northeast Brazil. Shrimp were tested by PCR for IMNV, WSSV, and infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Senapin et al. 2007, Nunan & Lightner 2011, OIE 2012), and only those shrimp groups that were PCR-positive for IMNV were used. Shrimp abdominal muscle (1:10 w/v) was homogenized in ice-cold saline buffer (330 mM NaCl, 10 mM Tris-HC1, pH 7.4), clarified by centrifugation at 4000 × *g* (30 min at 4°C), and the supernatant clarified again by centrifugation at 8000 × *g* (30 min at 4°C). The clarified homogenate was filtered sequentially through 0.45 µm and 0.22 µm membrane filters and stored in 2 ml aliquots at −80°C.

IMNV challenge bioassay

Juvenile *L. vannamei* (8 ± 1 g) collected from a farm cultivation tank in northeast Brazil were transported to the Center of Studies and Diagnosis of Aquatic Organism Diseases where they were acclimated for 30 d in 8 × 500 l tanks with continuous seawater renewal (100% d^{-1}). Seawater (30 ppt salinity) was maintained at 28°C, pH 7.2, and 5.5 mg l^{-1} dissolved oxygen. A 12:12 h light:dark photoperiod was used to mimic natural conditions. Shrimp were weighed and individuals placed into 5.5 l plastic tanks with constant aeration and 50% water renewal daily. Shrimp were allocated to 5 groups (72 shrimp $group^{-1}$), and IMNV infection load in hemolymph was quantified by TaqMan real-time RT-qPCR (Andrade et al. 2007). Tail muscle (third abdominal segment) of each shrimp was injected with saline or with 2 µg dsRNA g^{-1} body weight of dsRNA-IMNV-ORF1a, dsRNA-IMNV-ORF1b, dsRNA-IMNV-ORF2, or dsRNA-IGSF4D. Two days after injection, shrimp were injected with 100 µl IMNV inoculum containing ~1.02×10^6 IMNV RNA copies as determined by TaqMan real-time PCR.

Hemolymph (~150 µl) was collected from 12 shrimp selected randomly from each group on Days 0, 1, 3, 5, 8, 11, 15, 21, and 25 post-challenge with IMNV. After sampling hemolymph, shrimp were returned to their respective tanks and excluded from the subsequent collection. For histology, 5 shrimp $group^{-1}$ were sampled on Days 11 and 21 post-challenge. Shrimp were observed twice daily for gross signs of IMN and mortality. Over the 34 d bioassay period, shrimp were fed twice daily with commercial feed pellets containing 35% gross protein at a rate of 3.5% body weight $feed^{-1}$.

RNA extraction and cDNA synthesis

Total RNA was extracted from hemocytes centrifuged from hemolymph at 1200 × *g* (10 min at 4°C) using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was incubated with DNase I for 30 min at 37°C and quantified using a Qubit fluorometric RNA quantification assay kit. IMNV dsRNA was denatured by heating at 100°C for 5 min and chilled on ice before cDNA was synthesized using 1 µg total RNA and the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's protocol.

PCR quantification of IMNV RNA

IMNV RNA copies were quantified by TaqMan real-time RT-qPCR using a probe and PCR primers and reaction conditions described previously (Andrade et al. 2007). Briefly, each reaction (10 µl) contained 5 µl Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 0.3 µl IMNV412F and IMNV545R primers (10 µM), 0.15 µl TaqMan probe IMNVp1 (10 µM), 0.2 µl 50× Rox reference dye, 1 µl cDNA (25 ng), and 3.05 µl ddH_2O. Prepared similarly were negative control PCRs using ddH_2O to replace cDNA and positive control PCRs using 10-fold serial dilutions of pTOPO-IMNV.1 DNA corresponding to 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 IMNV RNA copies. The pTOPO-IMNV.1 plasmid contains a 593 bp IMNV PCR amplicon (GenBank AY570982, nt 197–789) cloned into the pCR 2.1-TOPO TA cloning vector (Invitrogen).

PCR tests were performed in duplicate using the thermal cycling conditions 50°C for 2 min followed by Platinum *Taq* DNA polymerase activation at 95°C for 2 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s using the Applied Biosystems 7500 Real-time PCR system. Data collected during the final 60°C extension step were analyzed using SDS version 1.3.1 software (Applied Biosystems) to generate regression curves from which IMNV RNA copy numbers could be quantified. Samples were considered IMNV-positive only if specific amplification occurred in both replicate tests.

Host gene expression analysis

Shrimp *sid1*, *dicer2*, *argonaute2*, β-*actin*, and *ef1α* mRNA transcripts were quantified using a Platinum SYBR Green qPCR SuperMix-UDG kit and Applied Biosystems 7500 Real-time PCR system. β-*actin* and *ef1α* mRNAs were analyzed as references for mRNAs transcribed from the RNAi-associated genes. PCR primer sequences used in the various tests are detailed in Table 1.

The RT-PCR efficiency for each mRNA was determined as described previously (Pfaffl et al. 2002), and melting curve analysis was employed to ensure PCR specificity. Each reaction (10 µl) was amplified in duplicate and contained 5 µl Platinum SYBR Green qPCR SuperMix-UDG, 0.3 µl of each primer (10 µM), 0.2 µl 50× Rox reference dye, 1 µl (20 ng) cDNA, and 3.2 µl ddH_2O. Thermal cycling conditions were 50°C for 2 min, 95°C for 2 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s. qPCR data were considered valid only when the mean cycle threshold (C_t) values of the replicate tests deviated by ≤0.3. Mean C_t values were converted to linear values and normalized using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001).

Histology

Each shrimp was fixed in Davidson's AFA solution (11.5% acetic acid, 22% formalin, 33% ethanol) for 48 h before being placed into 70% ethanol. Preserved tissue was dehydrated using increasing alcohol concentrations, clarified in xylene, and embedded into paraffin at 60°C using standard methods. Transverse and longitudinal sections (4 µm thick) of different regions (e.g. cephalothorax including gills, hepatopancreas, and lymphoid organ, third and sixth segments of the abdomen, and appendages) were prepared and stained with hematoxylin–eosin (Bell & Lightner 1988) to identify tissue pathology indicative of IMN as described previously (Lightner 2011).

Statistical analysis

A log-rank test based on the Kaplan-Meier survival curve estimates was used to verify differences in survival rates between various shrimp groups challenged with IMNV. Dunnett's test following 1-way ANOVA was conducted to analyze differences in IMNV RNA copy numbers and mRNA expression levels of RNAi-related genes. Values obtained for shrimp (n = 12) sampled from each experimental group at each time point following IMNV challenge were compared to values obtained at the time of dsRNA injection 2 d prior to IMNV challenge. GraphPad Prism 3.00 for Windows (GraphPad Software) was used for all statistical analyses.

RESULTS

Shrimp survival rates

Survival among groups of *Litopenaeus vannamei* (n = 72) with asymptomatic IMNV infections was tracked over a 34 d period following intramuscular injection of saline of various dsRNAs followed 2 d later by intramuscular injection challenge with an IMNV inoculum containing the equivalent of 1.02×10^6 IMNV RNA copies (Fig. 1). Injection of either dsRNA-IMNV-ORF1a or dsRNA-IMNV-ORF1b was

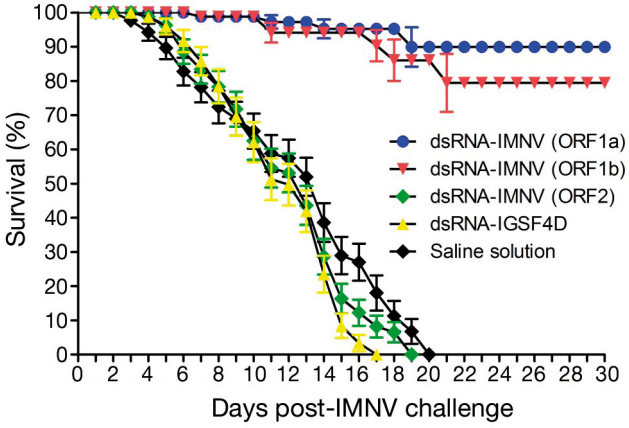

Fig. 1. Survival among groups of *Litopenaeus vannamei* injected intramuscularly with dsRNA-infectious myonecrosis virus (IMNV)-open reading frame (ORF)1a, dsRNA-IMNV-ORF1b, dsRNA-IMNV-ORF2, dsRNA-IGSF4D, or saline prior to challenge with IMNV by muscle injection at a dose equivalent to 1.02×10^6 IMNV RNA copies

associated with survival rates of 90 and 83%, respectively, at the end of bioassay ($p > 0.05$). In contrast, deaths accumulated at similar rates among shrimp groups injected with saline alone or with dsRNA-IMNV-ORF2 or the control dsRNA-IGSF4D or died at similar rates ($p > 0.05$), with 100% mortality occurring on Days 20, 17, and 19 post-challenge, respectively.

Histopathology

No histopathological changes indicative of IMN were detected in any IMNV-challenged shrimp examined from the dsRNA-IMNV-ORF1a group (Fig. 2). However, hemolytic infiltrations and edemas in muscle tissue were observed from Day 21 post-challenge among shrimp examined from the dsRNA-IMNV-ORF1b group. Between Days 15 and 21 following

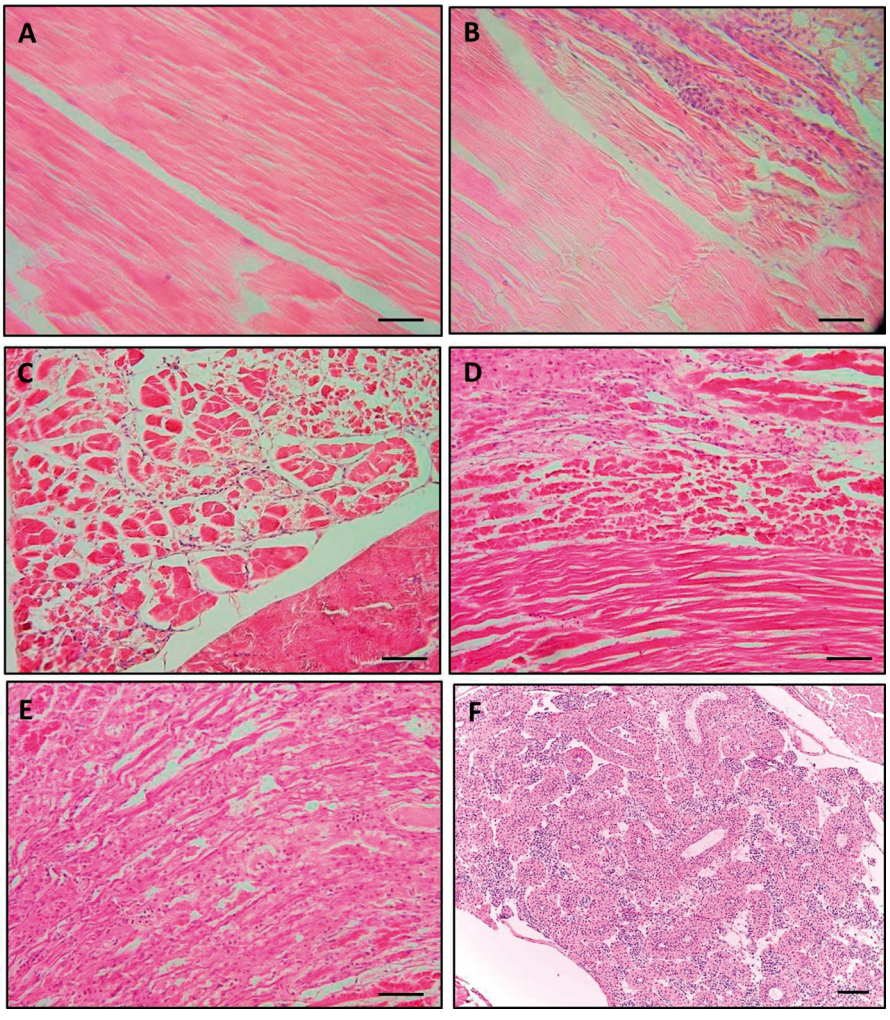

Fig. 2. Light microscopy of histological sections of IMNV-challenged *Litopenaeus vannamei* injected with (A) dsRNA-IMNV-ORF1a, (B) dsRNA-IMNV-ORF1b, or (C–F) saline (abbreviations as in Fig. 1). (A) Normal striated (skeletal) muscle; (B) focal hemocyte infiltration in muscle tissue; (C) coagulative muscle necrosis and hemocyte infiltrations; (D) coagulative and liquefactive muscle necrosis with replacement by fibrous tissue; (E) liquefactive necrosis of striated muscle fibers; and (F) lymphoid organ spheroids (LOS) interspersed among normal LO tubules. Scale bars = 100 µm

IMNV challenge, IMN muscle histopathology typified by hemolytic infiltrations, the presence of coagulated and liquefied necrotic areas, and the appearance of hypertrophied cells comprising lymphoid organ spheroids became evident among shrimp examined from groups injected with dsRNA-IMNV-ORF2, dsRNA-IGSF4D, or saline (Fig. 2).

IMNV infection loads following dsRNA injection and IMNV challenge

IMNV replication in hemocytes collected from representative *L. vannamei* injected with the various dsRNAs prior to IMNV challenge was quantified by TaqMan real-time RT-qPCR using a linear regression curve [r^2 = 0.9976; y = (–3.368 ± 0.048)x + (41.46 ± 0.26)] generated for a 10-fold dilution series of IMNV plasmid DNA of known copy number. Among the shrimp with subclinical IMNV infections tested 2 d prior to challenge at the time of dsRNA injection, IMNV RNA copy numbers detected were consistently low (mean ± SEM = 1.41 × 10^4 ± 480 IMNV RNA copies μg^{-1} RNA; Fig. 3). In contrast, increased IMNV replication levels were evident among shrimp sampled from all dsRNA-injected groups tested immediately prior to IMNV challenge, with the most significant increases occurring among shrimp in groups injected with dsRNA-IMNV-ORF1a (2.36 × 10^6 IMNV RNA copies μg^{-1} RNA, $p < 0.0001$) and dsRNA-IMNV-ORF1b (1.47 × 10^5 IMNV RNA copies μg^{-1} RNA, $p < 0.001$) (Fig. 3).

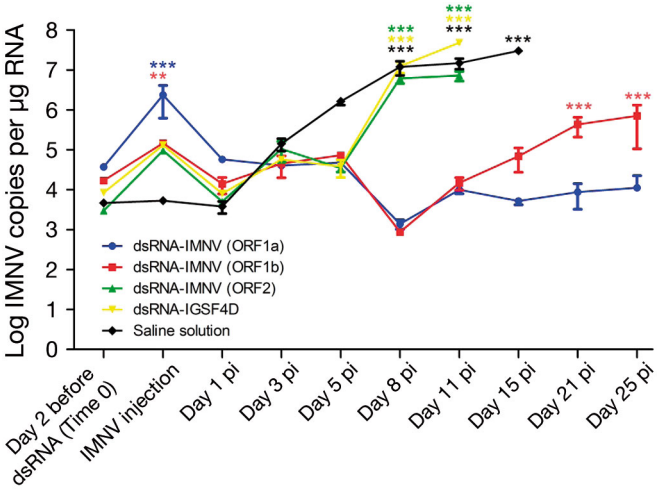

Fig. 3. IMNV RNA copy numbers in hemocytes of *Litopenaeus vannamei* injected with dsRNA-IMNV-ORF1a, dsRNA-IMNV-ORF1b, dsRNA-IMNV-ORF2, dsRNA-IGSF4D, or saline pre- and post-IMNV challenge (abbreviations as in Fig. 1). Data are presented as mean ± SEM log number of IMNV RNA copies (n = 12 shrimp at each time point). Here and in Figs. 4–6, asterisks indicate the statistical significance of differences in data values at different times post-challenge relative to when shrimp were injected with dsRNA 2 d prior to IMNV challenge (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$)

Among shrimp injected with saline, IMNV infection loads remained relatively low (3.81 × 10^3 IMNV RNA copies μg^{-1} RNA) on Day 1 following IMNV challenge but increased progressively thereafter, peaking on Day 15 (3.01 × 10^7 IMNV RNA copies μg^{-1} RNA; Fig. 3).

While not statistically significant, IMNV replication levels in shrimp groups injected with the various dsRNA appeared to dip slightly on Day 1 following IMNV challenge (Fig. 3). At subsequent sampling times, infection loads remained low and stable in shrimp injected with dsRNA-IMNV-ORF1a (e.g. Days 0 and 25: 3.73 × 10^4 and 1.12 × 10^4 MNV RNA copies μg^{-1} RNA, respectively). Among shrimp injected with dsRNA-IMNV-ORF1b, similarly low and stable IMNV infection loads were evident until Day 15, after which they increased significantly (e.g. Days 21 and 25: 4.30 × 10^5 and 7.09 × 10^5 IMNV RNA copies μg^{-1} RNA, respectively, $p < 0.0001$; Fig. 3). Post-challenge IMNV infection loads increased most rapidly among shrimp injected with either dsRNA-IMNV-ORF2 (e.g. Day 8: 6.11 × 10^6 IMNV RNA copies μg^{-1} RNA, $p < 0.0001$) or the control dsRNA-IGSF4D (e.g. Day 11: 1.23 × 10^7 IMNV RNA copies μg^{-1} RNA, $p < 0.0001$).

Host *sid1* mRNA expression

Two days after injection of shrimp groups with dsRNA-IMNV-ORF1a, dsRNA-IMNV-ORF1b, dsRNA-IMNV-ORF2, or dsRNA-IGSF4D, *sid1* mRNA expression levels in hemocytes increased in the order of 2.1-, 1.9-, 1.6-, and 2.1-fold, respectively (Fig. 4; $p < 0.0001$). In contrast, shrimp injected with saline alone showed no appreciable increase in expression levels. On Day 1 following IMNV challenge, *sid1* mRNA expression levels increased markedly among the saline-injected shrimp and remained at similar levels among shrimp injected with the various dsRNAs (Fig. 4).

On Days 3 and 5 following IMNV challenge, *sid1* mRNA expression decreased to basal levels among shrimp injected with dsRNA-IMNV-ORF1a or dsRNA-IMNV-ORF1b, but then spiked suddenly 3.5- and 6.3-fold, respectively ($p < 0.0001$), among shrimp tested from these groups on Day 8. Expression levels remained elevated until Day 11 post-challenge among shrimp injected with dsRNA-IMNV-ORF1a (1.7-fold;

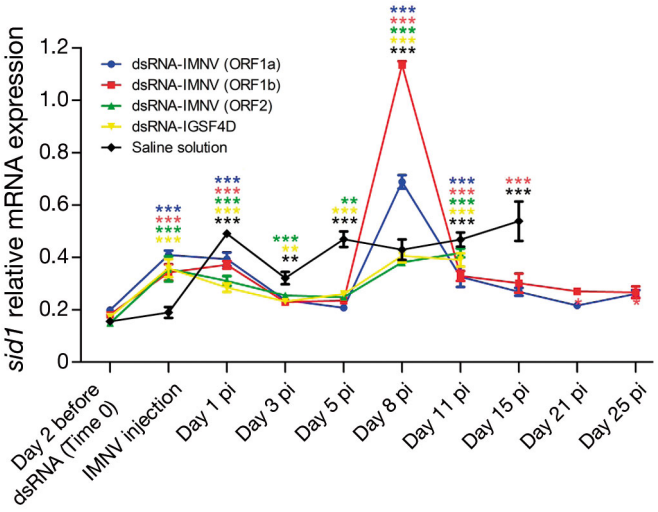

Fig. 4. Dynamics of *sid1* mRNA expression in hemocytes of *Litopenaeus vannamei* injected with dsRNA-IMNV-ORF1a, dsRNA-IMNV-ORF1b, dsRNA-IMNV-ORF2, dsRNA-IGSF4D, or saline pre- and post-IMNV challenge (abbreviations as in Fig. 1). Data are presented as mean ± SEM (n = 12 shrimp at each time point), and asterisks indicate statistical significance levels as described in Fig. 3

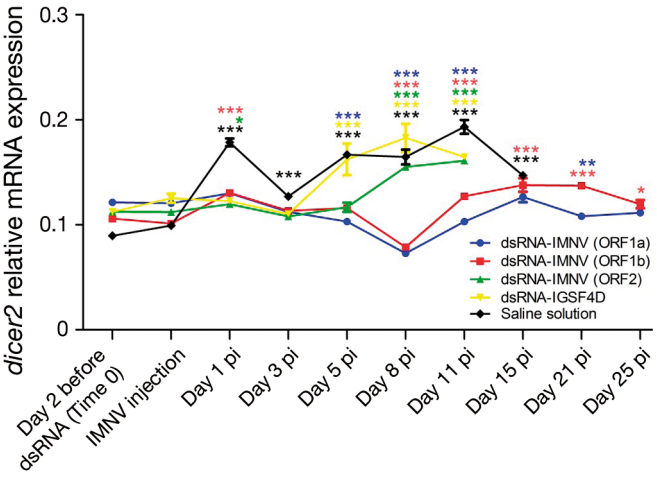

Fig. 5. Dynamics of *dicer2* mRNA expression in hemocytes of *Litopenaeus vannamei* injected with dsRNA-IMNV-ORF1a, dsRNA-IMNV-ORF1b, dsRNA-IMNV-ORF2, dsRNA-IGSF4D, or saline pre- and post-IMNV challenge (abbreviations as in Fig. 1). Data are presented as mean ± SEM (n = 12 shrimp at each time point), and asterisks indicate statistical significance levels as described in Fig. 3

$p < 0.0001$) and until Day 25 post-challenge among shrimp injected with dsRNA-IMNV-ORF1b (1.5-fold; $p < 0.05$; Fig. 4).

Among shrimp injected with saline, dsRNA-IMNV-ORF2, or dsRNA-IGSF4D, *sid1* mRNA expression increased most markedly (3.5-fold, $p < 0.0001$) by Day 15 post-challenge among those injected with saline (Fig. 4). Among shrimp in the other 2 cohorts, expression levels decreased slightly among shrimp examined on Days 3 and 5 post-challenge before increasing to approximately Day 1 post-challenge levels on Days 8 and 11 ($p < 0.001$).

Host *dicer2* mRNA expression

Two days after injection of shrimp groups with dsRNA, *dicer2* mRNA expression levels in hemocytes remained unchanged in all shrimp groups (Fig. 5). After IMNV challenge, expression levels reduced significantly among shrimp injected with dsRNA-IMNV-ORF1a (1.2-, 1.7-, and 1.2-fold decreases on Days 5, 8, and 11; $p < 0.0001$) and dsRNA-IMNV-ORF1b (1.4-fold decrease on Day 8; $p < 0.0001$). Among shrimp in the latter group, however, statistically significant increases in expression levels occurred from Day 11 onwards (Fig. 5). Even greater increases in *dicer2* mRNA expression occurred among shrimp injected with dsRNA-IMNV-ORF2 (1.1-fold, $p < 0.05$; 1.4-fold, $p < 0.0001$; 1.5-fold, $p < 0.0001$ on Days 1, 8, and 11) or dsRNA-IGSF4D (1.5-, 1.6-, and 1.5-fold increases, $p < 0.0001$, on Days 5, 8, and 11). *dicer2* mRNA expression also increased post-challenge among shrimp injected with saline, with levels fluctuating somewhat between sampling time points and being highest on Day 11 ($p < 0.0001$; Fig. 5).

Host *argonaute2* gene mRNA expression

Two days after injection of shrimp groups with dsRNA, *argonaute2* mRNA expression levels in hemocytes increased significantly by 1.6-, 1.8-, 2.4-, and 2.1-fold among shrimp injected with dsRNA-IMNV-ORF1a, dsRNA-IMNV-ORF1b, dsRNA-IMNV-ORF2, and dsRNA-IGSF4D, respectively ($p < 0.0001$; Fig. 6). In contrast, expression remained at basal levels among shrimp injected with saline but then increased startlingly (3.9-fold; $p < 0.0001$) on Day 1 following IMNV challenge, with a slightly lesser spike also occurring on Day 5 (Fig. 6). After the initial increase on Days 1 and 3 following IMNV challenge, expression declined back to basal levels among shrimp injected with dsRNA-IMNV-ORF1a (Days 5 and 11) or dsRNA-IMNV-ORF1b (Day 8) before increasing again on Days 15, 21, and 25. *argonaute2* mRNA expression levels remained slightly elevated over the course of the bioassay among shrimp injected with dsRNA-IMNV-ORF2. Among shrimp injected with dsRNA-

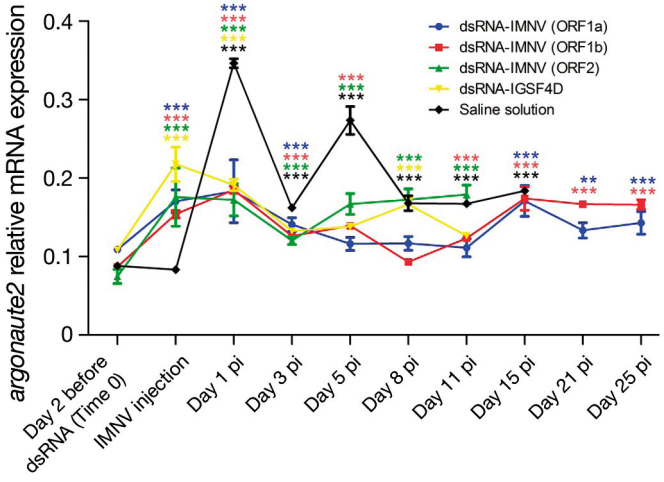

Fig. 6. Dynamics of *argonaute2* mRNA expression in hemocytes of *Litopenaeus vannamei* injected with dsRNA-IMNV-ORF1a, dsRNA-IMNV-ORF1b, dsRNA-IMNV-ORF2, dsRNA-IGSF4D, or saline pre- and post-IMNV challenge (abbreviations as in Fig. 1). Data are presented as mean ± SEM (n = 12 shrimp at each time point), and asterisks indicate statistical significance levels as described in Fig. 3

IGSF4D, expression levels were elevated slightly on Days 1 and 8 ($p < 0.0001$) but were detected at basal levels on Days 3, 5, and 11.

DISCUSSION

Long dsRNAs targeted to IMNV ORF1a and ORF1b gene sequences were identified here to be extremely effective in inhibiting IMNV replication, disease, and mortality in *Litopenaeus vannamei* over a 30 d period following virus challenge by intramuscular injection. These findings corroborate other recent reports of higher antiviral efficacy of dsRNAs targeted to IMNV ORF1 gene regions compared to ORF2 gene regions (Loy et al. 2012). Although no significant differences in survival rates were evident between shrimp groups injected with the ORF1a and ORF1b gene dsRNAs prior to IMNV challenge, TaqMan real-time RT-qPCR data indicated that the ORF1a gene dsRNA was most effective at inhibiting IMNV replication, particularly beyond Day 8 post-challenge. Reduced IMNV replication was confirmed by the complete absence of IMN disease histopathology in shrimp injected with ORF1a gene dsRNA, and by the presence of only minor muscle tissue hemolytic infiltrations and edemas from Day 21 post-challenge among shrimp injected with the ORF1b gene dsRNA. However, despite the obvious protective properties of these dsRNAs, PCR data showed that IMNV infection persisted over the 30 d trial period. It is thus not possible to predict, without undertaking longer-term challenge trials, to what level and over what time period shrimp would remain protected against developing acute IMNV infection and associated disease. Nonetheless, the promising results reported here and elsewhere (Tirasophon et al. 2007, Loy et al. 2012, 2013) clearly justify follow-up studies to examine whether RNAi approaches can be applied in breeding programs, hatcheries, or farms to effectively suppress IMNV infection over the life of *L. vannamei*, thus minimizing risks of IMN production losses occurring during culture.

The IMNV ORF1a gene encodes Protein 1 that contains a dsRNA-binding (DSRM) domain, which might play a role in suppressing the shrimp defense response, as well as Protein 2 with an as yet unknown function (Poulos et al. 2006, Nibert 2007). The ORF1b gene dsRNA is targeted to the viral MCP coding sequence in Protein 1, and the ORF2 gene dsRNA is targeted to the viral RdRp coding sequence (Nibert 2007). Various genome regions of different shrimp viruses have been identified to be more or less effective targets for dsRNA inhibition of viral replication and disease (Tirasophon et al. 2005, Attasart et al. 2009, Sarathi et al. 2010). In addition to known virus-specific factors dictating what viral RNAs will make more effective dsRNA targets, more arbitrary factors including RNA secondary structure stability, RISC accessibility, and asymmetry of the siRNAs generated randomly by the Dicer enzyme can also contribute to RNAi efficiency (Shao et al. 2007).

The *L. vannamei* selected for use in the challenge trials possessed subclinical IMNV infections as determined by PCR. Two days following dsRNA injection and immediately prior to IMNV challenge, IMNV RNA copy numbers quantified by TaqMan real-time RT-qPCR were increased among shrimp injected with the any of the various dsRNA. While generalized handling and injection stress factors might be implicated in these increased IMNV replication levels, as has been noted in chronic infections of shrimp with other viruses (de la Vega et al. 2004), it is possible that the capacity and or dispersion of RNAi responses induced by injected dsRNAs take time to reach optimal levels. The underlying molecular factors that are involved remain to be determined. However, it is clear that the virus-specific dsRNAs found to be highly effective at protecting shrimp against IMNV challenge were not effective during this period, possible due to limited cellular uptake and dispersion or down-regulating factors involved in maintaining infections at low and subclinical levels. In this regard, various virus-encoded proteins have been identified to possess func-

tions that suppress RNA silencing by impeding critical pathway elements such as viral RNA recognition and dicing and RISC assembly (Dong et al. 2003, Trinks et al. 2005, Voinnet 2005, Unterholzner & Bowie 2008). For example, similarly to IMNV (Nibert 2007), RNA viruses such as the insect Flock House virus (FHV) and the Nodamura virus (NoV) encode a non-structural DSRM-containing protein (Protein B2) that suppresses the RNAi process by ligating to long and small dsRNA chains in a sequence-independent manner (Johnson et al. 2004, Chao et al. 2005). Interestingly, both of these viruses have a rapid initial replication rate, possibly to maximize early viral RNA synthesis and thus translation of the Protein B2 to counteract host RNAi responses by interference with Dicer2-mediated cleavage of long dsRNA into siRNAs (Rodrigo et al. 2011, Jiang et al. 2012). Whether IMNV also possesses proteins that can confer similar functionality needs further investigation.

Shrimp groups injected with saline or dsRNAs targeted to IGSF4D or IMNV-ORF2 were not protected and died at similar rates following IMNV challenge. Compared to saline, however, IMNV replication was suppressed to a limited degree until Day 5 post-challenge in shrimp groups injected with either dsRNA. The data on the non-specific IGSF4D dsRNA is similar to that reported for IMNV challenge experiments employing a non-specific dsRNA targeted to green fluorescent protein (Loy et al. 2012), as well as for various non-specific dsRNAs used to examine RNAi responses against WSSV and TSV (Yodmuang et al. 2006, Kim et al. 2007). This accumulating evidence supports the generalized activation of the shrimp RNAi machinery providing some capacity to interfere with virus replication in the absence of it being primed by an effective virus-specific dsRNA. However, compared to the efficacy of RNAi responses primed by virus-specific dsRNA, the limited response capability stimulated by some non-specific dsRNAs appears to be far more easily overwhelmed depending on dsRNA delivery time and virus virulence and challenge dose (Robalino et al. 2004). For example, with TSV, shrimp have been found to be protected to a greater extent when a non-specific dsRNA was delivered 2 d rather than 3 d post-challenge, thus supporting the hypothesis of generalized RNAi responses being easily swamped as virus infection levels increase (Tirasophon et al. 2005).

Of the *sid1*, *dicer2,* and *argonaute2* host genes examined, *sid1* mRNA expression levels increased most markedly and peaked on Day 8 following IMNV challenge among shrimp injected with either IMNV-ORF1a or IMNV-ORF1b dsRNA. This corresponded with IMNV replication being suppressed most profoundly, and confirmed that the shrimp Sid1 protein plays a key role in RNAi-mediated gene silencing. This role was expected, as Sid1 has been identified in other species to function as a dsRNA-gated transmembrane channel mediating cellular import and export of dsRNA. For example, *Caenorhabditis elegans* Sid1 protein expression in *Drosophila* S2 cells causes changes in cell membrane conductance in response to dsRNA, with the intracellular retention of some dsRNAs being related to their direct involvement in RISC-mediated gene silencing (Shih & Hunter 2011). Consistently with their known roles in facilitating RNAi, increases in *dicer2* and *argonaute2* mRNA expression also occurred in *L. vannamei* injected with effective virus-specific dsRNA before challenge with IMNV.

Acknowledgements. This work was supported by the CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) (Proc. No. 405519/2012-5). R.G.F. holds a scholarship from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). L.F.M. is a CNPq research fellow (Proc. No. 304675/2011-3).

LITERATURE CITED

Andrade TPD, Srisuvan T, Tang KFJ, Lightner DV (2007) Real-time reverse transcriptase polymerase chain reaction assay using TaqMan probe for detection and quantification of Infectious myonecrosis virus (IMNV). Aquaculture 264:9–15

Attasart P, Kaewkhaw R, Chimwai C, Kongphoma U, Namramoon O, Panyim S (2009) Inhibition of white spot syndrome virus replication in *Penaeus monodon* by combined silencing of viral rr2 and shrimp PmRab7. Virus Res 145:127–133

Bell TA, Lightner DV (1988) A handbook of normal penaeid shrimp histology. World Aquaculture Society, Baton Rouge, LA

Chao JA, Lee JH, Chapados BR, Debler EW, Schneemann A, Williamson JR (2005) Dual modes of RNA-silencing suppression by Flock House virus protein B2. Nat Struct Mol Biol 12:952–957

Chen YH, Jia XT, Zhao L, Li CZ and others (2011) Identification and functional characterization of Dicer2 and five single VWC domain proteins of *Litopenaeus vannamei*. Dev Comp Immunol 35:661–671

de la Vega E, Degnan BM, Hall MR, Cowley JA, Wilson KJ (2004) Quantitative real-time RT-PCR demonstrates that handling stress can lead to rapid increases of gill-associated virus (GAV) infection levels in *Penaeus monodon*. Dis Aquat Org 59:195–203

Dong X, Wezel RV, Stanley J, Hong Y (2003) Functional characterization of the nuclear localization signal for a suppressor of posttranscriptional gene silencing. J Virol 77:7026–7033

Jiang L, Wei C, Li Y (2012) Viral suppression of RNA silencing. Sci China C Life Sci 55:109–118

Johnson KL, Price BD, Eckerle LD, Ball LA (2004) Nodamura virus nonstructural protein B2 can enhance viral RNA accumulation in both mammalian and insect cells. J Virol 78:6698–6704

Kim CS, Kosuke Z, Nam YK, Kim SK, Kim KH (2007) Protection of shrimp (*Penaeus chinensis*) against white spot syndrome virus (WSSV) challenge by double-stranded RNA. Fish Shellfish Immunol 23:242–246

Lightner DV (2011) Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): a review. J Invertebr Pathol 106:110–130

Lightner DV, Pantoja CR, Poulos BT, Tang KFJ, Redman RM, Andrade TPD, Bonami JR (2004) Infectious myonecrosis: new disease in Pacific white shrimp. Glob Aquacult Advocate 7:85

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408

Loy JD, Mogler MA, Loy DS, Janke BH, Kamrud KI, Harris DL, Bartholomay LC (2012) dsRNA provides sequence-dependent protection against infectious myonecrosis virus in *Litopenaeus vannamei*. J Gen Virol 93:880–888

Loy JD, Loy DS, Mogler MA, Janke B, Kamrud KI, Harris DLH, Bartholomay LC (2013) Sequence-optimized and targeted double-stranded RNA as a therapeutic antiviral treatment against infectious myonecrosis virus in *Litopenaeus vannamei*. Dis Aquat Org 105:57–64

Meister G, Tuschl T (2004) Mechanisms of gene silencing by double-stranded RNA. Nature 431:343–349

Nibert ML (2007) '2A-like' and 'shifty heptamer' motifs in penaeid shrimp infectious myonecrosis virus, a monosegmented double-stranded RNA virus. J Gen Virol 88: 1315–1318

Nunan LM, Lightner DV (2011) Optimized PCR assay for detection of white spot syndrome virus (WSSV). J Virol Methods 171:318–321

Nunes AJP, Martins PC, Gesteira TC (2004) Carcinicultura ameaçada. Panorama Aqüicult 14:37–57

OIE (World Organisation for Animal Health) (2012) Manual of diagnostic tests for aquatic animals. OIE, Paris. Available at www.oie.int/international-standard-setting/aquatic-manual/access-online/ (accessed 18 April 2014)

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36

Poulos BT, Tang KFJ, Pantoja CR, Bonami JR, Lightner DV (2006) Purification and characterization of infectious myonecrosis virus of penaeid shrimp. J Gen Virol 87:987–996

Prior S, Browdy CL, Shepard EF, Laramore R, Parnell PG (2003) Controlled bioassay systems for determination of lethal infective doses of tissue homogenates containing Taura syndrome or white spot syndrome virus. Dis Aquat Org 54:89–96

Robalino J, Browdy CL, Prior S, Metz A, Parnell P, Gross P, Warr G (2004) Induction of antiviral immunity by double-stranded RNA in a marine invertebrate. J Virol 78: 10442–10448

Robalino J, Bartlett T, Shepard E, Prior S, Jaramillo G, Scura E (2005) Double-stranded RNA induces sequence-specific antiviral silencing in addition to nonspecific immunity in a marine shrimp: convergence of RNA interference and innate immunity in the invertebrate antiviral response? J Virol 79:13561–13571

Rodrigo G, Carrera J, Jaramillo A, Elena SF (2011) Optimal viral strategies for bypassing RNA silencing. J R Soc Interface 8:257–268

Sarathi M, Simon MC, Venkatesan C, Thomas J and others (2010) Efficacy of bacterially expressed dsRNA specific to different structural genes of white spot syndrome virus (WSSV) in protection of shrimp from WSSV infection. J Fish Dis 33:603–607

Senapin S, Phewsaiya K, Briggs M, Flegel TW (2007) Outbreaks of infectious myonecrosis virus (IMNV) in Indonesia confirmed by genome sequencing and use of an alternative RT-PCR detection method. Aquaculture 266: 32–38

Senapin S, Phiwsalya K, Gangnonngiw W, Flegel TW (2011) False rumours of disease outbreaks caused by infectious myonecrosis virus (IMNV) in the whiteleg shrimp in Asia. J Negat Results Biomed 10:10–11

Shao Y, Chan CY, Maliyekkel A, Lawrence CE, Roninson IB, Ding I (2007) Effect of target secondary structure on RNAi efficiency. RNA 13:1631–1640

Shih JD, Hunter CP (2011) SID-1 is a dsRNA-selective dsRNA-gated channel. RNA 17:1057–1065

Tirasophon W, Roshorm Y, Panyim S (2005) Silencing of yellow head virus replication in penaeid shrimp cells by dsRNA. Biochem Biophys Res Commun 334:102–107

Tirasophon W, Yodmuanga S, Chinnirunvong W, Plongthongkum N, Panyim S (2007) Therapeutic inhibition of yellow head virus multiplication in infected shrimps by YHV-protease dsRNA. Antiviral Res 74:150–155

Trinks D, Rajeswaran R, Shivaprasad PV, Akbergenov R and others (2005) Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. J Virol 79:2517–2527

Unterholzner L, Bowie AG (2008) The interplay between viruses and innate immune signaling: recent insights and therapeutic opportunities. Biochem Pharmacol 75: 589–602

Voinnet O (2005) Induction and suppression of RNA silencing: insights from viral infections. Nat Rev Genet 6: 206–220

Yodmuang S, Tirasophon W, Roshorm Y, Chinnirunvong W, Panyim S (2006) YHV-protease dsRNA inhibits YHV replication in *Penaeus monodon* and prevents mortality. Biochem Biophys Res Commun 341:351–356

Editorial responsibility: Jeff Cowley, Brisbane, Queensland, Australia

Submitted: June 23, 2014; Accepted: January 29, 2015
Proofs received from author(s): April 22, 2015