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

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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...
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, and its N-terminal region encodes an RNA-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 (Lubreuche 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
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-HCl, 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 ddH2O. Prepared similarly were negative control PCRs using ddH2O 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, and 10^3 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 ddH2O. 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 (Ct) values of the replicate tests deviated by ≤0.3. Mean Ct values were converted to linear values and normalized using the 2^ΔΔCT method (Livak & Schmittgen 2001).

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
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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. 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 × 10⁶ IMNV RNA copies

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 \( r^2 = 0.9976; y = (-3.368 \pm 0.048)x + (41.46 \pm 0.26) \) 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 = \( 1.41 \times 10^4 \pm 480 \) 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 (\( 2.36 \times 10^6 \) IMNV RNA copies µg⁻¹ RNA, \( p < 0.0001 \)) and dsRNA-IMNV-ORF1b (\( 1.47 \times 10^5 \) IMNV RNA copies µg⁻¹ RNA, \( p < 0.001 \)) (Fig. 3).

Among shrimp injected with saline, IMNV infection loads remained relatively low (\( 3.81 \times 10^3 \) IMNV RNA copies µg⁻¹ RNA) on Day 1 following IMNV challenge but increased progressively thereafter, peaking on Day 15 (\( 3.01 \times 10^7 \) 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: \( 3.73 \times 10^4 \) and \( 1.12 \times 10^5 \) IMNV 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: \( 4.30 \times 10^5 \) and \( 7.09 \times 10^5 \) 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: \( 6.11 \times 10^6 \) IMNV RNA copies µg⁻¹ RNA, \( p < 0.0001 \)) or the control dsRNA-IGSF4D (e.g. Day 11: \( 1.23 \times 10^7 \) 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 levels increased markedly among the saline-injected shrimp and remained at similar levels among shrimp injected with the various dsRNAs (Fig. 4).

Host *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;
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-
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 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.

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