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Reduced loads of pre-existing *Gill-associated virus* (GAV) infection in juvenile

Penaeus monodon injected with single or multiple GAV-specific dsRNAs

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Abstract

The ability of RNA interference (RNAi) based on injected dsRNA was investigated here for its ability to reduce the severity of pre-existing subclinical *Gill-associated virus* (GAV) infections in farm stocks of juvenile Black Tiger shrimp (*Penaeus monodon*). Following tail muscle injection of single or multiple long dsRNAs targeted to sequences positioned across the GAV ORF1a/1b replicase genes, pleopods were sampled sequentially from individuals at regular intervals over a 2 week period to track changes in GAV RNA loads by quantitative real-time RT-PCR. Mean GAV RNA amounts showed statistically significant (P <0.05) declines from Day 6 post-injection in shrimp injected with a cocktail of 5 GAV dsRNAs or a single dsRNA (dsGAV1) that targeted a ~1.2 kb sequence at the extreme 5'-terminus of the GAV genome. GAV RNA amounts were not reduced significantly among shrimp injected with another single dsRNA (dsGAV6) targeted to a sequence just downstream of the helicase motif in the ORF1b gene. Based on these encouraging data, it will be useful to determine whether the RNAi approach can reduce GAV infection loads sufficiently in *P. monodon* broodstock used in commercial hatcheries in Australia to restrict vertical transmission of GAV to seedstock.

Introduction

Economic losses due to diseases mostly caused by viruses remain a major obstacle to realizing the production potential of shrimp aquaculture industries in most parts of the world. To help overcome these losses, targeted RNA interference (RNAi) approaches based on injected or ingested double-stranded (ds)RNAs specific to RNA sequences of various problematic viruses (Attasart et al., 2011; Kim et al., 2007; Robalino et al., 2004, 2005, 2007; Saksmerprome et al., 2009; Sarathi et al., 2008; Sellars et al., 2011; Tirasophon et al., 2005, 2006; Yodmuang et al., 2006), as well as to a mRNA

encoding a small GTPase protein (Rab7) involved in the cell endocytosis pathway in shrimp (Attasart et al., 2009; Ongvarrasopone et al., 2008; Posiri et al., 2011; Sanitt et al., 2014), have been investigated intensively over the past decade as means of protecting shrimp against disease and mortality following virus challenge. Despite clear experimental demonstration that such RNAi approaches can be highly effective in protecting shrimp against challenge, at least transiently, as yet none have been progressed to a point where the technology can be applied commercially.

Based on observational evidence, it has become generally accepted in the shrimp aquaculture industry that most shrimp viruses are transmitted vertically, and direct experimental evidence for transmission occurring from broodstock to progeny has been obtained for viruses including *Gill-associated virus* (GAV) (Cowley et al. 2002) and *White spot syndrome virus* (WSSV) (Lo et a. 1997). To avoid viral infections being carried into ponds with seedstock, PCR screening of wild-caught broodstock has been used widely by the larger and more sophisticated hatcheries to detect and cull those with higher-level infections (FAO Fisheries Technical Paper No 450, 2003; Coman et al., 2013). However, as such strategies have fallen short of providing the universal remedy for the major industries in Southeast Asia to be sustainable and profitable, in the mid 1990s, Pacific White shrimp (*Litopenaeus vannamei*) began to be trialled as an alternative aquaculture species to indigenous Black Tiger shrimp (*Penaeus monodon*) and Chinese White shrimp (*Penaeus chinensis*) that had become plagued by disease. With the successes seen in culturing *L. vannamei* in Taiwan, and upon the increasing availability of domesticated *L. vannamei* selected to be specific pathogen free (SPF) for viruses of most concern, the past decade has seen a massive shift to farming this species throughout the Asian region (FAO Fisheries Technical Paper No 476, 2005).

In countries like Australia where hatcheries remain reliant mostly on locally-caught wild stocks of *P. monodon* broodstock and where strict government quarantine regulations preclude the import of

non-indigenous shrimp species for aquaculture, hatchery screening exclusion approaches and the establishment and maintenance of virus-free breeding populations have been the only options available for avoiding vertical transmission of viral infections to seedstock. While some progress has been made in domesticating and selectively breeding GAV-free or GAV-tolerant stocks of *P. monodon* in Australia (Coman et al., 2013), many hatcheries rely on wild broodstock captured from regions where GAV is endemic and can occur at high prevalence (Cowley et al., 2000). However, as broodstock are generally managed quite intensively in Australian hatcheries and as numbers used are relatively low, opportunities exist to inject them with virus- and/or host-specific dsRNAs to induce RNAi responses that reduce viral infection loads prior to them being mated and/or spawned to generate seedstock.

To obtain initial evidence that such a strategy is feasible and might provide a means of reducing vertical transmission of GAV from broodstock to farmed progeny, here juvenile *P. monodon* with subclinical GAV infections collected from a farm in North Queensland were injected with either single dsRNAs or a cocktail of 5 dsRNAs targeted to sequences across the GAV ORF1a/1b replicase gene found previously to be highly effective at protecting shrimp against disease and mortality following GAV challenge (Sellars et al., 2011). By tracking GAV infection loads using real-time quantitative RT-PCR over a 2 week period following dsRNA injection, these were identified to be reduced significantly within 6 to 10 days among shrimp injected with the GAV dsRNA cocktail and 1 of the 2 single GAV dsRNAs examined.

Materials and Methods

dsRNA synthesis from PCR products containing terminal T7 RNA promoters

Target site positions for dsRNAs in the GAV ORF1a/1b gene, thus restricting their action to the

genomic-length ssRNA transcribed in low abundance and critical for translating enzymes essential

for generating genome replication machinery, were as reported previously (Sellars et al. 2011),

except that the dsGAV5 target region overlapping the helicase motif in ORF1b was substituted for

another region (dsGAV6; 17237-18014 nt; GenBank Acc. AF227196.2) positioned slightly further

downstream (**Table 1**). As nonspecific controls, dsRNAs were designed to *Penaeus merguiensis*

densovirus (*Pmerg*DNV) sequences used previously in RNAi bioassays (Attasart et al. 2010; 2011)

and to a firefly luciferase gene sequence (De Santis et al. 2011; GenBank Acc. EU754723).

DNA templates used to synthesize each dsRNA were amplified by PCR using forward and reverse

primers each containing T7 RNA polymerase promoter sequence extensions at their 5'-termini

(**Table 1**). The GAV-specific DNA products amplified using these primers were purified through

QIAquick PCR columns (QIAGEN) and dsRNA was synthesized using the MEGAscript® T7 kit

(Invitrogen) according to the manufacturer's instructions. Following quantification of a 1.5 uL

aliquot of each purified dsRNA using a Nanodrop® ND-1000 spectrophotometer (Thermo

Scientific) and electrophoresis of 800 ng each dsRNA in an agarose gel to confirm its size and

integrity (**Fig. 1**), they were stored at -80°C.

Shrimp and experimental tank systems

Pleopods were sampled from juvenile *Penaeus monodon* collected from a total of 42 ponds across 2

farms in Northern Queensland, Australia (10 shrimp per pond, weighing 6-8 g and ~180 days old).

Of these, 4 samples from each pond were tested using a GAV TaqMan RT-qPCR (de la Vega et al.,

2004) which identified 2 ponds with slightly higher GAV prevalence (data not shown). Testing of

pleopods sampled from the other 6 shrimp from each of these ponds identified one in which 1

shrimp possessed a low load GAV infection (20-200 RNA copies/ng RNA) and 2 others possessed

a low-moderate GAV infections (200-2,000 RNA copies/ng RNA). This pond was selected to collect additional shrimp for the bioassays. The shrimp (now 8-12 g) were collected by cast-netting and those visibly showing no gross signs or other evidence of compromised health were packed into oxygenated bags in styrofoam boxes (50 shrimp/box in 10 L cooled (~25°C) seawater) and transported by road and air on the same day (8 h transport duration) to aquarium facilities at the Bribie Island Research Centre in Southeast Queensland. Shrimp were acclimated for 22 h in 5 t circular bare-bottom fibreglass tanks (150 shrimp per tank) employing gentle aeration and flow-through filtered seawater (28 ± 2°C).

Shrimp collected from partially-drained 5 t tanks were weighed and stocked (10 per tank) into 100 L circular tanks filled to 80 L with 24 ppt salinity seawater that was aerated, maintained at 28 ± 2°C and trickle-fed with fresh seawater at a rate of ~0.6 L min^{-1}. The tanks had opaque white lids and were maintained in a facility providing alternating 12 h light and 12 h dark photoperiods. Replicates for each bioassay group were assigned to 4 tanks distributed randomly to accommodate for any position-related effects. Shrimp were fed commercial feed pellets (Ridley Aqua-feed, Melbourne, Australia) ad libitum twice each day at approximately 09:30 and 17:00 h, and waste was siphoned out 3 times per week or as required to maintain water quality. The number of shrimp alive in each tank was counted and recorded twice daily at the times of feeding, and at which times any dead shrimp observed were removed.

Tail-muscle injection of dsRNA and sampling

To deliver dsRNA, 25 μL shrimp saline solution (SSS) (450 mM NaCl, 10 mM KCl, 10 mM EDTA-2Na, 10 mM HEPES, pH to 7.3) containing each dsRNA formulation was injected into tail muscle of the 3rd abdominal segment of each shrimp between 08:30 and 11:00 h (bioassay Day 0) using a 100 μL Hamilton glass syringe fitted with a 26-guage needle. Injection doses were

15 µg GAV dsRNA cocktail (3 µg each of the 5 dsRNAs), 15 µg dsGAV1, 15 µg dsGAV6, 15 µg *Pmerg*DNV dsRNAs (7.5 µg each of the 2 dsRNAs) or 4 µg of the firefly luciferase dsRNA per shrimp.

A pleopod tip (~ 5 mm in length) was sampled from each shrimp on Days 0 and 14 of the bioassay. For shrimp in 2 of the 4 replicate tanks used for each treatment, a pleopod tip was also sampled on Days 3, 6 and 10. As GAV infection loads determined by TaqMan RT-qPCR have been identified to be relatively consistent across different pleopods of a shrimp (Do, 2012), this non-lethal sampling strategy provided opportunities for tracking changes in loads over time in individuals. Pleopod tips were removed using scissors alcohol-sterilized between collections, placed into 1 mL RNALater^TM solution (Applied Biosystems) and stored at 4°C. A different pleopod was sampled from each of the 10 shrimp in a tank on Day 0 to eliminate the need for eye tagging. At each subsequent sampling point, the next consecutive pleopod of each shrimp was sampled to ensure that it remained unique and identifiable. In brief, Shrimp 1 had its bottom left pleopod sampled on Day 0, its bottom right pleopod sampled on Day 3, its second bottom left pleopod sampled on Day 6, its second bottom right pleopod sampled on Day 9 and its third bottom left pleopod sampled on Day 14; Shrimp 2 had its bottom right pleopod sampled on Day 0, its second bottom left pleopod sampled on Day 3, its second bottom right pleopod sampled on Day 6 etc so that each of the 10 shrimp was unique.

Real-time quantitative (q)RT-PCR

Pleopod tissue was removed from RNALater^TM solution with sterile forceps, blotted dry briefly on sterile absorbent paper towel and homogenized in a tube containing 600 µL TRIzol^® reagent (Invitrogen) and 3 glass beads using a FastPrep FP120 tissue grinder (Savant). Total RNA was then extracted according to the standard TRIzol protocol. Dried RNA was resuspended in 15 µL RNase-free water and before being stored at -80°C, a 1.5 µL aliquot was examined using a Nanodrop^® ND-

1000 spectrophotometer to determine the RNA concentration and relative purity. cDNA was synthesized in a 10 µL reaction containing 500 ng total RNA, 50 ng random hexamers and 100 U SuperScriptTM-III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. A TaqMan RT-qPCR test for GAV (de la Vega et al., 2004) was performed as described except that 2 µL cDNA (equivalent to 100 ng total RNA) was used in a 20 µL reaction prepared using TaqMan® Universal PCR Master Mix (Applied Biosystems) and 900 nM each PCR primer. From this 20 µL mixture, 3 x 5 µL aliquots were placed into 3 wells of a 384-well PCR plate as plate replicates. PCR was performed in an ABI Prism® 9700HT Sequence Detection System (Applied Biosystems) using default thermal cycling conditions. To quantify GAV RNA copy numbers accurately, cDNA prepared to a 10-fold dilution series of synthetic GAV RNA of know copy number was amplified in the same plate to generate a linear regression plot of mean cycle threshold (Ct) value vs RNA copy number. Infection loads were expressed as GAV RNA copies per 1 ng total RNA to adjust for the use of cDNA prepared to 25 ng total RNA in each 5 µL reaction aliquot.

RT-qPCR was performed initially on RNA extracted from each Day 0 pleopod sample, and only those shrimp identified to possess higher GAV RNA amounts were selected to determine relative changes in infection loads over time following injection of the various dsRNAs. GAV infection loads were tracked over the 14 day bioassay period for 11 shrimp injected with the GAV dsRNA cocktail, 9 injected with dsGAV1, 9 injected with dsGAV6, 6 injected with *Pmerg*DNV dsRNA and 7 injected with luciferase dsRNA.

Statistical analyses

Shrimp survival among treatment groups was compared using repeated measures analysis of variance (ANOVA; PROC GLM; SAS Institute Software, 1999). GAV RNA copy number data were adjusted to $\log_{10}$ scale for standardization, tested for normality and homogeneity using Q-Q

plot analysis, and fitted to a linear regression model using R Software (V3.00). To compare changes between data collection points, a mixed-effects model one-way ANOVA with Tukey post-hoc test was used. P values ≤0.05 were considered statistically significant. Mean GAV RNA copy numbers were assessed to be significantly reduced at bioassay sampling points relative to mean RNA copy numbers detected among pleopods of shrimp sampled at Day 0 immediately prior to dsRNA injection.

Results & Discussion

Among the 5 groups of juvenile *Penaeus monodon* injected with the dsRNAs specific to GAV, *Pmerg*DNV or firefly luciferase, very few deaths occurred over the 14 day bioassay (**Fig. 2**). Similarly low numbers of mortalities also occurred among any of the shrimp handled and biopsied regularly over the 14 day period (**data not shown**). As RT-qPCR data on pleopods sampled from all shrimp immediately prior to dsRNA injection (Day 0) and from selected shrimp thereafter identified none with moderate to high GAV RNA copy numbers (**data not shown**), and as no gross signs of GAV disease became evident in any shrimp, the mortalities that did occur were most likely due to non-specific causes.

Among the 11 *P. monodon* injected with the cocktail of 5 GAV dsRNAs and tracked regularly by RT-qPCR over the 14 day bioassay, mean GAV RNA amounts detected on Day 0 dropped progressively to be significant (P <0.05) on Day 10 (**Fig. 3**). Similarly, among the 9 shrimp injected with dsGAV1 targeted to 5'-terminus of the GAV genome, mean GAV RNA amounts showed a slightly more dramatic decline to be significant (P <0.01) on Day 6 compared to Day 0, with further drops on Days 10 and 14 being statistically significant (P <0.01) compared to Days 0 and 3. While mean GAV RNA amounts appeared to decline somewhat over the 14 day trial among the 9 shrimp

tracked following injection with dsGAV6 targeted to an ORF1b gene region just downstream of the helicase motif, at no time point was the quantum of decline significant compared to Day 0. No significant declines in mean GAV loads became evident among the 6 shrimp tracked following injection of the 2 *Pmerg*DNV dsRNAs or the 7 shrimp tracked following injection of the firefly luciferase dsRNA (**Fig. 3**).

The GAV RT-qPCR data generated for pleopods biopsied progressively from shrimp indicated that GAV replication was being suppressed significantly following injection of the GAV dsRNA cocktail and dsGAV1. These trends were evident even amidst the variability in GAV infection loads among the individuals examined and them being an order of magnitude or more below what would have been ideal to accurately quantify changes above biological and technical noise. For example, at such low infection loads, how GAV is distributed in different pleopods might be expected to vary relatively more than in individuals with higher-level infections (Do 2012). The relative technical consistency in RT-qPCR test will also be lower when RNA template numbers are low. Having more juvenile *P. monodon* with higher-load GAV infections would have avoided these problems and moved RNAi-mediated effects further above the noise threshold. However, despite the limitations imposed by the shrimp available for use, the clear reductions in GAV RNA amounts noted from 6 to 10 days after injection of the GAV dsRNA cocktail and the single dsGAV1, and similar reports of the use of virus-specific dsRNA to reduce or clear pre-existing shrimp infections by densoviruses and YHV (Attasart et al., 2011; Ho et al., 2011; Posiri et al., 2011), provides encouragement for trialling this RNAi approach with wild broodstock used in commercial hatcheries to determine whether similar or greater reductions in GAV infection loads can be achieved.

The ability of tail muscle injection of a cocktail of 5 dsRNAs targeted to regions in the GAV ORF1a/1b gene, and thus the genomic-length RNA replicated in low abundance relative to the 2

sub-genomic mRNAs, to reduce loads of pre-existing subclinical GAV infections in juvenile *P. monodon* is consistent with its ability to protect *P. monodon* against disease following challenge with GAV (Sellars et al. 2011). The finding that the single dsGAV1 dsRNA targeted to the extreme 5'-terminus of the GAV genome reduced infection loads slightly more rapidly and substantially was interesting, and in a practical context would reduce complexity and cost of dsRNA production in any commercial application of technology. As the promoter for the viral RNA-dependant RNA polymerase needed to replicate the GAV genomic ssRNA is likely to reside at or near the 3'-terminus of the complementary full-length anti-genome RNA (Cowley et al 2002, Sawicki 2009), it is not unexpected that a dsRNA targeted to this part of the genome would profoundly inhibit transcription of genomic ssRNA as well as ribosomal translation of non-structural ORF1a/1b gene proteins critical for generating the functional enzymes needed to replicate the viral genome (Ziebuhr 2008). As dsRNA targeted to the *Pm*Rab7 mRNA of *P. monodon* has also been shown to be extremely effective in inhibiting replication of viruses such as YHV and WSSV (Attasart et al., 2009, Posiri et al 2011; Sanitt et al., 2014), and to enhance RNAi mediated by virus-specific dsRNAs, it will be worth exploring whether injection of dsGAV1 together with *Pm*Rab7 dsRNA will further improve its efficacy in inhibiting GAV infection.

The RNAi approach described here has potential to be applied commercially in hatcheries using wild broodstock captured from regions such as North Queensland where GAV is endemic and can occur at high prevalence (Cowley et al. 2000). If pre-existing GAV infections can be reduced appreciably prior to broodstock mating and/or spawning, this might provide a means of restricting vertical transmission of infections to progeny seeded into ponds. As GAV can be transmitted from males or females (Cowley et al. 2002), all broodstock would need to be treated. If used in combination with PCR screening to eliminate broodstock with higher level GAV infections, this RNAi approach might also assist in eliminating GAV infections from domesticated breeding

populations (Coman et al 2013). With these goals in mind, trials are now underway to determine whether injection of GAV dsRNA can reduce infection loads in *P. monodon* broodstock similarly without impacting their fecundity, and whether this approach can mitigate vertical transmission of GAV to progeny.

Acknowledgements

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Table 1: Sequences of PCR primers used to amplify DNA templates for dsRNA synthesis

Primer name	PCR primer sequence (5' - 3')	Position[a]
T7-dsGAV-1F	GGCC**TAATACGACTCACTATA**GGGACGTTACGTTCCACGTACTTATC	1-23
T7-dsGAV-1R	GGCC**TAATACGACTCACTATA**GGGTGATTCCACCTACAATCGTGAT	885-906
T7-dsGAV-2F	GGCC**TAATACGACTCACTATA**GGGATGATCAACCTCCAGAGCTTAGT	624-646
T7-dsGAV-2R	GGCC**TAATACGACTCACTATA**GGGACATTAACCACTGGCATGTAGTC	1446-1468
T7-dsGAV-3F	GGCC**TAATACGACTCACTATA**GGGAACGCATATGCCCAGGCAATCGA	8445-8467
T7-dsGAV-3R	GGCC**TAATACGACTCACTATA**GGGAGCAACGGAATCTGGTGAGAGG	9477-9498
T7-dsGAV-4F	GGCC**TAATACGACTCACTATA**GGGCGATGTTGCTGTTGAGCCT	13309-13327
T7-dsGAV-4R	GGCC**TAATACGACTCACTATA**GGGAGTGAGAGAACAGGATATGCCGGTGT	13818-13843
T7-dsGAV-6F	GGCC**TAATACGACTCACTATA**GGGCATTTCCCCCATAACACCAG	16489-16508
T7-dsGAV-6R	GGCC**TAATACGACTCACTATA**GGGGTGACGAGGATGGCAAAGTT	17267-17286
T7-dsDNV-ns1F	GGCC**TAATACGACTCACTATA**GGGTTACATTTTACTCTGGTATTATTGT	
T7-dsDNV-ns1R	GGCC**TAATACGACTCACTATA**GGGCCCTAGAACTGCTTAACCTTA	
T7-dsDNV-vp1F	GGCC**TAATACGACTCACTATA**GGGCCAAGGTAAACGAAAGAGTAAGC	
T7-dsDNV-vp1R	GGCC**TAATACGACTCACTATA**GGGATACTTCTTGGCGTTCTCTATCTG	
T7-Luc-dsRNA-F	GAATT**TAATACGACTCACTATA**GGGATCGCGCCATTCTATCCTCTA	
T7-Luc-dsDNA-R	GAATT**TAATACGACTCACTATA**GGGATCTACATCGACTGAAATCCCT	

[a] PCR primer positions in the GAV genome (GenBank Acc: AF227196) are indicated.

5'-terminal primer extensions (underlined) including T7 RNA promoter sequences (bold)

Figure legends

Fig. 1. Image of synthetic dsRNAs resolved by electrophoresis in a 1.5% agarose-TAE gel. M = 1 kb-PLUS DNA ladder (Invitrogen), lanes 1-7 = GAV-specific dsRNAs dsGAV1 (955 bp), dsGAV2 (893 bp), dsGAV3 (1093 bp), dsGAV4 (585 bp), dsGAV6 (860 bp) and *Pmerg*DNV dsRNAs targeted to vp1 (662 bp) and ns1 (671 bp) gene sequences, respectively.

Fig. 2. Mean survival (±SD) determined for the 2 tanks each containing 10 juvenile *Penaeus monodon* ($n = 20$) injected with the GAV dsRNA cocktail, the single GAV dsRNAs dsGAV1 or dsGAV6, the luciferase dsRNA and the *Pmerg*DNV dsRNAs,

Figure 3. Real-time RT-qPCR quantification of GAV RNA amounts present in pleopods biopsied progressively from *Penaeus monodon* injected with (A) the GAV dsRNA cocktail, (B) dsGAV1, (C) dsGAV6, (D) *Pmerg*DNV dsRNAs and (E) Luciferase dsRNA. Pleopods were sampled on Days 0, 3, 6, 10, and 14 of the bioassay, with data points labelled 'A' and 'B' being statistically different ($P < 0.05$) from GAV RNA amounts detected on Days 0 and 3, respectively.
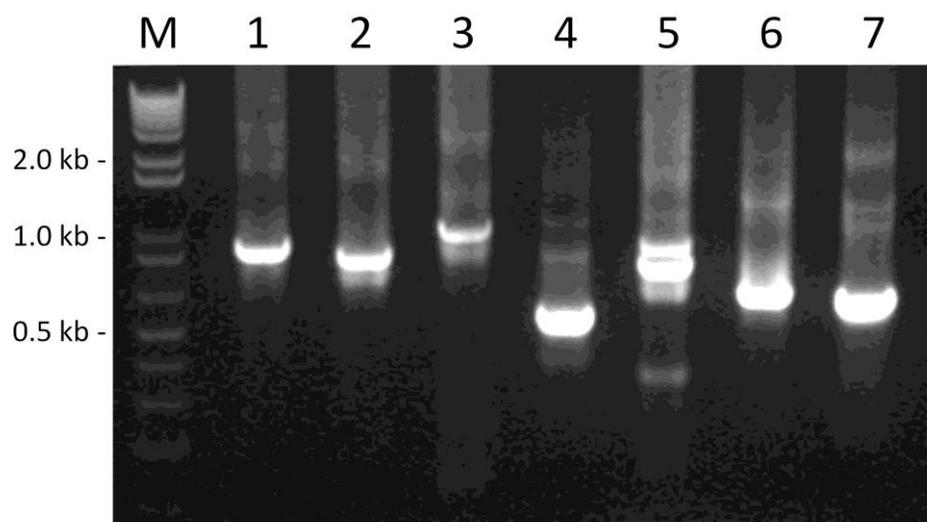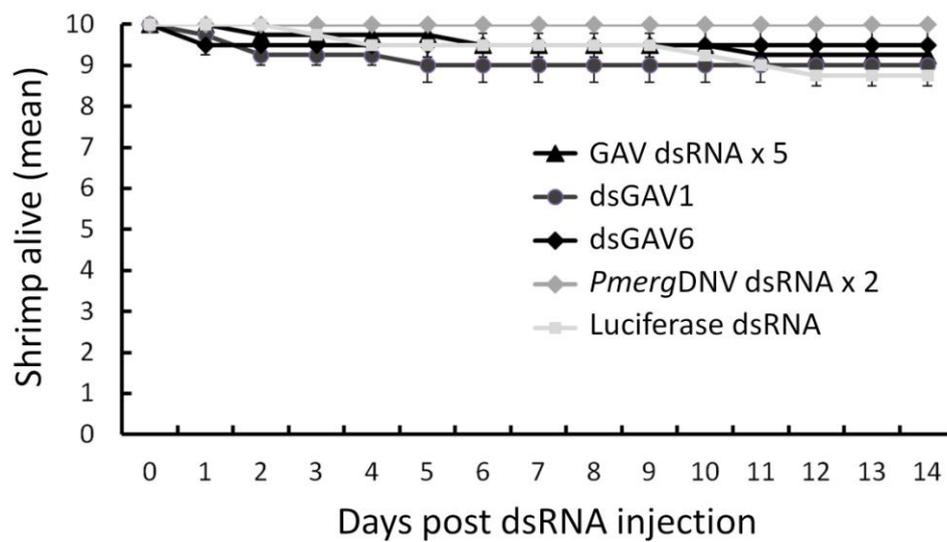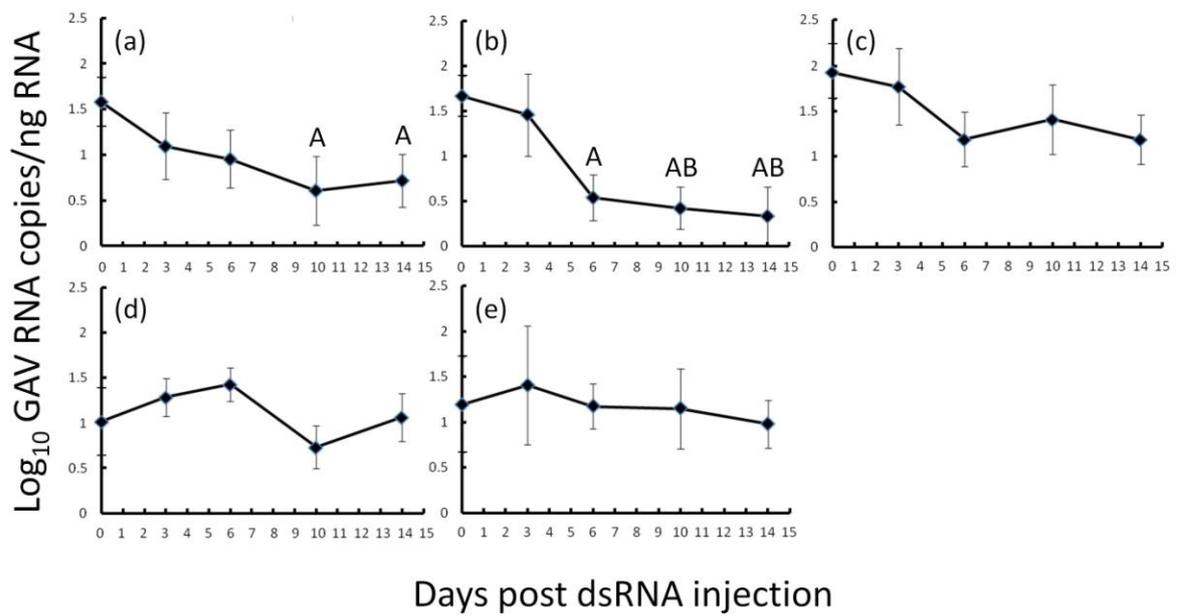
Figure 1.

Figure 2.

Figure 3.

Highlights

We demonstrate pre-existing (naturally acquired) GAV infection in juvenile Black Tiger shrimp can be reduced by injection of multiple or single dsRNAs for the first time.

We demonstrate dsRNA target site is critical to the level of viral reduction achieved when using RNAi in shrimp.

Results indicate that injection of 1 dsRNA instead of a cocktail of 5 in combination with administration to broodtsock shrimp has the potential to abrogate vertical transmission of GAV.