Optimization of viral clearance from broodstock prawns using targeted RNA interference

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Non-Technical Summary

2011/761 Optimization of viral clearance from broodstock prawns using targeted RNA interference

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PROJECT OBJECTIVES:

1. Develop a suite of dsRNAs targeted to *Gill-Associated* virus (GAV)
2. Determine the efficacy of muscle injection of the dsRNA suite in clearing or reducing GAV infection loads in *Penaus monodon* with GAV infection
3. Evaluate the spawning performance of *P. monodon* broodstock in which GAV infection loads have been reduced or cleared using the RNAi strategy

OUTCOMES/OUTPUTS ACHIEVED

The primary output of this project is a simple but robust procedure to effectively reduce GAV infection loads in domesticated and wild *P. monodon* broodstock that can be implemented easily within hatcheries.

A secondary output is the design of reagents and methods for producing dsRNAs to *Penaeus merguiensis* (Banana prawn) *densoviurs* (*Pmerg*DNV) with potential to generate similar RNAi-based reduction of *Pmerg*DNV infection loads in *Penaeus merguiensis* broodstock.

The CRC milestone outcomes have been achieved in full. The primary outcome of this project is knowledge of value to the Australian prawn farming industry on the potential of a simple RNAi injection procedure to reduce GAV infection loads in *P. monodon* broodstock that does not impair their ability to reproduce. With this knowledge, the Australian Prawns Farmers Association or commercial hatcheries can evaluate the potential risks and benefits of supporting pilot-scale hatchery trials to investigate whether the RNAi procedure can reduce GAV infections loads/prevalence in progeny and whether this can result in reduced disease problems and improved farm yields and profitability.

LIST OF SPECIFIC OUTPUTS PRODUCED

GAV dsRNAs with proven abilities to reduce viral loads in *P. monodon* juveniles.

GAV dsRNAs with abilities to reduce viral loads in *P. monodon* broodstock whilst not impeding their ability to spawn and produce nauplii.

Manuscript currently under review for publication in Aquaculture:
Sellars, M.J., Rao, M., O'Leary, Z., Wood, A., Degnan, B.M., Cowley, J.A. Submitted. Reduced loads of pre-existing Gill-associated virus (GAV) infection in juvenile *Penaeus monodon* injected with single or multiple GAV-specific dsRNAs.

Brief description of the project

Broodstock used in prawn hatcheries in Australia are generally managed quite intensively and numbers used are generally low. Opportunities thus exist to inject broodstock with virus-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 thus provide a potential means of reducing vertical transmission of *Gill-associated virus* (GAV) to progeny, juvenile *P. monodon* with subclinical GAV infections were collected from a farm in North Queensland. These juveniles were then injected with different non-specific dsRNAs prepared to sequences of *Penaeus merguiensis* (Banana prawn) *densoviurs* (*Pmerg*DNV), single dsRNAs targeted to GAV genome regions predicted to be highly efficacious, or a cocktail of 5 dsRNAs targeted to sequences spread across the ORF1a/1b replicase gene of GAV (Sellars et al., 2011). GAV infection loads in individuals were tracked over a 2 week period following dsRNA injection using quantitative real-time RT-PCR. The cocktail of 5 GAV dsRNAs and 1 of the 2 single GAV dsRNAs were found to reduce GAV infection loads significantly within 6 to 10 days. The finding that a single dsRNA targeted to a ~1.2 kb sequence at the extreme 5'-terminus of the GAV genome resulted in reductions in GAV loads equal or better than those obtained using the dsRNA cocktail, which also contained this dsRNA, offers potential to further simplify the procedure and markedly reduce its cost. Based on this encouraging data, the dsRNA injection approach was applied to female *P. monodon* broodstock obtained from a commercial hatchery. Although the number of broodstock examined was low, and GAV infection loads in many individuals were below ideal levels for the experiment, injection of the dsRNA cocktail was found to generally reduce infection loads compared to broodstock injected with saline. Reproductive assessment of egg and nauplii numbers and hatch rates showed that spawning performance of the females was not impaired by dsRNA injection. These findings provide encouragement for continuing these studies to determine whether the RNAi approach can reduce GAV vertical transmission from infected broodstock and thus reduce infection loads/prevalence in progeny, and whether injecting males as well as females prior to them mating might further enhance the efficacy of the process.

ACKNOWLEDGEMENTS
We thank Pacific Reef Fisheries, Australian Prawn Farms and Gold Coast Marine Aquaculture for providing experimental *Penaeus monodon* juveniles and broodstock. This research was funded by the Australian Prawn Farmers Association, the

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1. Introduction and Background

Economic losses due to diseases mostly caused by viruses remain a major obstacle to realizing the production potential of prawn aquaculture industries in many parts of the world. To overcome these losses, targeted RNA interference (RNAi) approaches based on injected or ingested virus-specific double-stranded (ds)RNA sequences have been investigated intensively over the past decade as means of protecting prawns against disease and mortality (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). Despite of the efficacy of such RNAi approaches in protecting prawns against viral challenge being demonstrated at least transiently in controlled experiments, little progress has been made in applying the technology commercially.

The RNAi pathway exists in many eukaryotes including prawns. The pathway functions in the cell cytoplasm and is activated by long double-stranded RNA (dsRNA) molecules. Upon recognition by the Dicer enzyme, these long dsRNAs are cleaved by into short dsRNAs ~20 nucleotide in length called short-interfering (si)RNAs (http://en.wikipedia.org/wiki/RNA_interference). These siRNAs then unwind into passenger- and guide-strand single-stranded (ss) ssRNAs, the former of which is degraded and the latter of which is incorporated into the RNA-induced silencing complex (RISC). The guide ssRNA component of RISC then hybridizes to its complementary sequence within a messenger (m)RNA or viral RNA and activates its cleavage by the Argonaute enzyme component of the complex. The outcome of this RNAi pathway is thus highly specific post-transcriptional gene silencing, or in the case of viral infection, specific inhibition of virus replication. In many organisms including prawns, evidence has been obtained for the RNAi pathway playing an important role in defending cells against viral infection. Moreover, the potential to silence genes specifically has resulted in much research interest in using synthetic siRNAs or long dsRNAs to activate the RNAi pathway to identify gene functions, alter biological processes or to protect an organism against parasites and pathogens such as viruses.

Accumulated evidence suggests that most prawn viruses are likely to be transmitted vertically from broodstock to progeny, although experimental evidence for transmission has only been reported for a few viruses including *Gill-associated virus* (GAV) (Cowley et al. 2002) and *White spot syndrome virus* (WSSV) (Lo et al., 1997). To avoid viruses being carried into ponds with seedstock, PCR screening of wild-

caught broodstock is often used to detect and exclude individuals with higher-level infections, thus reducing potential for virus to be transmitted vertically (FAO Fisheries Technical Paper No 450, 2003; Coman et al., 2013). However, such strategies have fallen short of providing a universal remedy against disease across the major aquaculture industries in Southeast Asia and Central America. The only effective disease prevention strategy for the sustainable profitability of these industries has been to replace farming of disease-prone Black Tiger prawns (*Penaeus monodon*) and Chinese White prawns (*Penaeus chinensis*) with domesticated Pacific White prawns (*Litopenaeus vannamei*) selected to be specific pathogen free (SPF) for all viruses of major concern (FAO Fisheries Technical Paper No 476, 2005).

In countries like Australia, however, where hatcheries remain mostly reliant on locally-caught wild stocks of *P. monodon* broodstock, and where quarantine regulations prohibit the import of live prawns of any species, screening exclusion approaches remain the only options for avoiding vertical transmission of viral infections to seedstock. While progress has been made in domesticating and selectively breeding GAV-free or GAV-tolerant stocks of *P. monodon* in Australia (Coman et al., 2013), most hatcheries remain reliant on wild broodstock captured from regions in Northern Queensland where, problematically, GAV is endemic and can occur at high prevalence (Cowley et al., 2000), or from the Northern Territory where GAV or related viruses also occur.

As broodstock are generally managed quite intensively in hatcheries, and as numbers used are usually relatively low, opportunities exist to inject them with virus-specific dsRNAs to induce RNAi responses capable of reducing viral infection loads prior to them being mated and/or spawned to generate seedstock. To obtain evidence that such a strategy is feasible, and thus provide a potential means of reducing vertical transmission of GAV to progeny, juvenile *P. monodon* with subclinical GAV infections collected from a farm in North Queensland were injected with a cocktail of 5 dsRNAs shown previously to protect against challenge with GAV (Sellars et al., 2011). Single GAV-specific dsRNA components of the cocktail were also examined to identify whether these might also reduced GAV infection loads effectively, non-specific dsRNA controls targeted to *Pmerg*DNV were generated for potential subsequent use in clearing *Pmerg*DNV infections from *Penaeus merguiensis* broodstock. By using real-time RT-PCR to quantify GAV infection loads in individual juveniles over a 2 week period following dsRNA injection, the GAV dsRNA cocktail and 1 of the 2 single GAV dsRNAs were found to be capable of reducing infection loads significantly within 6 to 10 days.

With the success of this preliminary study, female *P. monodon* broodstock identified to be infected naturally with GAV were injected with the GAV dsRNA cocktail to confirm that infection loads could be reduced in large prawns and that the procedure did not interfere with their ability to spawn and produce nauplii.

1.1 Need

Outbreaks of viral disease accompanied by morbidity and mortalities occur sporadically in Australian prawn farms and are generally worst when environmental conditions are less favourable, as in the 2010/2011 grow-out season. Poor survival and poor market quality caused by viral disease can impact significantly on farm profitability and even the larger and more sophisticated operations are vulnerable. Based on *Penaeus monodon* farmers commonly stating farm survival rates of 70% or lower, with poor survival in the latter period of grow-out often attributed to evidence of moribund and diseased prawns at pond edges, losses to annual production (around 5000 tonnes valued at $70M) due to GAV disease are estimated conservatively to be over $10M. As the Australian industry moves towards the use of domesticated lines of genetically-improved and virus-free/tolerant prawn species, there is an increasing need for hatchery-friendly methods that minimize the risks of viral infections being passed from broodstock to progeny. Development of a simple procedure for clearing or markedly reducing viral infection loads from valuable broodstock would thus have broad industry value either as part of broodstock breeding programs or for commercial seedstock production using wild-caught broodstock that often carry viral infections.

In Australia, disease caused by GAV infection results in the most substantial economic impacts to *P. monodon* farmers and Hepatopancreatic-parvovirus (HPV or more recently named *Penaeus merguiensis* densovirus, *Pmerg*DNV) is the most problematic virus for Banana prawn farmers. As such, this project aimed to optimise RNAi methods for clearing or reducing GAV infection loads in *P. monodon* broodstock and to produce RNAi reagents targeted to *Pmerg*DNV for use as non-specific controls in the GAV trials with potential for subsequent use for clearing or reducing *Pmerg*DNV infection loads in *P. merguiensis* broodstock. The industry value of this project has been demonstrated by the commitment of APFA funds to support this research together with donations of experimental juvenile and broodstock prawns from APFA members.

1.2 Objectives

1. Develop a suite of dsRNAs targeted to GAV
2. Determine the efficacy of muscle injection of the dsRNA cocktail in clearing or reducing GAV infection loads in *P. monodon* with GAV infection
3. Evaluate the spawning performance of *P. monodon* broodstock in which GAV infection loads have been reduced or cleared using the RNAi strategy

2. Methods

2.1 Juvenile prawn experiment

dsRNA synthesis from PCR products containing terminal T7 promoters

dsRNAs were targeted to positions in the GAV ORF1a/1b gene to restrict their activity to the lower-abundance genomic-length ssRNA to maximize inhibition of virus replication, and were as reported previously (Sellars et al. 2011) except for dsGAV5 targeted to the region overlapping the ORF1b gene helicase motif that was substituted by another dsRNA targeted to a nearby genome region (dsGAV6; 17237-18014 nt; GenBank Acc. AF227196.2) positioned slightly further downstream (**Table 1**). As nonspecific controls, dsRNAs were prepared to conserved sequences in *Pmerg*DNV used previously in RNAi bioassays for this virus (Attasart et al. 2010; 2011) and to a luciferase gene sequence (De Santis et al. 2011; GenBank Acc. EU754723).

DNA templates used to synthesise 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 1.5 uL of each purified dsRNA using a Nanodrop® ND-100 spectrophotometer (Thermo Scientific) and electrophoresis of 800 ng of each dsRNA in an agarose gel to confirm its size and integrity (**Figure 1**), they were stored at -80°C.

Prawns and experimental tank systems

Pleopods were biopsied from juvenile *Penaeus monodon* collected from 42 ponds across two different farms in Northern Queensland, Australia (10 prawns per pond, weighing 6-8 g and ~180 days old). Of these, 4 samples from each pond were tested using a GAV TaqMan real-time qRT-PCR (de la Vega et al., 2004) which identified 2 ponds with slightly higher GAV prevalence (data not shown). Testing of pleopods of the other 6 prawns from each of these two ponds identified, 1 prawn that possessed a low load GAV infection (20-200 GAV RNA copies/ng RNA) and 2 prawns that possessed a low-moderate GAV infection (200-2,000 GAV RNA copies/ng RNA) were identified from one of the ponds. This pond was selected to collect additional prawns for the bioassays. The prawns (now 8-12 g) were collected by cast-netting and those in good visible condition (n = 300) were packed into oxygenated bags in styrofoam boxes (50 prawns/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. Prawns were acclimated for 22 h in two 5 t circular bare-bottom fibreglass tanks (150 prawns per tank) employing gentle aeration and flow-through filtered seawater (28 ± 2°C).

Prawns 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. Prawns were fed commercial pellets *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 prawns alive in each tank was counted and recorded twice daily at the times of feeding.

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)

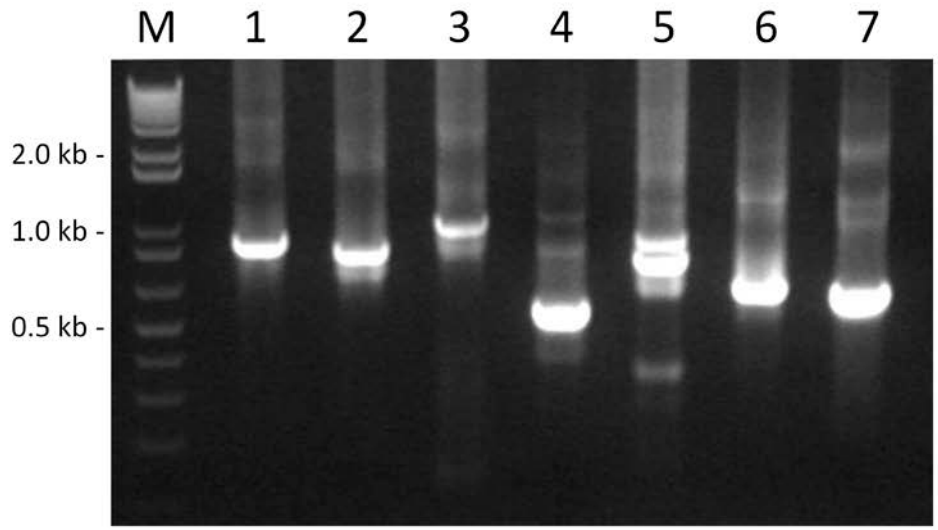

Figure 1. Image of synthetic dsRNAs resolved by electrophoresis in a 1.5% agarose-TAE gel. M = 1 kb-PLUS DNA ladder (Invitrogen), lanes 1-7 = GAV-specific dsRNAs dsGAV1 (955 bp), dsGAV2 (893 bp), dsGAV3 (1093 bp), dsGAV4 (585 bp), dsGAV6 (860 bp) and *Pmerg*DNV dsRNAs targeted to vp1 (662 bp) and ns1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

Tail-muscle injection of dsRNA and sampling

To deliver dsRNA by injection, 25 uL shrimp saline solution (SSS) containing each dsRNA formulation was injected into tail muscle of the 3rd abdominal segment of each prawn between 08:30 and 11:00 h (Bioassay Day 0) using a 100 uL 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 luciferase dsRNA per prawns. There were ten prawns per tank, and four replicate tanks per treatment.

A pleopod was sampled from each prawn on Days 0 and 14 of the bioassay. For prawns in 2 of the 4 replicate tanks used for each treatment, a pleopod was also sampled on Days 3, 6 and 10 to allow progressive tracking of changes in GAV infection loads in individuals over time. Pleopods were excised using scissors alcohol-sterilized between collections and placed into 1 mL RNALater^{TM} solution (Applied Biosystems) and stored at 4°C. To circumvent the need for eye tagging and as previous studies had identified GAV infection loads determined by TaqMan qRT-PCR to be relatively consistent across different pleopods of an individual (Do, 2012), a different pleopod was sampled from each of the 10 prawns in a tank on Day 0 of the

bioassay to allow individual identification in each tank. At each subsequent sampling point, the next consecutive pleopod of each prawn was sampled to ensure that each prawn remained unique and identifiable.

Real-time quantitative (q)RT-PCR

Pleopod tissue was removed from RNALater™ 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), and 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-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 qRT-PCR 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 which 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 the default thermal cycling conditions. To quantify GAV RNA copy numbers accurately, cDNA prepared to a 10-fold dilution series of synthetic GAV RNA of known copy number was amplified in the same plate to a generate linear regression plot of mean cycle threshold (Ct) value vs synthetic RNA copy number. Adjusting for the use of cDNA prepared to 25 ng total RNA in each 5 µL reaction aliquot analysed, infection loads were expressed as GAV RNA copies per 1 ng total RNA.

qRT-PCR was performed initially on RNA extracted from each Day 0 pleopod sample, and only the subset of prawns identified to possess higher GAV RNA amounts were selected to determine relative changes in infection loads over time following injection of the various dsRNA types. In total, 11 prawns injected with the cocktail of 5 GAV dsRNAs, 9 prawns injected with dsGAV1, 9 prawns injected with dsGAV6, 6 prawns injected with the 2 *Pmerg*DNV dsRNAs and 7 prawns injected with the Luciferase dsRNA had their GAV infection loads tracked over the 14 day bioassay period.

Statistical analyses

Prawn 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. Reductions in mean GAV RNA copy numbers at sampling points were assessed by changes relative to mean RNA copy numbers among pleopods of prawns sampled at Day 0 (i.e. immediately prior to dsRNA injection).

2.2 Broodstock prawn experiment

dsRNA synthesis from PCR products with terminal T7 promoters

The same methods as used in the juvenile prawn experiment were employed to produce the cocktail of 5 GAV dsRNAs.

Prawns and experimental tank systems

In an attempt to identify broodstock infected with GAV during the 2013 spawning season, pleopods were excised from over 500 *Penaeus monodon* broodstock originating from each of the geographical locations in the Northern Territory, the East Coast of Queensland and from a breeding program in Southeast Queensland. Testing of RNA isolated from these pleopods using a GAV real-time qRT-PCR (de la Vega et al., 2004) detected no GAV among the Northern Territory broodstock, a low prevalence of very low-level GAV infections among the QLD East Coast broodstock and the breeding program broodstock (data not shown). The low prevalence of moderate level GAV infection detected among these potential sources of broodstock precluded any value in using them in the experiment to assess whether injected GAV-specific dsRNAs could reduce GAV loads in large *P. monodon* with pre-existing infections and whether the procedure would be well tolerated and not impact their reproductive performance. While ideal, it proved difficult to obtain broodstock for use in this trial that originated from one or more wild population (where most hatcheries typically source their spawners) and that possessed moderate-level GAV infection at high prevalence.

At the end of the 2013 hatchery spawning season, a suitable number (n = 46) of ablated third-generation domesticated *P. monodon* females became available that had been held in the same tank as wild *P. monodon* and spawned commercially for 6 weeks. The pre-ablated broodstock were eye-tagged so that individuals were easily identified. Tips of 3 pleopods were excised from each female. Testing by qRT-PCR identified 46 individuals with GAV infection loads ranging from ‘really low’ to ‘high’ (**Table 2**). As these broodstock provided the last opportunity in the 2013 hatchery run to undertake the experiment, and as numbers of available broodstock were limited, it was decided to run only two experimental treatments, one injected with the cocktail of 5 GAV dsRNAs and the other injected with saline alone as a control.

The broodstock were collected from a hatchery in Southeast Queensland, packed into oxygenated bags in styrofoam boxes (10 prawns/box in 10 L cooled (~25°C) seawater) and transported by road on the same day (3 h transport duration) to aquarium facilities at the Bribie Island Research Centre. Prawns were acclimated for 2

h in 10 t circular fibreglass tanks (~25 prawns per tank) with a sub-sand circulation system (Crocos and Coman 1997) employing gentle aeration and flow-through (3.0 L min^{-1}) filtered seawater (28 ± 2°C, 24 ppt salinity). The tanks had black polygal® lids to reduce light intensity and were maintained in a facility providing alternating 14 h light and 10 h dark photoperiods. Prawns injected with either the GAV dsRNA cocktail or with saline were maintained in separate tanks. Prawns were fed commercial pellets, squid (*Loligo sp.*) and green-lip mussels *ad libitum* twice each day at approximately 09:30 and 17:00 h, and waste was removed 3 times per week. The number of prawns alive in each tank was counted and recorded daily.

Table 2. GAV infection loads in female *P. monodon* broodstock and numbers injected with either the GAV dsRNA cocktail or with saline

GAV infection load scale	GAV qPCR Ct	Number prawns	Percentage of population	Number of prawns allocated		Died during transport
				GAV dsRNA injection	Saline Injection	
Very High	≤ 16	0	0%			
High	17 - 19	2	4%	1	1	
Moderate High	20 - 22	5	11%	2	3	
Moderate	23 - 25	3	7%	3	0	
Moderate Low	26 - 28	3	7%	1	2	
Low	29 - 31	9	20%	6	2	1
Very Low	32 - 34	22	48%	10	11	1
Really Low	≥ 35	2	4%	2	0	
Not Detected	Undetermined	0	0%			

Ct = cycle threshold value which is the output value from the TaqMan qRT-PCR test

Tail-muscle injection of dsRNA and sampling

A standardised dose of 5 ug dsRNA per 10 g body weight (eg. 70 ug for a 140 g broodstock) from a 1 ug/uL stock solution containing equal amounts of each of the 5 GAV dsRNAs was injected into the 3rd abdominal segment of each prawn between 11:00 and 13:00 h (Bioassay Day 0) using a 100 uL Hamilton glass syringe fitted with a 26-guage needle. Saline control prawns were injected using the same standardised volume (eg. 70 uL for a 140 g broodstock) with shrimp saline solution (450 mM NaCl, 10 mM KCl, 10 mM Na_2-EDTA, 10 mM HEPES, pH to 7.3). In total, 25 prawns were injected with the GAV dsRNA cocktail and 19 were injected with saline. After injection, prawns were returned to their tank except for those displaying ripe stage IV ovaries which were placed immediately in individual spawning tanks.

Three pleopod tips were sampled from each prawn on Days 0 and 11 of the bioassay and from each prawn immediately after it had spawned. Each female was killed either after it had spawned or when the bioassay was terminated on Day 11. Pleopods were excised using scissors alcohol-sterilized between collections and placed into 1 mL RNALater™ solution (Applied Biosystems) and stored at 4°C. Three pleopods per

female were taken at each sampling time point to normalize any variations in GAV loads in different pleopods quantified by qRT-PCR.

Real-time quantitative (q)RT-PCR

Tissue extraction, reverse transcription of RNA and TaqMan real-time qRT-PCR quantification of GAV infection loads followed the same procedure as described for juveniles.

Reproductive performance assessment

Following tail-muscle injection of either GAV dsRNA or saline, the reproductive performance of female broodstock was assessed over an 11 day period. Females were examined daily for ovarian maturation. Ripe females (Tan-Fermin and Pudadera, 1989) were transferred to circular spawning tanks (0.66 m dia., water flow 0.7 L min^{-1}, water temperature 28°C, fitted with a 60 μM outlet screen) filled to 80 L and allowed to spawn. Spawning tanks were checked for eggs every day at ~09:00 h. After spawning, three pleopod samples were taken from each female and stored in 1 mL RNALater^{TM} solution (Applied Biosystems) as described above before it was culled. If ovaries of a female had regressed, the female was returned to the maturation tank. In instances where a female spawned partially, three pleopod samples were taken as described above and it was returned to the maturation tank. Eggs were allowed to hatch in the spawning tank and egg and nauplii numbers per spawning were estimated from the total number present in three 250 mL water samples taken 2-3 h after egg hatching was observation to have occurred (after thorough mixing to ensure eggs and nauplii were dispersed homogeneously within the water). Measures of reproductive performance included the numbers of females that spawned after injection, eggs per spawning, nauplii per spawning and the egg hatch rate per spawning.

3. Results and discussion

3.1 Juvenile prawn experiment

Among the five *Penaeus monodon* groups injected with dsRNAs specific to GAV, *Pmerg*DNV or Luciferase, very few deaths occurred over the 14 day bioassay period and no statistical differences were evident in survival (**Figure 2**). Among the 20 prawns from each group from which pleopods were collected at regular intervals to track changes in GAV infection loads, qRT-PCR analysis of samples taken immediately prior to dsRNA injection (Day 0) showed GAV RNA copy numbers to be low to undetected (**data not shown**). As no morbidity or gross signs of GAV disease became evident in any prawns, it is unlikely that the few mortalities recorded were due to acute GAV disease resulting from massively increased infection loads.

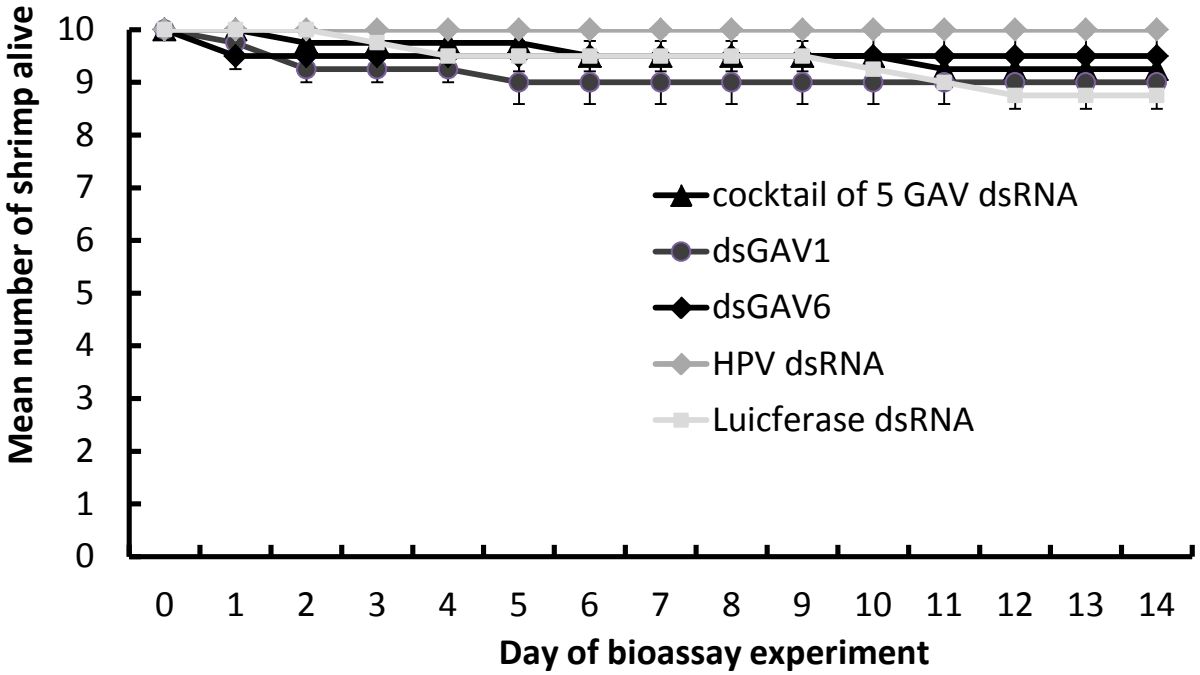

Figure 2. Mean survival (±SD) determined for the 2 tanks each containing 10 juvenile *Penaeus monodon* (*n* = 20) injected with the GAV dsRNA cocktail, the single GAV dsRNAs dsGAV1 or dsGAV6, the luciferase dsRNA and the *Pmerg*DNV dsRNAs.

Among the 11 *P. monodon* injected with the cocktail of 5 GAV dsRNAs and tracked regularly by qRT-PCR over the 14 day bioassay period, mean GAV RNA amounts in the prawns dropped progressively to be statistically significant ($P < 0.01$) at Day 10 compared to Day 0, and a further small drop on Day 14 was statistically significant ($P < 0.01$) compared to Days 0 and 3 (**Figure 3**). Similarly, among the 9 prawns injected with dsGAV1 targeted to 5'-terminal region of the GAV genome, mean GAV RNA amounts showed a slightly more dramatic decline to be statistically significant ($P < 0.01$) at Day 6 compared to Day 0. Mean GAV RNA amounts fluctuated among the 9 prawns tracked following injection with dsGAV6 and showed no significant decline over the bioassay period, although a trend to decline seems apparent. Similar patterns/results were observed among the 6 prawns tracked following injection of the 2 *Pmerg*DNV dsRNAs and the 7 prawns tracked following injection of the luciferase dsRNA (**Figure 3**).

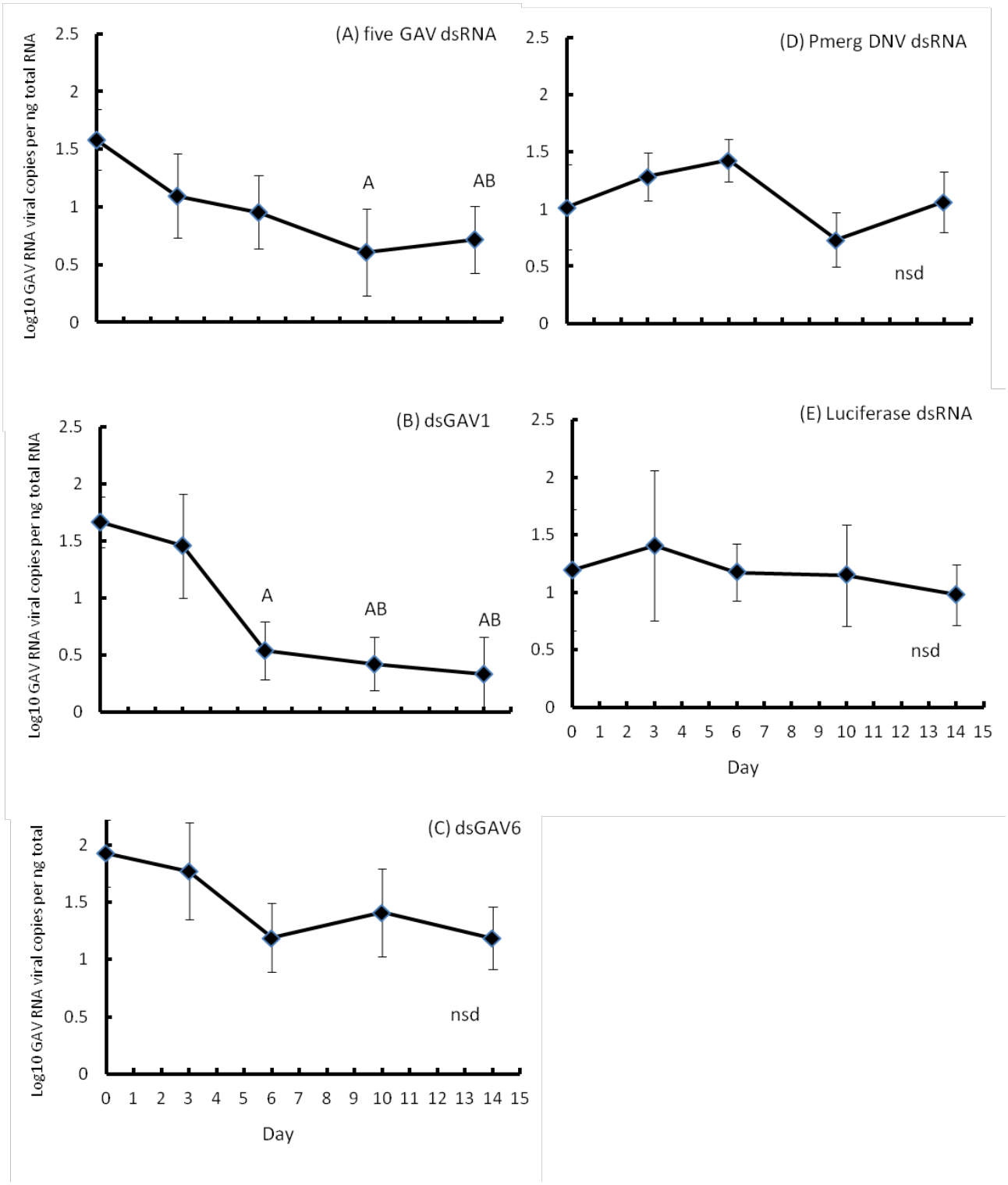

Figure 3. Real-time qRT-PCR quantification of GAV RNA amounts present in pleopods biopsied progressively from *Penaeus monodon* injected with (A) the GAV dsRNA cocktail, (B) dsGAV1, (C) dsGAV6, (D) *Pmerg*DNV dsRNAs and (E) Luciferase dsRNA. Pleopods were sampled on Days 0, 3, 6, 10, and 14 of the bioassay, with data points labelled 'A' and 'B' being statistically different ($P < 0.01$) from GAV RNA amounts detected on Days 0 and 3, respectively.

The mean data on GAV RNA amounts tracked by real-time qRT-PCR in different pleopods biopsied from individual prawns over the course of the bioassay indicated that GAV replication was being suppressed by the GAV dsRNA cocktail and by dsGAV1, but not dsGAV6 or the non-specific *Pmerg*DNV and Luciferase dsRNAs. These trends were evident amidst the variability among individuals that was somewhat expected due to GAV infection loads being less uniform and an order of magnitude or more below that considered ideal for the experiment (i.e. to clearly elevate the data 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. The relative technical variability in real-time qRT-PCR test will also be higher 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 limitation imposed by the viral status of the prawns available, the clear reductions in GAV RNA amounts noted from 6 to 10 days after injection of the GAV dsRNA cocktail and the single dsGAV1 was encouraging for trialling the RNAi approach on wild broodstock used in commercial hatcheries to determine whether similar or greater reductions in GAV infection loads could 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 observation that the single dsGAV1 dsRNA targeted to the extreme 5'-terminus of the GAV genome reduced infection loads even more rapidly than the 5 dsRNAs cocktail was interesting, and in a practical context would reduce complexity and cost of dsRNA production in any commercial application of the RNAi approach. 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 genome would profoundly inhibit transcription of genomic ssRNA and thus ribosomal translation of the non-structural ORF1a/1b gene proteins critical for virus replication (Ziebuhr 2008).

The ability of RNAi to reduce GAV infection loads in juvenile *P. monodon* injected with either a single or a cocktail of GAV-specific dsRNAs provided encouragement that the approach might be applicable 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). For broodstock with subclinical GAV infection, RNAi-mediated reduction of infection loads prior to mating or spawning might provide a means of restricting vertical transmission of viruses to progeny seeded into commercial 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, the RNAi approach might also assist in eliminating GAV infections from domesticated breeding populations (Coman et al 2013).

To investigate these possibilities, *P. monodon* broodstock with pre-existing subclinical GAV infections were sought to trial injections of GAV-specific dsRNA. Due to practical constraints, the experiment was restricted to confirming that dsRNAs could indeed induce similar reductions in GAV infection loads in broodstock as obtained using juveniles, and that the dsRNA injection procedure did not adversely affect reproductive fitness of female broodstock.

3.2 Broodstock prawn experiment

Over the 11 day bioassay period, 4 of the 19 female broodstock injected with saline (controls) spawned and 12 of the 25 female broodstock injected with a cocktail of 5 GAV-specific dsRNAs spawned, with 1 female spawning twice to give 13 spawnings in total (**Table 3**). Due to the fact that the females used in the experiment were obtained at the end of a commercial hatchery run, and thus had been ablated ~8 weeks and spawned for ~6 weeks, this total number of spawns was as good as could have been expected considering the additional handling and physiological stresses caused by capture and transport, acclimation to different tank systems, injection with saline or dsRNA and biopsy of 3 pleopod tips on Day 0 of the bioassay. The resilience of the broodstock to these stressors provides confidence that the dsRNA injection procedure alone will not negatively impact on the ability of females to spawn. With respect to the day of injection, there were 5 dsGAV spawnings on Day 1, 3 saline and 1 dsGAV spawnings on Day 3, 4 dsGAV spawnings on Day 4, 1 dsGAV spawning on Day 7 and on Day 8, 1 saline spawning on Day 9 and 1 dsGAV spawning on Day 10 (**Table 3**).

Fecundity varied markedly from 38,118 to 459,776 eggs/spawn among the 4 females injected with saline and from 6,224 to 231,793 among the 11 females injected the GAV dsRNA. These variations in numbers of eggs is normal for *P. monodon* broodstock given most of these domesticated females would have spawned one or more times in their prior 6 weeks in the commercial hatchery (Coman and Murphy, personal communication).

Egg hatch rates were also highly variable with only 1 batch of the 4 from saline-injected females hatching, and ranges of 1.58% to 51.26% (mean 32.89%) observed with the 9 of 13 batches that hatched from dsGAV-injected females (**Table 3**). As for egg numbers, high variations in hatch rates are normal for domesticated *P. monodon* broodstock. It is also important to note that as only females were available and stocked into tanks, that any female that moulted after injection on Day 0 of the bioassay and subsequently spawned would have discarded the male spermatophore resulting in no egg fertilisation and a 0% hatch rate. Despite the experimental

limitations, the spawning data demonstrate that the various stresses imposed on the *P. monodon* broodstock, including a single injection of the GAV dsRNA cocktail and the 3 pleopod biopsies needed to quantify GAV infection loads, did not have any profound adverse impacts on the ability of ablated females to spawn and produce viable nauplii.

The TaqMan real-time qPCR data were determined using a standard amount of total RNA isolated from each of the multiple pleopods sampled from each broodstock on Day 0 of the bioassay and at the time the female spawned between 1 and 9 days post-injection or when the bioassay was terminated for females that had not spawned on Day 11 post-injection. Testing of multiple pleopods from each individual was undertaken to improve statistical accuracy of the PCR data. Mean GAV infection loads detected in 2 of the 4 saline-injected females that spawned increased between Day 0 and time of spawning (**Table 3**). For Female Y4, GAV loads increased from very low to the low level infection category and for Female Y17, GAV loads increased from the moderate to moderate/high level infection category. Infection loads in the other 2 saline-injected females that spawned remained steady at either a moderate level for Female Y10 or a very low level for Female Y17. Among the 4 saline-injected females that survived to Day 11 and did not spawn, GAV infection loads increased in Female Y1 from very low on Day 0 to moderate on Day 11 and remained steady at either a very low in Females Y3 and Y8 or low in Female Y21.

Among the females injected with the GAV dsRNA cocktail, GAV infection loads remained stable between Day 0 and time of spawning in 7 of the 12 that spawned (Females B5, W5, W11 and W13 at very low infection category, Female W1 at low infection category, Female W18 at medium/low infection category and Female W8 at high infection category) (**Table 3**). The GAV infection loads among the other 5 females that spawned all decreased between Day 0 and the time of spawning (Female W3 low > really low infection category, W7 from low > very low infection category and W4, W15 and W20 from very low > really low infection category). Among the remaining 3 females injected with GAV dsRNA that did not spawn and remained alive at Day 11, 2 had reduced GAV infection loads (Females B2 and B17 were very low on Day 0 and really low on Day11), and one had a stable moderate low GAV infection load (Female W2).

In summary, tracking changes in GAV infection loads using the TaqMan real-time qPCR showed the loadings remained stable in most female *P. monodon* broodstock; increased in some of the saline-injected prawns; and decreased in some GAV dsRNA-infected prawns. The broodstock experiment thus demonstrated that the dsRNA injection procedure could be undertaken without apparent impact on the ability of females to produce progeny and that it provided the ability to reduce GAV infection loads in some individuals. Moreover, the absence of any increase in GAV infection loads in the dsRNA injected broodstock might also ‘suggest’ that increasing loads (due to the stress of handling, transport, spawning management etc) were constrained.

This experiment thus provides preliminary evidence that an RNAi approach based on tail muscle injection of dsRNA could realise commercial benefits when applied in hatcheries as a strategy for reducing viral infection loads in broodstock and thus provides potential to inhibit vertical viral transmission from broodstock to progeny in commercial aquaculture.

Table 3. GAV RT-qPCR data estimating infection loads female broodstock at Day 0 and 11 of the bioassay and after they spawned on various days post-dsRNA injection, and total egg, nauplii and egg hatch rates of each spawn

		GAV Pre-injection			GAV post-spawning			GAV end of experiment			Change	Spawning data		
Female	Treatment	Day	Ct Ave	SE	Day	Ct Ave	SE	Day	Ct Ave	SE	Ct	Total Egg	Total Naup	Hatch Rate (%)
Y 1	Saline	0	32.41	1.07				11	23.74	1.50	- 8.67			
Y 3	Saline	0	34.08	0.42				11	32.73	1.52	- 1.35			
Y 4	Saline	0	33.26	0.43	3	29.51	0.49	11	28.90	1.44	- 4.36	25,582	12,536	32.89
Y 8	Saline	0	33.41	0.37				11	32.17	1.46	- 1.24			
Y10	Saline	0	23.54	0.47	3	25.33	0.69				1.79	459,776	0	0.00
Y 14	Saline	0	34.24	0.21	9	34.08	0.11				- 0.16	82,785	0	0.00
Y17	Saline	0	22.77	0.91	3	20.91	1.86				- 1.81	367,587	0	0.00
Y 21	Saline	0	29.42	0.04				11	30.26	2.02	0.84			
B 2	dsGAV	0	33.74	0.22				11	34.58	0.14	0.84			
B 5	dsGAV	0	33.10	0.01	8	33.66	0.48				0.55	173,071	0	0.00
W 1	dsGAV	0	30.40	0.97	1	29.96	0.47				- 0.44	189,389	0	0.00
W 2	dsGAV	0	27.56	0.51				11	25.50	1.28	- 2.06			
W 3	dsGAV	0	31.42	0.28	4	34.91	0.05				3.49	213,857	3,440	1.58
W 4	dsGAV	0	33.64	16.82	3	35.77	0.67				2.13	72,052	68,294	48.66
W 5	dsGAV	0	32.64	0.32	4	34.37	0.07				1.73	63,904	60,060	48.45
W 7	dsGAV	0	31.84	0.35	10	34.03	0.36	11	33.99	0.33	2.15	23,323	0	0.00
W 8	dsGAV	0	19.82	0.25	1	18.70	0.11				- 1.12	111,373	38,602	25.74
W 11	dsGAV	0	33.36	0.50	1	34.35	0.31				0.99	210,363	0	0.00
W 13	dsGAV	0	33.25	0.49	1	34.19	0.13				0.94	194,849	36,944	15.94
W 15	dsGAV	0	33.31	0.43	1	34.71	0.14				1.40	136,980	17,326	11.23
W 17	dsGAV	0	34.52	0.11				11	34.74	0.26	0.22			
W 18	dsGAV	0	26.33	0.40	4	25.82	1.24				- 0.51	38,846	40,852	51.26
W 20	dsGAV	0	33.29	0.11	4	34.95	0.92				1.66	23,882	4,619	16.21
W 20	dsGAV				7	34.35	1.43				NA	4,876	1,349	21.67

Change Ct = Ct (Day 0) – Ct (Day 11 or Day female spawned); GAV infection load increase (negative value); GAV infection load decrease (Positive value), Ct 3.33 = 10-fold change;
Ct = qPCR cycle threshold; Ave = average of 3 replicate tests

5. Benefits and Adoption

Success in developing and implementing the RNAi-based GAV infection reduction or clearance methodology may derive increased industry profitability through maintained spawning performance of *P. monodon* broodstock with reduced potential to transmit infection to progeny resulting in reduced risks of GAV-induced morbidity and mortality occurring during grow-out. The impacts of increased survival during grow-out has the potential to add ~$12 million annually to the value of the industry (based on the 2009-10 data – QLD-DEEDI Report to Farmers: 5,115 tonnes, $78 million).

This project has demonstrated the possible benefits of dsRNA administration to broodstock used in hatcheries as a means of reducing GAV infection loads likely to result in reduced vertical transmission of GAV infection to seedstock. However, before this can be contemplated commercially, additional experimentation is needed to (i) optimize the dsRNA injection strategy to maximize reductions in GAV infection loads in broodstock prior to spawning (ii) confirm whether this strategy indeed restricts vertical transmission of GAV to progeny, (iii) quantify what improvements in survival and productivity can be derived from the use of such seedstock and (iv) quantify the costs-benefits of the process in terms of farm profitability. However, health risk assessments and regulatory permits will be required from the APVMA prior to undertaking RNAi viral clearance in commercial hatcheries to produce seedstock for commercial grow-out.

6. Further Development

There are several key steps to developing this technology further for commercial scale adoption;

- APVMA permit obtained to inject synthetic dsRNA into broodstock at a commercial hatchery supplying seedstock that will be grown for human consumption
- Administration of the antivirals to large numbers of commercial broodstock prawns with known GAV infection levels (known from PCR quantification), spawning of prawns on-farm, GAV testing of females after spawning (PCR quantification), and following of spawned families through the hatchery into farm ponds and GAV screening of progeny once they reach a minimum size of 8 g to determine if vertical transmission as been abrogated

The further development of this technology would be best achieved through an industry-supported tactical research project that spans 2 commercial production seasons to increase the likelihood of attaining a year in which broodstock possess low-moderate GAV infection loads needed to clearly demonstrate its potential. Access to good quality broodstock would also be essential to allow comprehensive

assessment of the technology and sound scientific replication of treatments and controls.

7. Planned Outcomes

Public Benefit Outcomes

Reduced viral infection loads resulting in increased and more predictable and stable supply of farmed prawns for human consumption.

Private Benefit Outcomes

Improved prawn farm productivity and profitability through reduced losses due to viral disease.

Linkages with CRC Milestone Outcomes and Outputs

The CRC milestone targeted has been achieved in full.
The proposal addresses outcomes and outputs in Seafood CRC Milestone 1.3.3 'Strategic disease management approaches and technologies developed for at least two aquaculture species'.

The primary output of this project is a simple but robust RNAi procedure with the ability to reduce pre-existing infection loads of GAV in *Penaeus monodon* juveniles as well as *P. monodon* broodstock that can be implemented easily as a commercial hatchery practice. The next phase of this research is to determine whether the RNAi procedure can restrict the vertical transmission of GAV to progeny and lessen the risks of GAV disease occurring during seedstock grow-out on farm.

A secondary output is the availability of PCR primers and methods for producing dsRNAs to *Pmerg*DNV to generate similar RNAi-based reductions in virus infection loads in *Penaeus merguiensis* broodstock.

8. Conclusion

This study has demonstrated that the injection of a cocktail of 5 GAV dsRNAs or a single dsRNA targeted to the extreme 5'-terminus of the GAV genome can reduce pre-existing GAV infection loads in juvenile *P. monodon*. Similarly, the injection of a cocktail of 5 GAV dsRNAs in broodstock prawns appeared to reduce pre-existing GAV infection loads without any adverse impacts on the ability of females to produce viable nauplii. This experiment provides the first evidence that an RNAi approach applied in hatcheries might be able to contribute to mitigation viral disease impacts in prawn aquaculture that are vertically transmitted to improve farm productivity. The next phase of this research needs to explore whether the resulting reduced viral

infection levels can indeed reduce or eliminate vertical transmission of GAV infection to progeny, thus reducing disease risks associated with infection being carried with postlarvae (PL) into ponds.

9. Acknowledgements

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