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In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

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Abbreviations

APFA	Australian Prawn Farmers Association
APVMA	Australian Pesticides and Veterinary Medicines Authority
BIRC	Bribie Island Research Centre
bp	base pairs
cDNA	complementary DNA
CSIRO	Commonwealth Scientific and Industrial Research Organisation
dsRNA	Double stranded RNA
DNA	deoxyribonucleic acid
FRDC	Fisheries Research and Development Corporation
GAV	Gill-associated virus
HPV	Hepatopancreatic parvovirus
IHHNV	Infectious hypodermal and haematopoietic necrosis virus
IMNV	Infectious myonecrosis virus
JBG	Joseph Bonaparte Gulf
MBV	Monodon baculovirus
MCMS	Mid-crop mortality syndrome
MoV	Mourilyan virus
nPCR	nested PCR
nt	nucleotides
PCR	polymerase chain reaction
PL	postlarvae
RNAi	RNA interference
QBP	Queensland Bioscience Precinct
QDAF	Queensland Department of Agriculture and Fisheries
QLD	Queensland
RNA	ribonucleic acid
RT-PCR	Reverse transcription-PCR
RT-nPCR	RT-nested PCR
RT-qPCR	RT-quantitative PCR
TNA	total nucleic acid
YHV	Yellow head virus

Executive Summary

What the report is about

Reported here are the outcomes of a project with original objectives to assess (i) the ability of injected double-stranded (ds)RNA antivirals to reduce Gill-associated virus (GAV) infection loads in Black Tiger prawn (*Penaeus monodon*) broodstock and whether this can (ii) reduce GAV infection prevalence/loads in progeny and (iii) result in improved growth performance and survival of progeny reared in research ponds under simulated commercial conditions.

As these project objectives were revised due to difficulties in sourcing wild broodstock infected with suitably high loads of GAV, also reported are data from agreed alternative project objectives showing that (i) Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is transmitted vertically from infected female broodstock to progeny and that the IHHNV prevalence and infection loads in progeny are influenced by infection loads in their parental female and (ii) the high-load infections that develop in progeny spawned from females with higher-level IHHNV infection result in substantially reduced growth performance and survival of progeny reared in 0.16 ha ponds under simulated commercial conditions.

The screening of batches of wild *P. monodon* broodstock to identify locations where these might be infected with GAV at moderately-high loads suitable for the original project objectives identified GAV to be present at very low prevalence among prawns captured at various locations in the vicinity of Innisfail between May and June 2016. Similar screening also identified the absence of Yellow head virus genotype 7 (YHV7) in these broodstock.

Further to these objectives and data, it was agreed to include another project variation objective to investigate whether (i) dsRNA(s) injected into tail-muscle of female broodstock at the time eyestalks were ablated to induce ovary maturation/spawning could be detected by TaqMan real-time RT-qPCR in various tissues (i.e. pleopod, ovary and lymphoid organ) several days later when the female spawned and (ii) dsRNA might transfer from injected females to spawned eggs and be maintained or amplified through larval life stages (i.e. nauplii, protozoea, mysis) to an early post-larvae (PL) stage.

Background

Globally, pathogens have been estimated to cause USD$6B in annual economic losses to prawn aquaculture. To try to ameliorate these losses, dsRNA antivirals that activate the prawn RNAi interference (RNAi) mechanism have been investigated intensely over the past 15 years. Experimentally, such dsRNA antivirals have been demonstrated to protect prawns against disease and mortality (or at least markedly slow progression to disease) following viral challenge, and to reduce infection loads in prawns with naturally-acquired infections. While dsRNA antivirals have been demonstrated to be effective against all viruses of commercial concern and there are reports of them being used in prawn hatcheries overseas, no therapies based on the use of dsRNA antivirals are available commercially.

In CSIRO, it has been demonstrated experimentally that muscle-injected dsRNA antivirals can interfere with and markedly slow the rate of GAV replication, and that a single dsRNA dose can completely protect prawns against disease and mortality over a 3 week period during which all non-injected prawns had died (Sellars et al., 2011). It has also been demonstrated that injected dsRNA antivirals can substantially reduce loads of naturally-acquired sub-clinical GAV infections in juveniles and broodstock (Sellars et al., 2011; 2014). Moreover, when injected into female broodstock, dsRNA antivirals do not impede spawning of fertile eggs (Sellars et al., 2016).

As vertical transmission of viruses from broodstock to progeny is the primary route by which viral infections enter commercial grow-out ponds (e.g. GAV, Cowley et al., 2002), and as injected dsRNA can reduce viral infection loads in broodstock, injecting broodstock in hatcheries prior to them spawning is attractive as an intervention point at which to minimize risks of virus-induced production losses for three fundamental reasons; (i) the numbers of broodstock needed to be treated is low compared to numbers of prawns that would need to be treated in ponds, (ii) injecting a single broodstock offers the opportunity to markedly reduce viral infection prevalence and/or loads in the up to 450,000 progeny commonly produced each time a female spawns, and (iii) injections can occur during standard operating procedures where females are handled for eye-tagging and weighing.

This project was thus conceived to identify whether the application of dsRNA antivirals in prawn hatcheries could generate commercial benefit. Specifically the experiment aimed to determine whether muscle-injected dsRNA could reduce GAV infection loads in broodstock sufficiently to reduce virus loads vertically transmitted to progeny using simulated commercial hatchery methods and whether the process could improve prawn health, growth performance and survival under simulated commercial rearing conditions.

Generating such data would provide the Australian prawn farming industry with confidence that using dsRNA antivirals in their hatcheries could improve farm productivity without compromising the spawning performance of expensive broodstock. Moreover, as injected dsRNA will be considered a therapeutic drug and have conditions for its commercial use assessed and regulated by the Australian Pesticides and Veterinary Medicines Authority (APVMA, https://apvma.gov.au/), an additional objective to assess human health risks of the dsRNA antiviral injection process by undertaking an additional experiment to investigate whether dsRNA injected into female broodstock is transferred to and maintained/amplified in their progeny was undertaken.

Project objectives

The original project objectives were to:

1. Demonstrate that injected dsRNA antivirals can abrogate or reduce GAV transmission from broodstock to progeny, and
2. Quantify the health, survival and growth performance of progeny derived from GAV-infected female broodstock injected with GAV-specific dsRNA antivirals when reared in ponds under simulated commercial conditions

Original project objectives were amended following consultation with FRDC, SIEF and APFA representatives according to risk mitigation strategies outlined in the Project Agreement. These alternative project objectives were to:

3. Quantify the production benefits of rearing progeny derived from female broodstock with lower-load IHHNV infections compared to females with higher-load IHHNV infections, and
4. Determine whether dsRNAs injected into female broodstock at the time of eyestalk ablation are detectable in tissues several days later at spawning and whether this dsRNA is transferred to progeny and maintained/amplified through larval life stages up until an early post-larvae age

Methodology

Objectives 1, 2 and 3. In May-June 2016, a batch of wild *P. monodon* broodstock captured near Innisfail, Qld, Australia were screened by real-time qPCR to assess the prevalence and infection loads of GAV (as well as IHHNV and YHV7 to identify whether these viruses might compromise data) to determine whether these were sufficiently high to meet Project Objectives 1 and 2 as described above. As both GAV prevalence and infections loads were below that considered useable, the commercial broodstock supplier was asked to trawl different locations in June-Juy 2016 for stocking this experiment in the hope of finding more suitable experimental prawns. However, due to difficulties at this time in finding broodstock in numbers needed for the project, and to time constraints/commitments on when the trial needed to start to meet commercial hatchery and grow-out time-lines, a second broodstock batch caught from the same general location was provided. As a few of these prawns possessed GAV infections at loads considered marginal but potentially useful, agreements were sought to proceed with the GAV dsRNA injection and progeny grow-out project objectives as planned. Based on the GAV-load data, broodstock considered more suitable for the trial were segregated into treatment/control tanks and fed a conditioning diet for 16 days followed by a maturation diet for 22 days. At this time, females were eyestalk ablated to promote spawning and either injected with the GAV dsRNA antiviral or with saline. Females began spawning from 6 days after eyestalk ablation. Larvae were reared in tanks containing progeny from between 1 and 4 females for 37 days, at which time selected families of postlarvae stage 20 (PL20) were pooled, stocked at 44 PL20/m^2 into 0.16 ha research ponds (2 dsRNA-injected, 2 control ponds) and reared for 160 days. All procedures followed those typically used in commercial hatcheries and farms as closely as practical, and to ensure Australian industry relevance,

were undertaken either directly or under the direct guidance of Brian Murphy who had previously managed large prawn hatcheries in Queensland, Australia for 20 years.

Tissues were sampled from (i) broodstock before and after dsRNA antiviral injection and at the time each female spawned, (ii) eggs and larval life stages (ie. nauplii, zoea, mysis, early PL) reared in each hatchery tank and (iii) prawns reared in each of the 4 ponds at 30-40 day intervals throughout the grow-out period in numbers (n = 144) selected to allow GAV (or IHHNV and YHV7) to be detected by RT-qPCR testing down to a 2% prevalence level. The sex and weights of prawns were also recorded at these times, and total prawn biomass and survival levels were estimated when ponds were harvested.

Objective 4. A group of 8 female *P. monodon* broodstock, some identified later to be carrying a low-level naturally-acquired GAV infection, were co-injected with dsRNAs designed to be specific to the GAV RNA genome (dsGAV1) and the firefly luciferase gene mRNA (dsLuc). Another group of 8 non-injected females were used as controls. Egg batches spawned from each of 3 injected and non-injected females were reared separately to an early PL age. Broodstock tissues (pleopod, ovary, lymphoid organ) were sampled immediately after each female spawned, and pools of eggs and larval life stages (nauplii, protozoea, mysis), as well as a large number of early-stage PL, were sampled in a manner that would allow each to be tested individually. Loads of GAV genomic RNA and the dsGAV1 and dsLuc dsRNAs in these samples were quantified using 3 RT-qPCR tests designed to detect GAV genomic RNA, dsGAV1 dsRNA (which also co-detect GAV genomic RNA) and dsLuc dsRNA.

Prawns were maintained, matured, spawned and reared in tank systems and 0.16 ha research pond under simulated commercial hatchery and farm conditions at the CSIRO Bribie Island Research Centre (BIRC), Bribie Island, Queensland, Australia. The dsRNA antivirals were synthesised and prawn tissues were processed and testedby RT-qPCR at CSIRO Agriculture & Food, Queensland Bioscience Precinct, St Lucia, Queensland, Australia.

Results/key findings

1. At the very low GAV levels present in broodstock, GAV RNA became undetectable in some of the prawns injected with dsGAV1 dsRNA, and these decreases (3 to 4 GAV RT-qPCR Ct values) equated to 8-fold to 16-fold reductions in GAV RNA load. Whether GAV-load reductions of this magnitude or substantially larger could be achieved by a single injection of the dsRNA antiviral into broodstock infected at higher loads with GAV is not known. It is also unknown if complete clearance of GAV can be achieved, noteing that all sampling methods and testing methods have limitations. In optimising this process, it will thus be important to investigate the effects on GAV infection load and of repeated injections of dsRNA antivirals.

2. Simulated commercial hatchery and farm pond rearing of progeny from dsRNA-injected female broodstock was not compromised, and indeed harvest yields achieved were well above the average for the Australian *P. monodon* farming industry.

3. Data were generated that confirmed IHHNV to be transmitted vertically and demonstrating that IHHNV loads in progeny were well correlated to, and could be predicted from, loads detected in the female from which they were spawned.

4. Ponds stocked with PL20 derived from female broodstock with low-load IHHNV infections had significantly improved growth, survival and total prawn biomass at harvest (equivalent to a 3.72

tonne/ha pond improvement) compared to ponds stocked with PL20 derived from females with high-load IHHNV infections. With the average weight (35-38 pieces/kg) of prawns that maintained low-load IHHNV infection and a current wholesale value of cooked prawns of AUD$18/kg, improved gross value was estimated to be ~AUD$67K for a typical 1 ha commercial pond.

5. Between May and June 2016, *P. monodon* broodstock in the Innisfail region of North Queensland, Australia had a very low prevalence of GAV, and when detected, it was present only at very low infection loads.

6. When injected into the tail-muscle of female broodstock at the time of eyestalk ablation, dsRNA spreads and can be detected several days later (when the female spawns) in pleopod, lymphoid organ and potentially importantly, for the purpose of it reducing virus loads in spawned eggs, in ovarian tissue. Despite being detected in ovary, there is no evidence from this study of the injected dsRNA being transferred from females to their eggs, or it being either maintained or amplified during larval growth through the nauplii, protozoea or mysis life stages up to early-stage PL.

Implications for stakeholders

There is now sufficient scientific literature and first-hand experimental experience in CSIRO Agriculture & Food to state with confidence that injected dsRNA antivirals will have the capacity to reduce viral infection loads in broodstock, and that the dsRNA injection procedure itself does not compromise female spawning capacity or the viability/robustness of progeny reared using standard commercial systems. If the procedure can significantly and reliably reduce virus prevalence and/or loads in progeny, it should assist hatcheries produce higher-health PL for either pond stocking or that command a premium price. Injecting dsRNA antivirals into broodstock might also find application in prawn breeding programs, either to eliminate viral infections in progeny derived from infected wild founder stocks and if this is not absolute, by employing it at each breeding cycle to pragmatically and systematically reduce viral infection prevalence to the point of freedom, whereby breeding lines can be certified unequivocally as specific pathogen free (SPF).

Data showing that IHHNV is transmitted vertically and that infection loads in progeny correlate with that present in the female from which they were spawned indicate that qPCR screening to select for either IHHNV-low broodstock or IHHNV-low PL batches will be valuable. Just how valuable was clearly demonstrated by improved growth performance, survival, prawn biomass yields at harvest and the estimated gross net value of these increased yields (~AUD$67K per 1 ha pond) realized in ponds stocked with progeny of IHHNV-low females.

Hurdles that remain to trial the technology in commercial hatcheries include granting of a permit/licence/exception for its use by the APVMA and CSIRO licencing RNAi IP to a company willing to synthesise and make dsRNAs available for use. To support an application to the APVMA to consider how they might regulate the use of dsRNA antivirals in hatcheries, data was obtained in this protect to demonstrate unequivocally that dsRNA injected into a female broodstock at the time of eyestalk ablation is not transferred to eggs. As progeny reared commercially for human consumption from such eggs should thus not represent a health risk, these data will be persuasive in application to the AVPMA to evaluate and define conditions under which it might be used.

Recommendations

1. Based on the outcomes of this project, we recommend that a small consultative working group be established to champion discussions with the APVMA to define conditions under which the dsRNA antiviral injection process can be trialled in commercial prawn hatcheries to reduce virus vertical transmission. This working group would include key CSIRO researchers, industry technical advisers and APVMA staff.

2. Scientific rigorous data sets that quantify the impacts to production of pathogens such as this study with IHHNV are scarce and not commonly published. Efforts within the Australian industry should look at establishing these data sets for other pathogens so as to identify the biggest pain points and thus greatest opportunities for intervention with antivirals and pathogen management strategies throughout the culture phase. Such data sets would be best obtained by scientific team(s) and farm operator(s) coming together to monitor prawn health, growth and survival on specified ponds during commercial culture.

Keywords

Black tiger prawn, Giant tiger prawn, Leader prawn, *Penaeus monodon*, prawns, gill-associated virus, Infectious hypodermal and haematopoietic necrosis virus, antivirals, RNA interference, RNAi aquaculture

Introduction

Pathogens in global prawn aquaculture cause economic losses of $6B annually. To help ameliorate these losses, dsRNA-based antivirals that activate the natural RNA interference (RNAi) mechanism of prawns have been investigated intensively over the past decade (Shekhar and Lu, 2009). Such dsRNA antivirals have been demonstrated experimentally to protect prawns, at least transiently, against disease and mortality following virus challenge and/or to reduce virus loads in prawns with naturally-acquired subclinical infections. Viruses for which RNAi has proved effective include those with double-stranded (ds)DNA genomes such as White spot syndrome virus (WSSV; Robalino et al., 2004; Mejía-Ruíz et al., 2011; Sarathi et al., 2008; Thammasorn et al., 2015; Rattanarojpong et al., 2016; Nilsen et al., 2017; Sanjuktha et al., 2012), single-stranded (ss)DNA genomes and IHHNV and hepatopancreatic parvovirus (HPV; Attasart et al., 2010, 2011, Chimwai et al., 2016, Ho et al., 2011, Dhar et al., 2014); single-stranded (ss)RNA genomes such as Yellow-head virus (YHV) and Gill-associated virus (GAV) (Saksmerprome et al., 2009; Somchai et al., 2016; Tirasophon et al., 2005; Yodmuang et al., 2006, Sellars et al., 2011, 2014, 2016), Taura syndrome virus (TSV; Ongvarrasopone et al., 2011) and Laem Singh virus (LSNV; Saksmerprome et al., 2013; Thammasorn et al., 2013). As yet, however, no dsRNA-based antivirals are available for commercial use.

Vertical transmission of viruses from broodstock to progeny in hatcheries is the primary route by which viral infections enter commercial grow-out ponds (e.g. GAV, Cowley et al., 2002). As such, the use of dsRNA antivirals in hatcheries to abrogate or reduce vertical transmission levels offers an attractive intervention point for limiting the risks of viral disease occurring during prawn grow-out, particularly as each female can spawn up to 450,000 eggs that would be far more challenging to treat effectively as individuals.

In CSIRO, it has demonstrated that dsRNA antivirals targeted to GAV and interfere with and slow the rate of virus replication, thus transiently protecting prawns against disease and mortality following experimental challenge (Sellars et al., 2011). The dsRNA antivirals have also been shown to be able to reduce loads of pre-existing sub-clinical GAV infections both in juveniles and adult broodstock (Sellars et al., 2011; 2014). Moreover, when injected into female broodstock, dsRNA was not identified to impede spawning of fertile eggs (Sellars et al., 2016). In combination these data look promising for applying dsRNA treatments in prawn hatcheries. However, until this study, whether or not the grow-out performance of progeny from dsRNA-injected broodstock might be compromised has not been reported.

In Australia, the culture of Black Tiger prawns (*Penaeus monodon*) relies mostly on seedstock produced from wild broodstock of unknown pathogen infection status and found commonly to carry viruses including GAV, YHV7, IHHNV, HPV and MBV as subclinical infections (Cowley et al., 2015). The reliance on such broodstock substantially adds to the risks of disease occurring during grow-out, and such risks have recently been elevated further by detections of WSSV-positive wild prawns in a systematic survey instigated in response to the WSSV incursion (Scott-Orr et al., 2018). While substantial progress had been made at one farm over the past 15 years in selectively breeding broodstock, these advanced-generation domesticated breeding lines had to be destroyed in early

2017 in response to the incursion of WSSV in Southeast Queensland, Australia (Landos 2017a, 2017b). Thus with no domesticated and/or guaranteed pathogen-free broodstock as a resource, many farms have expressed keenness to have their wild broodstock screened by PCR for pathogens, and in cases when a virus is detected at high prevalence to preclude broodstock culling as risk avoidance strategy, to trial dsRNA antivirals as a means of reducing infection loads prior to spawning and thus minimizing the levels of virus transmission to progeny.

Hindering the use of dsRNA in broodstock that produce progeny grown for human consumption is it being considered a therapeutic drug by the Australian Pesticides and Veterinary Medicines Authority (APVMA, https://apvma.gov.au/) and as such its use needs rigorous safety evaluation to decide upon appropriate regulatory controls. To generate data to assist the APVMA assess the risks of injecting broodstock with dsRNA, an objective was included in this project to assess whether dsRNAinjected in broodstock females is transferred to and amplified in their progeny.

Objectives

The original project objectives were to:

1. Demonstrate that injected dsRNA antivirals can abrogate or reduce GAV transmission from broodstock to progeny, and
2. Quantify the health, survival and growth performance of progeny derived from GAV-infected female broodstock injected with GAV-specific dsRNA antivirals when reared in ponds under simulated commercial conditions

Original project objectives were amended following consultation with FRDC, SIEF and APFA representatives according to risk mitigation strategies outlined in the Project Agreement. These alternative project objectives were to:

3. Quantify the production benefits of rearing progeny derived from female broodstock with lower-load IHHNV infections compared to females with higher-load IHHNV infections, and
4. Determine whether dsRNAs injected into female broodstock at the time of eyestalk ablation are detectable in tissues several days later at spawning and whether this dsRNA is transferred to progeny and maintained/amplified through larval life stages up until an early post-larvae age

Methods

Antivirals experiment – Objectives 1, 2 and 3

Wild-broodstock preliminary screening
On 25 May 2016, a batch of 79 wild *Penaeus monodon* broodstock was captured from Etty Bay in North Queensland by a commercial broodstock supplier. On 26 May 2016, a pleopod from each broodstock was sampled, placed in a tube containing 1.0 mL RNALater® preservative (Ambion, Thermo Scientific) and sent to CSIRO. At CSIRO, TNA was extracted using the MagJet RNA Kit (Thermo Scientific), following the manufacturers protocol except for omitting the DNase I digestion step using a KingFisher Flex 96 (Thermo Scientific) as described in detail below. Each TNA was tested by TaqMan real-time quantitative (q)PCR to determine the infection status and loads of GAV, YHV7 and IHHNV (see methods below and Cowley et al., 2015). On 4 June 2016, a second batch of 87 wild *P. monodon* broodstock was captured from Bramston Beach (n = 71), Etty Bay (n = 12) and a nearby 'position x' location (n = 4) in North Queensland by the same broodstock supplier. Pleopods were sampled and preserved as above from each prawn on 5 June 2016 and sent to CSIRO where they were tested by qPCR to determine GAV and IHHNV infection status and loads.

Broodstock source and pre-maturation period (16 days)
Wild *Penaeus monodon* broodstock were caught by the same commercial trawler operator from coastal waters off Bramston Beach and Mission Beach in North Queensland and airfreighted to CSIRO Bribie Island Research Centre, Southeast Queensland within 48 h of capture. Broodstock were transported in airline-approved broodstock transport bags (6/bag) containing oxygenated seawater at 23.5 ± 1°C on 11 July 2016.

Upon arrival at CSIRO, 2 bags each containing 6 broodstock were placed in 120 L aerated tubs and acclimated using flow through water to 25.0 ± 1°C. Once acclimated, broodstock were eye-tagged to identify each individual and a pleopod tip was removed using sterile scissors and placed into a tube containing 1.0 mL RNALater®. Once sampled, broodstock were placed into circular 10,000 L fibreglass maturation tanks. The maturation tanks were stocked with 32 males and 32 females and maintained at 25.0 ± 1°C using heated flow-through seawater at a rate of 3.5 L^{-min}. The tank bottom was covered with a 3 mm thick layer of sand and a 10 mm walled polycarbonate (Polygal®, York Precision Plastics, Riverwood, NSW, Australia) lid was used to reduce light intensity. Prawns were subjected to a 12 h light: 12 h dark photoperiod for the pre-maturation period.

Daily at 0700 h during pre-maturation, moults and mortalities were recorded, excess feed and dead prawns were removed and BREED-S Prawns maturation pellets (INVE, Dendermonde, Belgium) was supplied at ~0.5% estimated total bodyweight (ETB) of the prawns (~5% daily requirement). At 1000 h, raw chopped green lipped mussel (*Perna canaliculus*) meat was fed at ~3% ETB (~ 30% daily requirement). At 1600 h, raw chopped New Zealand arrow squid (*Nototodarus sloanii*) was fed at ~6% ETB (~ 60% daily requirement). Feed rates were adjusted on a daily basis depending on the amount found uneaten the following morning.

Maturation period (22 days)

After 16 days of pre-maturation, tank water temperatures were increased by 1°C each day until it reached 28°C (~5 days) and the same moult, mortality, excess feed and tank cleaning protocol as described above was continued, but at 0600 h. A full maturation diet was initiated for which prawns were fed at 0600 h with BREED-Prawn maturation pellets (INVE, Dendermonde, Belgium) at ~0.5% ETB (~5% daily requirement). At 0800 h, live blood worms (*Marphysa sanquinea*) were fed at ~2% ETB (~20% daily requirement). At 1030 h, raw chopped mussel meat was fed at ~2.5% ETB (~25% daily requirement). At 1400 h, raw finely chopped beef liver was fed at ~0.5% ETB (~5% daily requirement). At 1700 h, raw chopped squid was fed at ~5% ETB (~45% daily requirement). Feed rates were adjusted on a daily basis depending on the amount found uneaten at the time of the subsequent feed. The light regime was continued at a 12 h light: 12 h dark photoperiod.

After being matured for 3 days (29 July 2017), 40 male/female pairs allocated to each of a control and treatment 10,000 L tanks based on their GAV load determined by TaqMan real-time RT-qPCR. Preliminary RT-qPCR screening of broodstock pleopods sampled soon after capture revealed GAV to be present at only a low prevalence, and where detected was presents at low loads. As such, broodstock with highest-level detections of GAV were selected preferentially to be injected with the anti-GAV dsRNA antiviral and as non-injected controls for this group of broodstock.

Spawning period (6 days) and dsRNA injection

After being matured for 22 days, a pleopod tip was sampled and preserved from all males and females as described above. All females had their ovary stage recorded (Tan-Fermin and Pudadera, 1989) and were unilaterally eye-stalk ablated (Sellars et al., 2013). Muscle at the 4th abdominal segment of broodstock was injected with either the GAV dsRNA antiviral (Treatment group) or saline (Control group) using a 100 µL Hamilton glass syringe fitted with a 26-guage needle. dsRNA was injected at a standardized dose of 5 µg/10 g body weight (eg. for dsRNA stock concentration of 1 µg/mL, 70 µL [= 70 µg dsRNA] was injected for a 140 g prawn). Control prawns received equivalent volumes of shrimp saline solution (SSS; 450 mM NaCl, 10 mM KCl, 10 mM Na_2-EDTA, 10 mM HEPES, pH 7.3). The same feeding, tank cleaning, moult and mortality recording regime as for the maturation period was maintained throughout the spawning period.

At 1600 h each day, females were checked for ovarian maturation. Once assessed as ripe (Tan-Fermin and Pudadera, 1989), they were placed in tanks (0.66 m dia.) filled to 80 L using flow-through seawater (0.7 L min^{-1} flow rate, 28°C) and fitted with a 120 µM outlet screen. Tanks were checked for eggs daily at 0630 h. If ovaries of a female had regressed, she was returned to her maturation tank. When a female had spawned, a pleopod tip was sampled as described above and it was removed from the experiment. A sample of ~50 eggs from each spawning was placed in 1.0 mL RNALater, noting that they were consider to have been 'washed' as a result of the water flowing through the spawning tanks. Eggs from each spawning were then placed in a 100 L tank (0.66 m dia.) with flow through seawater (0.7 L min^{-1} flow rate, 29°C) until they hatched. Nauplii were then harvested the following morning by siphoning into a submerged 120 µm screen and concentrated into a 7 L volume of seawater. The total number of nauplii was calculated volumetrically from 3 x 1.0 mL subsamples to calculate the water volume needed to stock a larval rearing tank at the desired density.

Larval rearing period (37 days)

Nauplii were stocked (at 50-100 individuals/L depending on number of nauplii) into 6,000 L parabolic larval-rearing tanks filled to ~4,000 L with 30°C, 10 µm filtered, UV sterilized seawater. In instances where multiple families were spawned within a 48 h period, Control or Treatment families of nauplii were pooled into larval rearing tanks. Tank temperatures were maintained using 3 kW titanium submersible heaters and aeration was supplied by 10 x 50 mm weighted air stones distributed evenly along the tank bottom. The tank was covered with a black 200 micron black plastic cover to reduce light intensity and the risk of aerosols cross-contaminating tanks. Prior to pond stocking, 10 ppm Na_2-EDTA was added to the tank water to chelate any potential heavy metals.

Larvae were examined visually each day for activity, fouling, mortalities, deformities and other physical attributes as per standard commercial hatchery operating procedures using a glass beaker designated to each tank to avoid water cross-contamination. Tank temperatures were checked twice daily.

When protozoea underwent metamorphosis into mysis stage 1, the solid standpipe in the centre of each tank was replaced with a slotted 100 mm PVC standpipe fitted with a 500 µm filter cover. When mysis underwent metamorphosis into postlarval stage 1 (PL1), tanks were topped up (if required) to 6,000 L with 10 µm filtered, UV-sterilized seawater. At PL2, tanks received a 500 L water exchange, and each subsequent day, exchange rates were increased by an additional 500 L until they were receiving ~2,500 L (40%) exchange per day by the time prawns reached PL6. All water exchanges were undertaken at 0700 h prior to the morning artemia feed.

At 0600 h, both on the morning following nauplii stocking and just prior to them undergoing metamorphosis into protozoea, prawns were fed ~4 L concentrated microalgae, *Chaetoceros mueleri*. Additional concentrated microalgae was fed at ~1800 h if the majority of nauplii had undergone metamorphosis into protozoea and/or if their consumption rate was high as evident from faecal trails on protozoea. Microalgae was fed to larvae until they reached PL1 (i.e. approx. the first 10 days of culture) as a concentrate for the first 3 days (~4-8 L/day) then pumped directly from culture tanks to increase tank water volume (~150-200 L/day).

At 0500, 1100, 1700 and 2200 h, protozoea stage 2 and 3 were fed commercial prawn diet (Frippak CD#2, Sap International Corporation, Zoersel, Belgium) at 1 g/100,000 larvae. At 0800 and 1900 h, frozen (-22°C) artemia nauplii were fed from protozoea stage 3 to PL20, at ~50 g artemia per tank. At 0500, 1100, 1700 and 2200 h, mysis stage 1, 2 and 3 and PL1 were fed commercial prawn diet (Frippak CD#3, Sap International Corporation, Zoersel, Belgium) at 2 g/100,000 prawns. When prawns underwent metamorphosis into PL2, at the same times they were fed alternatively on a larger and the same commercial prawn diet (Frippak PL+150 and CD#3, Sap International Corporation, Zoersel, Belgium) at 2 g/100,000 prawns with each feed increased daily by ~0.5 g/feed. At PL5, Frippak CD#3 was replaced with LANSY® Prawn PL (Primo Aquaculture, Narangba, Queensland, Australia), and at PL8, Frippak PL+150 was replaced with Frippak PL+300. Once prawns reached PL10, they were fed additional feeds at 0800, 1400, 1930 and 0200 h of crushed LANSY® Power Flake (Primo Aquaculture, Narangba, Queensland, Australia) at 5 g/tank. Feed consumption was checked visually prior to every feed and the quantity fed was adjusted based on the amount of feed not consumed.

Post-larvae harvesting and counts

In preparation for harvesting when PL reached PL17, an additional 40% water exchange was undertaken at 1900 h and again the following 2 days when prawns were PL18 and PL19. This water exchange was to induce a night time (dark hours) moult so that prawns did not moult during or immediately following their transfer to ponds.

When prawns reached PL20 they were harvested from hatchery tanks between 0900 and 1100 h. To harvest tanks, 80% of the water was removed through the screened standpipe. A polystyrene catching box was placed in the drain, filled using the discharge water from the dropping tank and a 700 µm screen was placed in the catching box. Once the PL20 were concentrated, the remaining 20% of the water was drained with the outlet valve turned down slow the water flow and the standpipe was removed to allowing water containing PL20 to flow into the 700 µm screen. PL20 were continually scooped out of the screen using a 2 L plastic container and placed gently into 40 L of aerated seawater to maintain low densities in the 700 µm screen. The parabolic tank sides were washed regularly with seawater to detach stuck PL20 and ensure all were collected. Once the parabolic tank was empty, remaining prawns in the 700 um screen were rinsed into the 40 L aerated seawater. The amount of seawater in the collection tank was maintained at 40 L by submerging a second 700 µM screen and continually scooping out excess. Multiple holding tanks were used to ensure that no more than 80-100,000 PL20 were held in the 40 L volume at any one time.

Once all PL20 in a parabolic tank were harvested in exactly 40 L seawater, they were gently mixed by hand to be distributed evenly and then 6 samples of 37 mL (Schott bottle lid volume) were taken to count PL. The average of these 6 counts was used to estimate the total number of PL20 harvested from each tank and number/L of seawater. Based on these numbers, volumes were determined to stock each pond at a density of 44 PL20/m^2. PL received constant aeration during the harvest process until transported to each pond by vehicle (~2 min trip time) in 20 L volumes. Water containing the PL20 was aerated immediately upon receipt pond-side, and the PL were acclimated by the adding 2 x 9 L volumes of pond water over a 5 min period before being gently released into the pond.

Two of the 4 ponds used were stocked with PL20 spawned from females identified by real-time qPCR to possess IHHNV loads generally much lower (IHHNV-low families) than the females that spawned progeny stocked into the other 2 ponds (IHHNV-high families). About 1,000 PL20 from each parabolic tank determined volumetrically were concentrated onto a mesh screen and preserved in 250 mL RNALater®. A sub-sample of 16 pools of 10 x PL20 prawn cephalothoraxes was taken from each hatchery rearing tank for later TNA extraction and viral load quantification.

Pond design and preparation

The 0.16 ha research ponds used were 40 m x 40 m x 2 m deep and fully lined with 1.5mm HDPE. Each pond had a 100 mm PVC standpipe central drain fitted with a threaded cap, 3 mm oyster mesh screened outlet weir and a 100 mm screened (70% shade cloth) inlet. The group of 4 ponds was completely enclosed with bird-netting with its entire perimeter tucked under a 0.8 m high corrugated iron ground level surround to prevent Kangaroos entering and falling into ponds.

Pond surfaces were scraped and washed and left to dry for 24 days after which time 30 kg super-fine agricultural lime (Flinders Dolomite), 1 kg Omnia MAP 39, 5 kg Incitec Pivot granular Urea, 5 kg Yarra Krista K and 5 kg Organic Xtra were spread evenly over the pond bottom by hand broadcasting. Ponds were then filled over a 4 day period and water aeration/circulation was then supplied by a corner-located 2 HP paddle wheel. Twice weekly, follow up doses of fertiliser were added at 20% of original rates to encourage algal growth. As no blooms had stabilized after 10 days, 500 g pond dye (Blue Horizon, Primo Aquaculture, Narangba, Queensland, Australia) was added to each pond to reduce light penetration to the bottom to prevent benthic algae from establishing and to reduce light stress on the prawns to be stocked the following day.

Grow-out period (160 days)

Pond water quality (dissolved oxygen, pH, temperature, salinity and secchi depth) was measured daily during the grow-out period at 0600 h when dissolved oxygen was lowest. An algal bloom of *Coccolithophorid spp.* was established in all ponds by 17 days of culture (DOC). As secchi depth dropped quickly to 20 cm at this time, pond fertilization was ceased. Algal bloom density was controlled by flow-through seawater as required and secchi depth was maintained at 20 to 40 cm. At 26 DOC, the *Coccolithophorid spp.* algal bloom dropped out of Ponds 2 and 3, being replaced by a think green bloom. By 30 DOC, Pond 2 secchi depth was back to 40 cm while Pond 3 remained relatively clear (secchi depth of 80 cm) and was therefore fertilised at 20% of the original rates to encourage more vigorous algal growth. Throughout the grow-out period, paddlewheels were turned off and ropes removed and cleared of filamentous algae as required.

At ~53 DOC, pH and alkalinity were decreasing. To rectify this, a combination of dolomite, hydrated lime and sodium bicarbonate was applied. Dolomite was added at a rate of 10 kg/pond when pH dropped below 7.8 by broadcasting small amounts at a time into the current in front of paddlewheels. Hydrated lime was added at a rate of 2-5 kg/pond when dolomite was not increasing the pH (applied the same as dolomite). Sodium bicarbonate was added at a rate of 3-5 kg/pond when alkalinity dropped below 100 ppm (applied the same as dolomite).

Due to increasing feed (organic matter) inputs by 60 DOC, bloom densities were continually increasing. Water exchanges were used to control this. Usually only 30 cm exchanges were undertaken to avoid any significant altering of the water chemistry to upset the prawns. This was done by removing one weir board and allowing water levels to drop before pulling a second board. Once the required depth drop had been achieved, the boards were returned and the pond filled with fresh seawater from the inlet.

At 120 DOC, pH levels and alkalinity were dropping rapidly even with the addition of agricultural lime (Dolomite). Hydrated lime was thus added at a rate of 5 kg/pond/application in conjunction with weekly 30 cm water exchanges. Application frequency was dependent on pH. Sodium bicarbonate was added to the hydrated lime at 5 kg/pond when alkalinity dropped below 100 ppm.

During the first 50 DOC, prawns were fed 3 times daily (0600, 1200 and 1800 h) with Starter #1 MR pellet feed (Ridley Aqua Feeds, Narangba, Queensland, Australia) at rates calculated from “Australian Prawn Farming Manual – Health Management for Profit” (2006). Feed rates were adjusted (reduced) as cold water temperatures (minimums around 21°C and maximums around 23°C) resulted in slower

growth than those predicted in the manual. At 44 DOC (PL64), 3 belt-feeders were installed on each pond allowing an additional nightly (dark hours) feed. At 53 DOC, PL73 prawns began to use feed trays which allowed feed amounts to be adjusted thereafter based on "tray scores" according to methods outlined in the Australian Prawn Farming, Health Management for Profit manual (2006). At each feeding time, 3 g/kg of feed provided was placed on a tray and checked/scored 2.5 h later. At 86 DOC, feed tray volumes were increased to 4 g/kg of feed provided and the inspection time was reduced to 2 h post-feeding. At 100 DOC, an additional belt-feeder was installed on each pond to allow nightly feeding of the higher quantities of feed pellets. At 110 DOC, pond feed rates were capped at 12 kg/feed/pond due to concerns about nitrogen discharge levels exceeding site licence levels set by the Environmental Protection Agency (EPA, Brisbane, Australia). At 110 DOC (once prawns in all ponds have an average weight >15 g), feed tray volumes were increased to 5 g/kg of feed provided and the inspection time was extended to 2.5 h post-feed.

At 51, 86, 120, 133 and 155 DOC, 144 prawns were collected randomly by cast-netting from each of the 4 ponds. Each prawn was weighed and sexed a pleopod was sampled into RNALater as described above. A random set of 48 prawns sampled from each pond at 51, 86, 120 and 155 DOC had a pleopod tested by real-time qPCR for IHHNV and GAV as described below.

At 140 DOC, lymphoid organ (LO) tissue was sampled from 30 prawns collected randomly from each pond. LO tissue was preserved in RNALater the sample number was selected to allow IHHNV, GAV and yellow-head virus genotype 7 (YHV7; a GAV-related virus endemic in some populations of *P. monodon* inhabiting Northern Australia; Cowley et al., 2015; Mohr et al., 2017) to be detected by qPCR down to a 10% prevalence level (OIE, 2017).

Pond harvesting

Ponds were harvested using a partial harvest technique whereby small amounts (500-800 kg) of the larger prawns were removed using 'wing traps' on 3 or 4 days (depending on numbers of large prawns in each pond) between 150 and 170 DOC. These were followed by complete harvest which involved draining the ponds completely and collecting prawns in seine nets as well as by hand.

TNA extraction and qPCR to quantify IHHNV and GAV prevalence and loads

Larval stages and tissues of juvenile shrimp in RNALater were removed from 96-well deep-well plates or tubes using either sterile forceps or by pouring onto absorbent paper towel to remove excess liquid. With the PL20, cephalothorax severed from the abdomen with a scalpel was pooled in lots of 10 PL for extraction. Tissue was placed in 600 µL RLT lysis buffer (QIAGEN), homogenised using a bead beater and TNA was extracted using a MagJET RNA Kit (Thermo Scientific) and a KingFisher Flex 96 (Thermo Scientific) extraction robot following the manufacturer's protocols, except that the DNase 1 digestion step was omitted and an additional Wash Buffer 2 step was included. TNA quantity and purity was assessed by examining a 1.5 µL aliquot of each extract using a ND-8000 UV spectrophotometer (Thermo Fisher Scientific). To detect gill-associated virus (GAV) and yellow head virus genotype 7 (YHV7) by real-time qPCR, cDNA was synthesized in a 10 µL reaction containing 500 ng TNA and prepared using SensiFAST™ cDNA Synthesis Kit (Bioline) reagent containing an optimized mix of random hexamers and anchored oligo-dT primers as described in the manufacturers protocol.

Primer and probe sequences used in the qPCR tests to detect IHHNV (Cowley et al., 2018) as well as GAV (de la Vega et al., 2004) and YHV7 (Cowley et al., 2015) are described in detail elsewhere. For each test, reactions (20 µL) were prepared to contain 1 x SensiFAST™ Probe Lo-ROX mastermix (Bioline), 0.9 µM each primer, 0.25 µM probe and a normalized amount (100 ng) of either TNA or cDNA. Reaction aliquots (5 µL containing 25 TNA/cDNA) were dispensed into each of 3 wells of a 384-well real-time PCR plate as technical replicates using an EpMotion 5075 liquid handling robot (Eppendorf). DNA was amplified using a ViiA 7 Real-Time PCR System (Applied Biosystems) and a thermal cycling profile (95°C/2 min for polymerase activation, 40 cycles of 95°C/15 s, 60°C/30 s) within the parameters recommended in the SensiFAST™ Probe Lo-ROX Kit instructions (Bioline).

To quantify IHHNV DNA and GAV/YHV7 RNA copy numbers accurately in clinical samples, 10-fold dilution series of IHHNV plasmid DNA, GAV cDNA or YHV7 cDNA of predetermined copy number was amplified in the same plate to determine a linear regression plot of mean cycle threshold (Ct) values vs. copy number (Sellars et al., 2014).

Statistical analyses

Individual prawn weights and IHHNV loads quantified in prawn pleopod tissue sampled progressively during pond rearing were assessed by factorial ANOVA with model factors including RNAi treatment, pond nested within RNAi treatment, and sex when this was possible to determine from 86 DOC onwards (SAS Institute Software, 1999). IHHNV DNA copy number was log-transformed prior to analysis of IHHNV DNA loads.

dsRNA residuals experiment – Objective 4

dsRNA preparation

A 954 nt region (GAV1) at the extreme 5' terminus of the GAV genome (Sellars et al., 2014) and a 464 nt region of the firefly luciferase (Luc) gene were amplified by PCR using standard methods and primers containing 5' T7 RNA promoter extensions (**Table 1**). Amplified DNA was purified using a QIAquick PCR column (QIAGEN) and quantified using a NanoDrop™ ND8000 UV spectrophotometer (Thermo Fisher Scientific). Using 500 ng each template DNA, large amounts of dsGAV1 and dsLuc dsRNA were synthesised at 37°C for 4 h in 100 µL reactions using MEGAscipt® T7 Transcription Kit (Thermo Fisher Scientific) reagents according to the manufacturer's protocol. To digest DNA, 5 µL Turbo DNase 1 was added and the reaction incubated at 37°C for 30 min. Each dsRNA was then purified by binding to a MEGAclear™ column (Thermo Fisher Scientific) and eluting in EB Buffer (QIAGEN) preheated to 90°C. The eluates were then made to 1 x DNase digestion buffer (Thermo Fisher Scientific) and incubated again with 10 µL Turbo DNase 1 at 37°C for 30 min followed by heating at 70°C for 10 min to denature the enzyme. DNase-treated dsRNA batches were recovered as above using MEGAclear columns. Batches of dsGAV1 dsRNA and batches of dsLuc dsRNA were pooled, and the amounts and purity ($A_{260/280nm}$ 2.02-2.05, $A_{260/230nm}$ 2.02-2.20) of each batch were determined using a NanoDrop ND8000. The purity and integrity of the dsRNA pools were confirmed by electrophoresis in a 1.5% agarose-TAE (data not shown). Each pool of dsGAV1 and dsLuc dsRNA was normalized to a concentration of 1.5 µg µl-1 using EB buffer (QIAGEN), portions of which were then mixed 1:1 before being stored in aliquots at -80°C.

Table 1. Sequences of primers and probes used in qPCR tests and PCR primers used to amplify DNA templates to synthesise dsRNA using T7 RNA polymerase

Purpose	Primer/probe	Tm (°C)	Sequence 5'-3'	amplicon (bp)	genome position
qPCR	dsGAV1-qF2	60.9	GTCAGATTGACTCTCAGGACCACTT	112	165 - 276
	dsGAV1-qR2	61.2	CCATCCTGGTGGTAGTCTTGCTA		
	dsGAV1-qPr1	70.1	[6FAM]CCAGCCCCGCTAGATGGTTCGA[BHQ1]		
qPCR	Luc-qF1	60.4	GCGCGTTATTTATCGGAGTTG	76	
	Luc-qR1	60.7	TTCATACTGTTGAGCAATTCACGTT		
	Luc-qPr1	71.6	[6FAM]AGTTGCGCCCGCGAACGACAT[BHQ1]		
qPCR[a]	GAV-qPF1	60.9	GGGATCCTAACATCGTCAACGT	81	17427 - 17507
	GAV-qPR1	59.0	GTAGTATGGATTACCCTGGTGCAT		
	GAV-qPr1	71.1	[6FAM]TCAGCCGCTTCCGCTTCCAATG[BHQ1]		
dsRNA	T7-dsGAV1-F		GGCC**TAATACGACTCACTATAGGG** ACGTTACGTTCCACGTACTTATC	954	1 - 907
	T7-dsGAV1-R		GGCC**TAATACGACTCACTATAGGG** TGATTCCACCTACAATCGTGAT		
dsRNA	T7-dsLuc-F		GAATT**TAATACGACTCACTATAGGG**ATCGCGCCATTCTATCCTCTA	464	
	T7-dsLuc-R		GAATT**TAATACGACTCACTATAGGG** ATCTACATCGACTGAAATCCC		

T7 RNA polymerase promoter sequence is in boldface

GAV genome sequence position (GenBank Acc: AF227196)

[a] de la Vega et al. (2004) except for the use of BHQ1 instead of TAMRA for quenching GAV-qPr1 probe fluorescence

Prawns and dsRNA injection

Broodstock used were selected from a Generation 1 (G1) cohort reared from postlarvae (PL) to sexual maturity initially in 0.16 ha lined ponds covered with bird netting and then in passively-heated 200 m2 covered nursery ponds at the Bribie Island Research Centre, Queensland. On 1 Aug 2017, broodstock were moved to 10 tonne maturation tanks employing heating and a sand substrate and feeding was commenced on a typical hatchery maturation diet for *P. monodon*. At this time, a pleopod was also sampled from each shrimp and preserved in 1.0 mL RNALater® Solution (Thermo Fisher Scientific) for real-time PCR analysis to determine the infection status of GAV (de la Vega et al., 2004), YHV genotype 7 (YHV7, Cowley et al., 2015, Mohr et al., 2015) and IHHNV (Cowley et al., 2015, 2018). Using TNA extracted from each pleopod of the 96 G1 broodstock as described below, the real-time RT-qPCR (GAV and YHV7) and qPCR (IHHNV) analyses detected no YHV7, GAV at low levels in 60/96 (66%) shrimp and moderate levels in 3 shrimp and IHHNV in most (91/96 = 95%) shrimp, although generally at low levels except for 10 at more moderate levels and 3 at levels considered to be high (data not shown).

On 5 Sep 2017, females displaying evidence of being inseminated with male spermatophores were weighed and eyestalk ablated using heated forceps to induce spawning. Immediately prior to this procedure, muscle tissue at the 2nd abdominal segment was injected either with 60 µL sterile saline (group of 8 females) using a 100 µL glass Hamilton syringe fitted with a 26-gauge disposable needle as described previously (Sellars et al., 2014), or with a 1:1 mixture of dsGAV1:dsLuc dsRNA (another group of 8 females) using a standardised dose of 0.5 µg g-1 shrimp weight of each dsRNA (e.g. 60 µL 1.5 µg µl-1 stock dsRNA for a 90 g shrimp, representing 45 µg dsGAV1 dsRNA + 45 µg dsLuc dsRNA). Following ablation, control, saline-injected and dsRNA-injected females were returned to their respective tanks and monitored each afternoon to assess their readiness to spawn.

Spawning, sampling and larval rearing

Females were spawned in individual 100 L tanks (0.66 m dia.) with flow through seawater (0.7 L/min flow rate, 29°C) that were fitted with a 120 µM mesh screen. The morning after spawning, each female was euthanized in an ice slurry, and two pleopods, two pieces of ovary and the two lymphoid organs were sampled into five separate tubes (lymphoid organs pooled together) containing 1.0 mL RNALater® Solution (Thermo Fisher Scientific). Once the females were removed, aeration within the tanks was set high, flow through seawater remained and eggs were left to develop and hatch.

A pool of 50-100 eggs was sampled from each spawning into RNALater at 2-6 h prior to when they were expected to hatch. As the spawning tanks incorporated water flow-through, the duration between spawning and egg sampling (8-12 h) was considered to have washed the eggs. At the time nauplii had visibly exhausted their yolk sacs and prior to metamorphosing into protozoea, a pool of approx. 50 nauplii were sampled from each spawning into RNALater. Similarly, a pool of approx. 50 protozoea, two pools of approx. 50 mysis and 32 individual post-larvae (PL) were sampled from each spawning into RNALater. The age of PL when sampled was PL7 (Female 1), PL8 (Females 2 and 5), PL10 (Female 6), PL11 (Female 3) and PL12 (Female 4). All tissue samples were preserved in RNAlater solution initially at 4°C for 2-5 days before storage at -20°C.

Nauplii were harvested from spawning tanks and stocked into 100 L rearing tanks (0.66 m dia.) with flow through seawater (0.7 L min-1 flow rate, 29°C, 34 ppt salinity) at 100 prawns/L. Tanks were fitted with mesh screen sized suitably to stop the different life-history stages from escaping, had light aeration and an opaque white lid to reduce light intensity. Nauplii received a 12 h light: 12 h dark photoperiod. Late stage nauplii, protozoea, mysis and early PL were fed a range of feeds four times a day (0500, 1100, 1700 and 2200 h) suitable to their life-history stage including live algae (*Chaetoceros muelleri*), live *Artemia spp*. and artificial diets (Frippak, Sap International Corporation, Zoersel, Belgium) as done by Sellars et al., 2018. Tanks were cleaned by siphoning as required.

TNA extraction

Preserved tissues and pooled larvae were blotted briefly on paper towel to remove excess RNAlater and placed in 0.6 mL RLT buffer (QIAGEN) in wells of a 96-well deep-well pate each containing 2 glass and 2 ceramic beads. Tissue was disrupted by beating for 2 min at maximum speed using a Retsch MM300 TissueLyser (Metrohm Australia) and plates were then centrifuged briefly. To extract total nucleic acid (TNA = RNA + DNA), lysate free of particulate matter was transferred to another 96-well plate and extracted using MagJET™ RNA Kit reagents (Thermo Fisher Scientific) and a KingFisher™ Flex Purification System (Thermo Fisher Scientific) using the manufacturer's recommended protocols, except for omitting the DNase I digestion step and including an additional Wash Buffer 2 step. TNA was eluted in 50 µL RNase/DNase-free water, its concentration and relative purity (A260/280nm, A260/230nm) were estimated using a NanoDrop™ ND8000 UV spectrophotometer and aliquots were normalized to 100 ng/µL by diluting with RNase-free water. TNA was stored at -80°C until analysed.

qRT-PCR optimization for dsGAV1 and dsLuc quantification

Primer and probe sequences used in the 3 TaqMan real-time quantitative (q)PCR tests are detailed in Table 1. Bio-RP cartridge purified PCR primers and HPLC-purified [6FAM]-BHQ1 labelled TaqMan

probes were purchased from Bioneer Pacific. The qPCR tests included one described previously to detect an ORF1b gene region in the GAV genome (de la Vega et al., 2004), except that BHQ1 was used instead of TAMRA to quench [6FAM]-probe fluorescence. The other 2 tests were designed to detect (i) an ORF1a gene region close to the extreme 5'-end of the GAV genome internal to the 954 bp dsGAV1 dsRNA sequence and (ii) a region internal to the 464 bp firefly luciferase gene sequence amplified to prepare the dsLuc dsRNA. PCR primer and probe sequences were selected using the default TaqMan test design parameters in Primer Express V3 (Applied Biosystems). Before purchase, PCR primer sequence conformity was rechecked using Primer 3 V0.4.0 (Untergasser et al., 2012).

To help ensure high qPCR test sensitivity, 2 primer sets were evaluated with the dsGAV1 test probe and 3 primer sets were evaluated with the dsLuc test probe. The amplification characteristics and detection sensitivity limits of qPCR tests using these primer sets were evaluated using the reaction and thermal cycling conditions described below and serial 10-fold dilutions of either purified pGAV1 plasmid DNA and purified dsLuc DNA amplified by PCR down to a lower limit of 0.1 dsDNA copies per reaction (data not shown). Copy numbers of each dsDNA template were calculated based on A260nm data and masses determined from their sequences using OligoCalc V3.27 (Kibbe, 2007). For completeness, the efficiency of the modified GAV qPCR test (de la Vega et al., 2004) using the BHQ1-labelled probe was evaluated at the same time using serial 10-fold dilutions of GAV cDNA prepared to a synthetic GAV ssRNA of calculated copy number. These pilot analyses resulted in the selection of the PCR primer sets detailed in **Table 1**.

Quantification of GAV, dsGAV1 and dsLuc in tissue and life-history samples

cDNA was synthesized in reactions (10 µL) containing 500 ng TNA, 0.5 µL reverse transcriptase and 1 x SensiFAST™ cDNA Synthesis Kit buffer (Bioline) containing both random hexamer and oligo-dT primers. Reactions were incubated at 25°C for 10 min, 42°C for 15 min, 85°C for 5 min and then held at 4°C. In some tests, TNA was heated at 95°C for 2 min and chilled on ice for 2 min to strand-separate dsRNA prior to cDNA synthesis reagents being added and reacted as described above. In tests to assess RT-qPCR test efficiencies and limits of detection (LOD), cDNA was prepared to serial 10-fold dilutions of either synthetic ssRNA or dsRNA of known copy number. In the case of dsGAV1 and dsLuc RT-qPCR tests, cDNA was tested that had been synthesised to dilutions of either native or heat-denatured (HD) dsRNA.

For each qPCR test, reactions (20 µL) were prepared to contain 1 x SensiFAST™ Probe Lo-ROX mastermix (Bioline), 0.9 µM each primer, 0.25 µM probe and normalized amounts of either TNA (100 ng) or cDNA synthesized from 100 ng TNA. Reaction aliquots (5 µL) were dispensed into each of 3 wells of a 384-well real-time PCR plate as technical replicates using an EpMotion 5075 liquid handling robot (Eppendorf). DNA was amplified using a ViiA 7 Real-Time PCR System (Applied Biosystems) and a thermal cycling profile (95°C for 2 min for polymerase activation, 40 cycles of 95°C for 15 s, 60°C for 30 s) conforming to parameters recommended in the SensiFAST™ Probe Lo-ROX Kit instructions (Bioline). For accuracy and ease in comparing qPCR data, cycle threshold (Ct) values were determined at a standardised threshold set to 0.09.

To generate standard curves to accurately quantify RNA amounts in each TNA extract (Svec et al., 2015), all qPCR runs included 3 technical replicates of cDNA prepared to serial 10-fold dilutions of appropriate synthetic ssRNA for the GAV qPCR test (de la Vega et al., 2004) or dsRNA (either native or heat-denatured as described above) for the dsGAV1 and dsLuc qPCR tests.

Results

Antivirals experiment

Preliminary screening of wild broodstock

In May to June 2016, wild *P. monodon* broodstock caught from the North Queensland, qPCR testing identified GAV and IHHNV to be present at low prevalence and low loads and YHV7 to be absent (**Table 2**). Among 79 broodstock caught on 25 May 2016 from Etty Bay, 44 tested GAV-negative, 62 tested IHHNV-negative, and all 79 tested YHV7 negative. In those individuals in which GAV (n = 23) and/or IHHNV (n = 12) were detected, viral loads were low. Among 71 broodstock caught on 4 June 2016 from Bramston Beach, 38 tested GAV-negative and 61 tested IHHNV-negative. When either virus was detected, loads again were generally low. Among the 252 broodstock caught on 11 Jul 2016 from the same regions and sent for BIRC for experimental use, 141 tested GAV-negative, and of the remainder, 100 were scored as having GAV at only really-low levels, 7 at very low levels and 4 at low levels. None possessed GAV at moderately-low to moderate levels considered ideal for the experimental purpose of demonstrating improved grow-out performance of progeny spawned from broodstock injected with GAV-specific dsRNA antivirals.

GAV infection loads in experimental female broodstock

GAV RNA amounts (pseudo measure of infection loads) detected by RT-qPCR in pleopod tips sampled from Treatment and Control female broodstock were generally very low and often at the detection sensitivity limits of the GAV RT-qPCR test. For ease of describing qPCR data of potential biological significance, and as at very low GAV detection levels, tissue selection and differences across individual pleopod tips make RT-qPCR data less reliable than when infection loads are higher, detections at ≤100 GAV RNA copies/µg total nucleic acid (TNA) were considered undetermined (UD). When such extremely low-level detections were discounted, the 4 females that contributed progeny families to be reared as a Treatment cohort were identified to have 8-fold to 16-fold reduced GAV loads following injection of the dsGAV1 dsRNA antiviral (**Table 3**).

With respect to the 3 females contributing progeny to the on-reared Control cohort, GAV infection loads increased marginally in 1 female between when it was injected with saline and spawned, reduced marginally in another female and remained below levels (ie. 100 GAV RNA copies/µg TNA) where qPCR data was considered meaningful in the other (**Table 3**). The GAV RT-qPCR test was unable to detect GAV RNA either in any egg pools from each family or in any of the pooled PL20 cephalothorax batches reared from the 7 females contributing progeny to the Treatment and Control family groups on-reared in ponds. The total numbers of eggs and PL20 cephalothorax samples tested from each family (n ≥150) were selected allowed GAV to be detected down to a 2% prevalence level.

Table 2. Summary of GAV and IHHNV real time PCR data using TNA extracted from pleopods of *Penaeus monodon* caught in the May to July period of 2016 from North Queensland, Australia.

Infection load	Viral RNA or DNA copies/µg TNA	25 May 2016			4 June 2016						11 July 2016
		Etty Bay			Bramston Beach		Etty Bay		Position X		Bramston Beach
		GAV	IHHNV	YHV7	GAV	IHHNV	GAV	IHHNV	GAV	IHHNV	GAV
Very High	10^8 - 10^9										
High	10^7 - 10^8										
Moderate High	10^6 - 10^7										
Moderate	10^5 - 10^6										
Moderate Low	10^4 - 10^5		5		1	2		2			
Low	10^3 - 10^4	2			2	1	1	1			4
Very Low	100 - 1000				30	4		1			7
Really Low	1 - 100	23	12						2	1	100
Not Detected	0	44	62	79	38	61	11	8	2	3	141
Total prawns		79	79	79	71	71	12	12	4	4	252

Supplementary Tables 1, 2 and 3 in this report detail the real-time qPCR data used to generate this summary table

Table 3. RT-qPCR quantification of GAV RNA loads in pleopods of female broodstock that contributed progeny to the Control and Treatment grow-out ponds

Female eye tag	Hatchery tank	Grow-out pond allocation	GAV RNA copies/µg TNA			
			Day of arrival	Day of injection and ablation	After spawning	Egg pool
O26	4	Control	-	223	504	-
Y62	6	Ponds	828	2,070	1,080	-
Y77	6	1 and 4	177	-	-	-
O15	1	Treatment	-	2,190	-	-
O19	1	Ponds	-	370	-	-
O29	1	2 and 3	4,260	3,680	-	-
Y80	1		577	3,810	-	-

Note: Only detections above 100 GAV RNA copies/µg TNA were considered significant and reported

At regular intervals during grow-out, 144 prawns collected randomly from each of the 4 ponds were weighed, sexed and had pleopod tissue preserved in RNAlater. This number was selected for plate-based processing efficiency and to approach the number needed (n=150) to detect GAV down to close to a 2% prevalence level. Prawns were sampled after 51, 86, 120, 133 and 155 days of culture (DOC). TNA extracted from each pleopod sample was tested by RT-qPCR for GAV. All prawns sampled at 51, 86, 120 and 133 DOC, irrespective of them being from the Control or Treatment cohort ponds, tested negative for GAV. After 133 DOC, due to a lower average weight and poorer overt health being clearly evident with the prawns sampled from the 2 Control cohort ponds (see **Figure 2**, Pond Performance Section below), it was decided, at 140 DOC, to destructively sampled lymphoid organ (LO) tissue of 30 prawns. LO was sampled as it has been identified previously to sequester and concentrate GAV-infected cells within spheroids when GAV infection loads are low. This sample size was selected to detect GAV down to a 10% prevalence level. The LO samples were also tested by RT-qPCR for YHV7 and by qPCR for IHHNV to identify whether either of these viruses might be contributing to the reduced condition of the Control cohort prawns. LO tissue from all 120 prawns tested negative for both GAV and YHV7, but positive for IHHNV. Moreover, IHHNV DNA

amounts detected in the Control cohort prawns were in general significantly higher than in the Treatment cohort prawns (**Figure 1**, 140 DOC).

GAV infection prevalence and loads in *Penaeus monodon* generally increase as commercial grow-out progresses, and have been found to spike and result in acute infection and disease following environmental stressors such as pond water discharge and replenishing with fresh water to undertake a partial harvest. Based on this knowledge and after consulting the APFA R&D committee, it was decided to mimic a severe stress event in the Control and Treatment ponds. This stress constituted a 40% water exchange in all 4 ponds between 141-144 DOC. At 155 DOC, pleopod tips were again sampled from 144 prawns selected at random from each pond. The samples were tested by RT-qPCR for GAV and by qPCR for IHHNV. GAV was not detected in any of the prawns sampled from either Control Pond 1 or Treatment Pond 2, and in only a single prawn from Control Pond 4 at a relatively low load (2,044 GAV RNA copies/µg TNA). However, it was detected in ~70% of prawns sampled from Treatment Pond 3, although again only at relatively low loads (ie. average 639 GAV RNA copies/µg TNA).

In contrast to GAV, IHHNV was detected in all prawns sampled from all 4 ponds. Moreover, the relative IHHNV loads in Control cohort prawns were far higher than in Treatment cohort prawns, confirming data obtained at 140 DOC using LO tissue, with the exception that IHHNV levels were far higher in pleopod tissue than in LO tissue (**Figure 1**, 155 DOC). These differences in IHHNV loads strongly suggested a role in the poorer condition, growth and survival of the Control prawn cohort relative to the Treatment prawn cohort (see **Figure 2**, Pond Performance Section below).

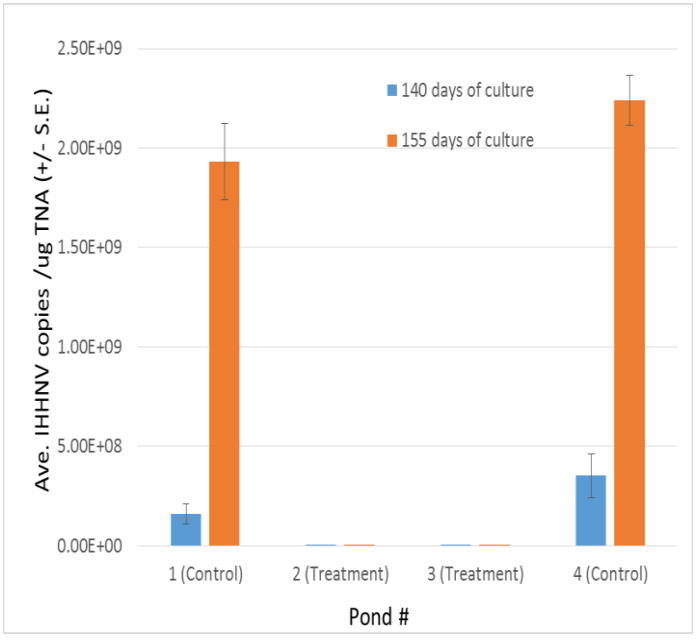

Figure 1. IHHNV DNA amounts detected by qPCR in LO tissue sampled from 30 prawns selected at random from each of the 4 ponds after 140 DOC and in pleopod tissue sampled from 144 prawns selected at random from each pond after 155 DOC (qPCR data presented as mean IHHNV DNA copies/µg TNA ± SE). IHHNV was detected by qPCR in either tissue from every prawn sampled at either time point.

Based on the detection of IHHNV and its potential association with the poorer health of the Control prawn cohort, TNA extracted from pleopod tissue from female broodstock that contributed families for grow-out was tested by qPCR for IHHNV. These qPCR data identified mean IHHNV infection loads to be markedly higher in the Control cohort females compared to Treatment cohort females when they were sampled the morning after each spawning (**Table 4**). IHHNV was also detected at markedly-higher loads in a pooled egg batch from 1 of the 3 Control cohort females (**Table 4**) as well as among 16 pools of 10 x PL20 reared in 2 tanks containing progeny of Control females compared to pools of PL from the tank containing progeny of Treatment females (**Table 5**). These data confirmed that IHHNV was being transmitted vertically and demonstrating that the prevalence and loads of IHHNV was higher in the Control cohort families compared to the Treatment cohort families at the time PL20 were stocked into grow-out ponds. Although the family numbers were low and genetic factors or other pathogens not tested for might have been involved, the IHHNV qPCR data on the broodstock, progeny and prawns after 140 and 155 DOC lend support to acute IHHNV infection being involved in the poorer relative condition, growth and survival of the Control cohort prawns.

Table 4. qPCR quantification of IHHNV RNA loads in pleopods of female broodstock that contributed progeny to the Control and Treatment grow-out ponds

Female eye tag	Hatchery tank	Grow-out pond allocation	GAV RNA copies/µg TNA			
			Day of arrival	Day of injection and ablation	After spawning	Egg pool
O26	4	Control	-	709	4.65×10^{8}	1,030
Y62	6	Ponds	-	2,450	2.25×10^{7}	225
Y77	6	1 and 4	6.71×10^{5}	3.56×10^{7}	1.10×10^{9}	2.80×10^{5}
O15	1	Treatment	-	4,270	4,070	-
O19	1	Ponds	-	-	790	-
O29	1	2 and 3	5.44×10^{4}	7.39×10^{4}	1.83×10^{5}	255
Y80	1		-	-	9.51×10^{6}	193

Note: Only detections above 100 GAV RNA copies/µg TNA were considered significant and reported

Table 5. Mean GAV and IHHNV loads quantified by RT-qPCR/qPCR in 16 pools of 10 x PL20 sampled from each hatchery tank on the day ponds were stocked

PL20 Group	Female (eye tag)	Hatchery tank	Ponds stocked	Mean RNA or DNA copies/µg TNA ± SD	
				GAV RT-qPCR	IHHNV qPCR
Control	O26	4	1 and 4	-	$2.14 \pm 8.49 \times 10^{6}$
Control	Y62, Y77	6	1 and 4	-	$4.16 \pm 3.99 \times 10^{7}$
Treatment	Q15, O19, O29, Y80	1	2 and 3	-	$4.81 \pm 8.36 \times 10^{4}$

Note: Only detections >100 IHHNV DNA copies/µg TNA were considered significant and reported; SD = standard deviation

IHHNV, GAV and YHV7 load and prevalence during pond grow-out

IHHNV prevalence was lower and increased more slowly during grow-out in the 'IHHNV-low' Treatment cohort prawns reared in Ponds 2 and 3 and only reached 100% prevalence when being harvested at 155 DOC (**Table 6, Figure 2B**). Consistently with this, IHHNV loads detected in infected prawns from both of these ponds were maintained at significantly lower levels throughout grow-out compared to 'IHHNV-high' Control cohort prawns reared in Ponds 1 and 4 (**Table 6, Figure 2B**). In addition, no differences in IHHNV loads were found between males and females sampled from any of the 4 ponds after the time (86 DOC) at which sex could be determined accurately by eye (data not shown).

In the 2 ponds (2 and 3) stocked with Treatment cohort prawns, the only time GAV was detected of at any sampling time point throughout grow-out was in prawns sampled from Pond 3 at harvest at 155 DOC and after prawns had been purposely stressed by undertaking a significant water exchange several days early. However, while detected in 71% of the 48 prawns tested from this pond, GAV loads were low (**Table 6**). In the 2 ponds (1 and 4) stocked with Control cohort prawns, GAV was only detected in a single prawn tested at either 140 or 155 DOC, and GAV levels so low that not all 3 technical replicates of the RT-qPCR tests were identified as GAV-positive (**Table 6**).

Lymphoid organ tissue of all prawns sampled from the 4 ponds at 140 DOC tested RT-qPCR-negative for YHV7.

Growth rate during pond culture and commercial production metrics

At the first sampling point at 51 DOC, the mean weight of juveniles in one of the 2 ponds (Pond 3) stocked with Control cohort prawns was significantly lower (P <0.001) than those of prawns from either of the 2 ponds (1 and 4) stocked with Treatment cohort prawns (**Figure 2**). While no differences in mean weights were evident among prawns from any of the 4 ponds at the next sampling time point (86 DOC), at sampling times thereafter, mean weights of Control cohort prawns in Ponds 1 and 4 began to significantly and progressively lag further behind the Treatment cohort prawns in Ponds 2 and 3 (**Figure 2**). Differences in mean male:female weights (with females being heavier) were evident from the 133 DOC sampling point onwards (data not shown). However, male:fermale ratios of the prawns sampled from each of the 4 ponds after this time point were relatively uniform, and thus were unlikely to have contributed significantly to differences observed in overall mean weights (**Table 6**).

Prawns in the 2 IHHNV-low ponds (2 and 3) had collectively consumed ~36% more feed than prawns in the 2 IHHNV-high ponds by the end of the grow-out period (**Table 7**). Collective harvest weights of prawns from Ponds 2 and 3 were also ~39% higher than Ponds 1 and 4. Extrapolated from the 0.16 ha research ponds to 1 ha sized commercial grow-out ponds as used commonly in Australia, yields from Ponds 2 and 3 ranged between ~14.2 and ~12.2 tonne/ha compared to between ~10.1 and ~9.0 tonne/ha for Ponds 1 and 4). Survival was also improved by 9.4% to 19.9% in Ponds 2 and 3 compared to Ponds 1 and 4 (**Table 7**).

Table 6. Prevalence and loads of IHHNV, GAV and YHV7 determined by qPCR or RT-qPCR for Treatment and Control shrimp sampled at different times over grow-out in 0.16 ha research ponds

Group	Pond		Shrimp			Tissue		IHHNV qPCR		GAV RT-qPCR		YHV7 RT-qPCR	
	No.	DOC	Number weighed	Male (%)	Female (%)	Type	Number tested	Prevalence (%)	mean IHHNV DNA copies/µg TNA ± SD	Prevalence (%)	mean GAV RNA copies/µg TNA ± SD	Prevalence (%)	mean YHV7 RNA copies/µg TNA ± SD
Treatment	2	51	144			Pleopod	48	31	$1.86 \pm 2.12 \times 10^{4}$	0	-		
		86	144	44.4	55.6	Pleopod	48	52	$0.80 \pm 1.34 \times 10^{5}$	0	-		
		120	144	37.5	62.5	Pleopod	48	79	$0.62 \pm 1.29 \times 10^{5}$	0	-		
		140	30	43.3	56.7	LO	30	97	$0.38 \pm 1.67 \times 10^{5}$	0	-	0	-
		155	143	41.2	58.8	Pleopod	48	100	$0.24 \pm 1.85 \times 10^{6}$	0	-		
	3	51	144			Pleopod	48	56	$0.97 \pm 1.73 \times 10^{4}$	0	-		
		86	144	50.7	49.3	Pleopod	48	90	$0.70 \pm 1.26 \times 10^{5}$	0	-		
		120	144	48.6	51.4	Pleopod	48	75	$0.42 \pm 1.03 \times 10^{5}$	0	-		
		140	30	53.3	46.7	LO	30	97	$0.51 \pm 1.46 \times 10^{4}$	0	-	0	-
		155	142	47.2	52.8	Pleopod	48	100	$0.58 \pm 1.43 \times 10^{5}$	71	$7.88 \pm 2.07 \times 10^{2}$		
Control	1	51	144			Pleopod	48	67	$0.50 \pm 1.84 \times 10^{6}$	0	-		
		86	144	44.4	55.6	Pleopod	48	100	$3.37 \pm 7.37 \times 10^{8}$	0	-		
		120	144	50.0	50.0	Pleopod	48	100	$4.71 \pm 8.27 \times 10^{8}$	0	-		
		140	30	46.7	53.3	LO	30	100	$1.58 \pm 2.76 \times 10^{8}$	0	-	0	-
		155	48	45.8	54.2	Pleopod	48	100	$1.94 \pm 2.30 \times 10^{9}$	0	-		
	4	51	144			Pleopod	48	83	$0.67 \pm 2.33 \times 10^{6}$	0	-		
		86	144	56.9	43.1	Pleopod	48	100	$3.66 \pm 8.01 \times 10^{8}$	0	-		
		120	144	39.6	60.4	Pleopod	48	100	$2.44 \pm 5.92 \times 10^{8}$	0	-		
		140	30	63.3	36.7	LO	30	100	$3.53 \pm 6.08 \times 10^{8}$	3	8.58×10^{1}*	0	-
		155	142	40.1	59.9	Pleopod	48	100	$2.24 \pm 1.52 \times 10^{9}$	1	2.04×10^{3}*		

* Value only obtained with1/3 technical replicates; - = not detected in all 3 technical replicates of all 30-48 TNA samples tested; DOC = day of culture, SD = standard deviation; at 51, 86, 120 and 155 DOC, pleopod tips were sampled from 48 to 144 random shrimp/pond; at 140 DOC, LO tissue was sampled from 30 random shrimp/pond

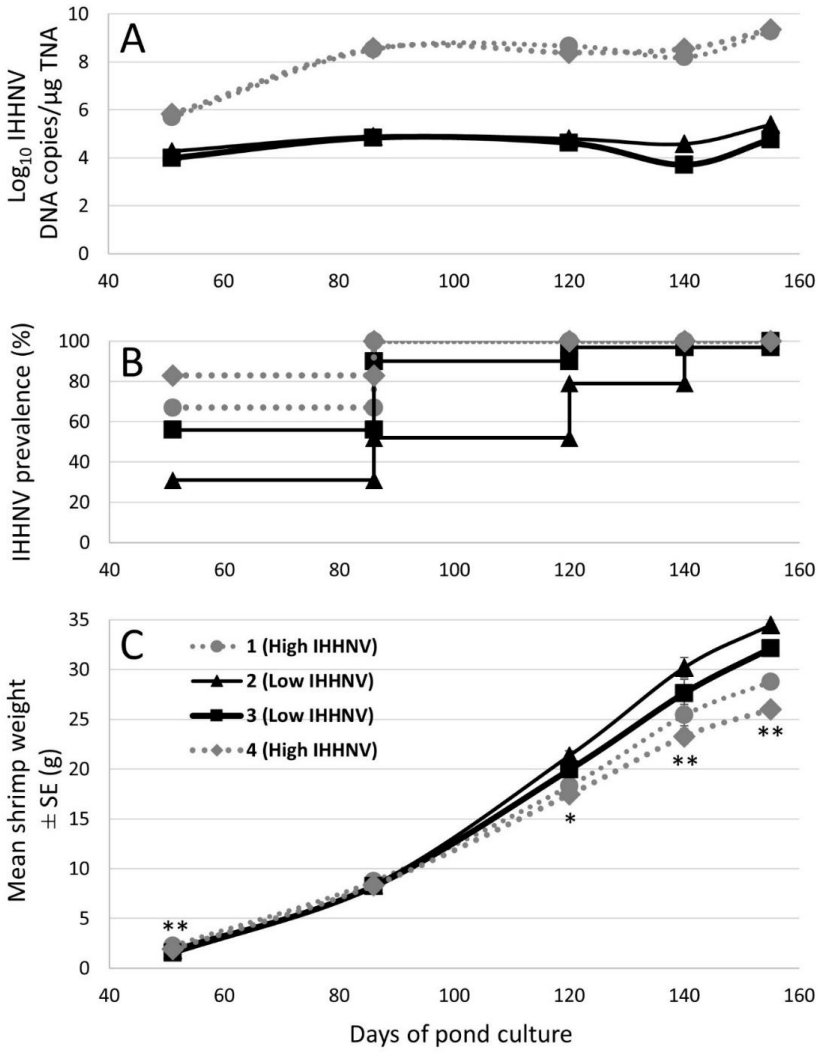

Figure 2. (A) Log_{10} mean IHHNV DNA copies/µg TNA as determined by real-time qPCR using pleopod tissue from 48 of the 144 shrimp sampled from each of the 4 ponds at each time point, except at 140 DOC when lymphoid organ tissue from 30 shrimp was tested. (B) Prevalence (%) at which IHHNV as detected by real-time qPCR in the 48 shrimp examined from each pond at each time point, except at 140 DOC when prevalence was assessed for only 30 shrimp. **(**C) Mean shrimp weight ± SE (g) of 144 shrimp/pond sampled progressively throughout grow-out except at 140 DOC when only 30 shrimp/pond were weighed. Levels of statistical significance (* P <0.05; ** P <0.001) were determined using logarithmic-transformed mean weights of shrimp from Ponds 1 and 4 combined and Ponds 2 and 3 combined at each time point. Pond codes are indicated, Pond 1 (●), Pond 4 (♦), Pond 2 (▲) and Pond 3 (■).

Table 7. Pond production metrics

Pond #	IHHNV	Total feed consumed (kg)	Harvest weight (kg)	Feed conversion ratio (FCR)	Tonnes/ha	Survival (%)
1	High	2434	1613	1.51	10.08	84.5
4	High	2388	1436	1.66	8.98	79.9
2	Low	3245	2273	1.43	14.21	95.9
3	Low	3337	1966	1.70	12.29	99.8

dsRNA residuals experiment

Of the 8 saline-injected and 8 dsRNA-injected female broodstock used in the experiment, none of the saline-injected females spawned. Thus 3 females from a same broodstock batch that were being spawned at the time but had not been injected were used as controls for 3 dsRNA-injected females that spawned. Each of these 6 females spawned fertile embryos between 6 and 14 days post-ablation/injection. Progeny from each of these spawns were reared as separate families to post-larval (PL) age, with representatives of each larval life stage preserved in RNAlater for real-time RT-qPCR analysis to detect residual dsRNA that might have been transferred to eggs from the parental female.

RT-qPCR detection of GAV RNA and dsGAV1/dsLuc dsRNA in broodstock tissues

Tissues of *P. monodon* broodstock (3 non-injected and 3 dsRNA-injected females that spawned fertile embryos between 6 and 14 days post-ablation/dsRNA injection) were collected at the time of spawning to identify residual dsRNA remaining from that injected at the time their eye stalks were ablated. Initially, cDNA prepared to native TNA extracted from pleopod, LO and ovary tissues was tested using RT-qPCR tests designed to detect GAV genomic ssRNA, dsGAV1 dsRNA and dsLuc dsRNA (**Table 8**). Subsequently, the dsGAV1 dsRNA and dsLuc dsRNA tests were repeated using cDNA prepared to TNA heat denaturated (HD) at 95°C to strand-separate dsRNA prior to cDNA synthesis to maximise the detection sensitivity of the qPCR tests.

With tissues of the 3 non-injected Females G90, B107 and P84, the GAV RT-qPCR test detected low amounts of GAV genomic ssRNA in LO tissue and the 2 pleopods, but not in ovary tissues. The dsGAV1 RT-qPCR test data correlated well with GAV RT-qPCR test data on the same samples (as expected due to it also detecting GAV genomic ssRNA), with the exception that low-level detections also occurred non-reproducibly with some ovary tissue samples of Females G90 and P84. As was expected, the dsLuc qPCR tested negative with all tissues examined from the 3 non-injected females (**Table 8**). With tissue of these females, the use of HD TNA to prepare cDNA had no significant effect on Ct values generated by the dsGAV1 and dsLuc qPCR tests.

With the 3 females co-injected with the dsGAV1 and dsLuc dsRNAs, the GAV RT-qPCR test was negative for all tissues tested from Female P83. However, Ct values were generated with the 2 pleopods but not LO tissue of Female B111 and with LO tissue but not the 2 pleopods of Female B120 (**Table 8**). The substantially lower Ct value (25.3) generated with LO tissue of Female B120 identified it to be infected at moderate loads with GAV. Using native TNA, data obtained using the

dsGAV1 RT-qPCR test showed good consistency with the GAV RT-qPCR test. However, it also generated Ct values with LO tissue samples from Females P83 and B111 that were undetermined (-) with the GAV RT-qPCR and a slightly reduced Ct value (23.9) with the LO tissue sample from Female B120 (Table 4). Using HD TNA, however, dsGAV1 qPCR test Ct values were markedly reduced (up to 9 Ct shift) compared to those obtained using native TNA. Moreover, in the case of Females B111 and B120 for which pleopod and ovary tissue samples that were undetermined (-) using native TNA, these same samples generated unequivocal Ct values indicative of dsGAV1 dsRNA being clearly detected when HD TNA was tested (**Table 8**). The exception was the LO sample from Female B120 identified using the GAV qPCR to be infected at moderate loads with GAV, where dsGAV1 qPCR test Ct values did not vary considerably, presumably due to this shrimp being infected at moderate loads with GAV and thus its LO containing GAV genomic ssRNA in substantial excess compared to residual injected dsGAV1 dsRNA. Detections of dsLuc dsRNA using the dsLuc RT-qPCR test in general mimicked detections of dsGASV1 dsRNA using the dsGAV1 RT-qPCR test using both native and HD TNA (**Table 8**). Exceptions to this were dsGAV1 RT-qPCR test detections of GAV genomic ssRNA in addition to dsGAV1 dsRNA that slightly complicated the interpretation of data generated using this test.

Direct testing of the DNA component of TNA sampled using the dsGAV1 and dsLuc qPCR tests failed to generate any Ct values with any tissues sampled from the 3 dsRNA-injected female broodstock, confirming that none of the qPCR detections were due to remnants of DNA template in the injected synthetic dsRNAs (data not shown, samples tested as in **Table 8**).

RT-qPCR detection of GAV RNA and dsGAV1/dsLuc dsRNA in progeny

To determine whether the dsGAV1 and dsLuc dsRNAs injected into tail muscle of females and detected subsequently in ovary tissue (see **Table 8**) might be transmitted vertically to progeny, eggs spawned from each female were reared separately to collect various larval life stages for analysis. GAV, dsGAV1 and dsLuc real-time qPCR data generated using cDNA prepared to either native or HD TNA extracted from pools of washed eggs, nauplii, mysis and protozoea and from 35 individual postlarvae (PL7 to PL11) reared from each of 3 non-injected and 3 dsRNA-injected females are shown in **Table 9**. A few qPCR tests (7/150) on pools of eggs, nauplii, protozoea or mysis generated at Ct value. However, except for a dsLuc RT-qPCR test using HD TNA that generated a Ct value from 1 of 2 zoea pools from dsRNA-injected Female B111 (Ct = 33.7), Ct values were invariably high (Ct 35.3 - 37.2) and in the range where qPCR data becomes equivocal. In support of this, none of the suspect detections were confirmed in companion tests using cDNA prepared to the same TNA treated differently or to TNA extracted from a second pool of larval from the same batch. Among the 192 PL examined from the 6 females, only a single individual among the 32 tested from dsRNA-injected Female B120 generated a high Ct value with both the GAV and dsGAV1 qPCR tests consistent with this PL being infected at low levels with GAV (**Table 9**).

As with the tissue samples from the dsRNA-injected females, direct dsGAV1 and dsLuc qPCR testing of the DNA component of TNA samples prepared to the progeny life stages tested failed to find any evidence of remnants of DNA template in the injected synthetic dsRNAs (data not shown, samples tested as in **Table 9**).

Table 8. GAV, dsGAV1 and dsLuc qPCR data on cDNA prepared to either native or heat-denatured TNA extracted from tissues sampled at the time of spawning from groups of 3 female *P. monodon* broodstock either not injected or injected with dsGAV1 and dsLuc dsRNA

Injection	Female	Day p.i.	qPCR test	qPCR Ct mean ± SD using cDNA prepared to either native or HD TNA									
				Pleopod #1 native	HD	Pleopod #2 native	HD	Lymphoid organ native	HD	Ovary 1 native	HD	Ovary 2 native	HD
none	G90	11	GAV	33.4 ± 0.3		34.5 ± 0.4		33.8 ± 0.3		-		-	
			dsGAV1	34.1 ± 0.8	34.3 ± 1.3	34.7 ± 0.8	34.4 ± 1.1	34.0 ± 0.6	33.2 ± 0.1	-	36.1 ± 1.8	36.4 ± 0.0	-
			dsLuc	-	-	-	-	-	-	-	-	-	-
	B107	14	GAV	33.5 ± 0.4		34.3 ± 0.1		30.4 ± 0.1		-		-	
			dsGAV1	34.4 ± 0.7	33.9 ± 0.9	34.9 ± 1.5	35.1 ± 0.3	29.6 ± 0.1	30.8 ± 0.3	-	-	-	-
			dsLuc	-	-	-	-	-	-	-	-	-	-
	P84	6	GAV	33.0 ± 0.0		36.3 ± 0.6		31.9 ± 0.4		-		-	
			dsGAV1	32.4 ± 0.3	33.7 ± 0.5	36.3 ± 0.4	36.9 ± 0.0	32.5 ± 0.1	31.0 ± 0.3	-	-	-	36.7 ± 1.2
			dsLuc	-	-	-	-	-	-	-	-	-	-
dsGAV1 dsLuc dsRNA	P83	6	GAV	-		-		-		-		-	
			dsGAV1	-	26.1 ± 0.0	-	27.4 ± 0.2	32.7 ± 0.3	25.7 ± 0.0	-	29.8 ± 0.1	-	29.9 ± 0.3
			dsLuc	-	25.2 ± 0.6	-	27.1 ± 0.1	32.2 ± 0.4	26.8 ± 0.1	-	31.6 ± 0.4	-	30.2 ± 0.4
	B111	10	GAV	33.4 ± 0.1		34.3 ± 0.4		-		-		-	
			dsGAV1	34.3 ± 0.6	29.7 ± 0.1	34.9 ± 1.3	30.3 ± 0.1	32.8 ± 0.3	26.3 ± 0.0	-	36.3 ± 0.7	-	35.8 ± 2.2
			dsLuc	34.6 ± 0.2	29.1 ± 0.1	-	30.1 ± 0.3	32.3 ± 0.7	25.5 ± 0.1	-	35.4 ± 0.8	-	36.0 ± 0.1
	B120	8	GAV	-		-		25.3 ± 0.1		36.6 ± 1.0		-	
			dsGAV1	-	27.9 ± 0.4	-	27.7 ± 0.1	23.9 ± 0.0	24.0 ± 0.1	-	30.2 ± 0.2	34.6 ± 0.6	31.3 ± 0.6
			dsLuc	36.1 ± 0.1	26.9 ± 0.1	35.9 ± 0.1	27.0 ± 0.1	35.4 ± 0.6	26.2 ± 0.1	-	30.1 ± 0.1	33.9 ± 1.0	31.7 ± 0.1

HD = heat-denatured, - = not detected, p.i. = post-injection, Ct mean determined for 3 technical replicates

Table 9. GAV, dsGAV1 and dsLuc qPCR data on cDNA prepared to either native or heat-denatured TNA extracted from larvae life stages reared separately from 3 female *P. monodon* broodstock not injected and 3 females injected with dsGAV1 and dsLuc dsRNA

dsRNA injection	Female	qPCR test	qPCR Ct mean ± SD using cDNA prepared to either native or HD TNA														
			Egg		Nauplii #1		Nauplii #2		Zoea #1		Zoea #2		Mysis #1		Mysis #2		Postlarvae[1]
			native	HD	native	HD	native	HD	native	HD	native	HD	native	HD	native	HD	native
none	G90	GAV	-		-		*		-		*		-		-		-
		dsGAV1	36.2 ± 0.2	-	-	-	*	*	-	-	*	*	-	-	-	-	-
		dsLuc	-	-	-	-	*	*	-	-	*	*	-	-	-	-	-
	B107	GAV	-		-		*		-		-		-		-		-
		dsGAV1	-	-	-	-	*	*	-	-	-	-	-	-	-	-	-
		dsLuc	-	-	-	-	*	*	-	-	35.2 ± 1.2	-	-	-	-	-	-
	P84	GAV	*		*		*		-		*		-		-		-
		dsGAV1	*	*	*	*	*	*	-	-	*	*	-	-	-	-	-
		dsLuc	*	*	*	*	*	*	-	-	*	*	-	-	-	-	-
dsGAV1 + dsLuc	P83	GAV	*		-		*		-		-		-		-		-
		dsGAV1	*	*	-	37.2 ± 0.4	*	*	-	37.2 ± 0.4	-	-	-	-	-	-	-
		dsLuc	*	*	-	-	*	*	36.3 ± 0.7	-	-	-	-	-	-	-	-
	B111	GAV	-		-		-		-		-		-		-		-
		dsGAV1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		dsLuc	-	-	-	-	-	-	-	33.7 ± 0.5	-	-	-	-	-	-	-
	B120	GAV	-		*		*		-		*		-		-		- 34.2 ± 0.2[2]
		dsGAV1	-	-	*	*	*	*	-	-	*	*	-	-	-	-	- 36.1 ± 0.6[2]
		dsLuc	-	-	*	*	*	*	-	35.9 ± 2.4	*	*	-	-	-	-	-

HD = heat-denatured, - = not detected, * No sample or TNA extraction failed, [1] 32 individual PL tested (UD = undetermined for all 32), Ct mean determined for 3 technical replicates

Discussion

Antivirals experiment

Reported here are data associating the sustained presence of high-level IHHNV infection with reduced growth performance and survival of *Penaeus monodon* reared under simulated commercial conditions in 0.16 ha research ponds. While the pond trial was designed and run for another purpose, the role of IHHNV in reduced growth performance was investigated due its detection and to the absence of both GAV, which has been associated most commonly over that past 2 decades with reduced survival and harvest yields of *P. monodon* farmed in eastern Australia (Spann et al., 1997; Callinan et al., 2003; Callinan and Jiang 2003; Munro et al., 2011), and YHV7, a genotypic variant of GAV which has also been associated with the similar stock losses since being introduced into Queensland more recently through the use of wild broodstock sourced from remote locations in northern Australia (Cowley et al., 2015, Mohr et al., 2015; J.A. Cowley et al., unpublished). Pivotal to investigating IHHNV was (i) its detection by TaqMan real-time qPCR (Cowley et al., 2018) at differing loads in the wild female *P. monodon* from North Queensland used to generate the experimental postlarvae (PL) and (ii) detection loads in the 3 females that contributed PL to the 2 ponds in which growth performance and survival was compromised being substantially higher than in the 4 females that contributed PL stocked into the other 2 ponds.

Using single pleopod tips as a source of TNA for qPCR analysis, IHHNV loads in the 7 wild female broodstock were noted to increase significantly, in some cases by up to 10^7-fold between when they were tested upon receipt compared to when each spawning ~6-7 weeks later. While factors contributing to these increased IHHNV infection loads might have included stresses induced by capture, tank rearing, dietary changes, handling and eyestalk ablation (de la Vega et al., 2004), variability in virus loads in different pleopods (Noble et al., 2018) and/or virus horizontal transmission occurring over the maturation period (Walker et al., 2001), the exact causes are unknown. The presence of IHHNV in the 7 female broodstock resulted in it also being detected in pools of washed eggs spawned from each, confirming the well-recognised propensity for IHHNV to be transmitted vertically from infected female *L. vannamei* (Lotz 1997; Lightner, 1999; Motte et al., 2003) and *P. monodon* (Withyachumnarnkul et al., 2006).

While data on nauplii produced from large numbers of female *L. vannamei* determined to either nested PCR negative or positive for IHHNV identified a likely association between infection and compromised egg hatch rates and larval survival and growth performance (Motte et al., 2003), no differences in egg hatch rates or larval growth were evident among progeny the 7 female *P. monodon* broodstock examined here. Despite not knowing the IHHNV infection status of males that inseminated the females, the role of males in IHHNV transmission has been discounted previously based on the rarity of detecting IHHNV DNA in *L. vannamei* spermatophores by nested PCR (Motte et al., 2003). As reported previously (Motte et al., 2003), the qPCR data highlight the potential value in undertaking testing in hatcheries to identify and select for either IHHNV-free or IHHNV-low wild broodstock, when sufficient are available to entertain this option, as is recommended commercial practice to preclude virus from being transmitted to seedstock at high efficiency The data also

highlight potential value in pursuing RNA interference (RNAi) approaches, as examined more extensively with other viruses (Sellars et al., 2011; 2016; Rao et al., 2018; Escobedo-Bonilla 2013; Itsathitphaisarn et al., 2017), as a means of reducing or more ideally clearing viral infections prior to broodstock spawning, thus further reducing the potential for IHHNV transmission.

At PL20 when seedstock from 3 larval-rearing (LR) tanks were stocked into the grow-out ponds, mean IHHNV loads were 45-fold to 865-fold higher in the Control PL20 in LR Tanks 4 and 6, respectively, that were combined in equal numbers and stocked into Ponds 1 and 4, compared to the Treatment PL20 from LR Tank 1 stocked into Ponds 2 and 3. However, these were tested using TNA extracted from 16 pools of 10 PL (total = 160) from each tank for logistics of testing of >150, IHHNV loads varied substantially among the pools. While this might have been due to PL in 2 of the 3 LR tanks each originating from 3 different females that transmitted IHHNV at variable levels, significant variability was also evident among the 16 PL pools from LR Tank 4 that contained progeny of a single female (O26). While both vertical and horizontal transmission likely contributed to the variability in IHHNV loads, the findings that Female Y77 was (i) infected with IHHNV at moderate loads upon receipt, (ii) maintaining this infection through to spawning and (iii) spawning eggs in which IHHNV was detected at loads 280-fold to 9,333-fold higher than eggs spawned from any of the other 6 females suggested a clear role in it contributing to the higher IHHNV loads in the PL20 reared in Tank 6. While associations between the duration of IHHNV infection and the propensity of a female to spawns eggs carrying IHHNV at substantial loads will need to be demonstrated, our findings suggest that wild broodstock infected at moderate to high loads with IHHNV present an elevated risk of transmitting infection to progeny. Thus early PCR screening to identify and cull such wild broodstock before being matured and spawned is recommended (Motte et al., 2003). To further reduce production impacts, as IHHNV can be reliably detected by PCR at earlier PL life stages (i.e. PL8, Lightner, 1983; M.J. Sellars et al., unpublished), opportunities also exist in hatcheries to select at this stage for IHHNV-free/low seedstock to either culture or sell at a premium price.

At 51 DOC when juvenile *P. monodon* could be captured at pond edges using a cast net, mean IHHNV loads of qPCR-positive juveniles detected among the 48 sampled at random and tested from each of the 4 ponds remained comparable those detected in the Control PL20 stocked into Ponds 1 and 4 and the Treatment PL20 stocked into Ponds 2 and 3. However, between then and 86 DOC, IHHNV loads increased by ~300-fold among the shrimp in Ponds 1 and 4 compared to only ~10-fold among the shrimp in Ponds 2 and 3. As IHHNV prevalence in the 48 shrimp tested from either Pond 1 or 4 also reached 100% at this time, infection severity and prevalence, mediated presumably via horizontal transmission, was escalating more rapidly than in Ponds 2 and 3 in which IHHNV prevalence and loads were lower. These findings are consistent with transmission cycle dynamics expected in ponds as infection loads increase to acute levels capable of compromising shrimp health (Walker et al., 2001). IHHNV infection loads progressed upwards until 155 DOC when sampling was discontinued and became more uniformly high among shrimp tested from Ponds 1 and 4, presumably due to prolonged exposure to the high IHHNV infection burden in the pond. In contrast, it took until 120-140 DOC before IHHNV was detected by qPCR in all shrimp tested from Ponds 2 and 3, indicative of these ponds being subjected to a much lower infection burden.

From 120 DOC onwards, mean weights of juveniles being reared in Pond 1 and particularly Pond 4 began to lag significantly behind those in Ponds 2 and 3. qPCR tracking of IHHNV loads, and testing of

lymphoid organ tissue at 133 DOC that identified no evidence of either GAV or YHV7, strongly implicated the sustained higher IHHNV infection burden as the most likely cause of this reduced growth performance, as well as survival determined at the time shrimp were harvested. While IHHNV infection has been noted previously to not adversely impact *P. monodon* growth or general health (Bell and Lightner 1984. Flegel 2006, Flegel et al., 2008; Chayaburakul et al., 2005; Withyachumnarnkul et al., 2006), such associations between reduced pond growth performance and viral infection burden have been made in commercial ponds stocked with *L. vannamei* PL generated from IHHNV-infected broodstock compared to IHHNV-free broodstock (Castille et al., 1993). Such affects are thus likely to be influenced by both IHHNV infection level and duration that prawns must withstand high-level IHHNV infection, and are well supported by observed reductions in survival and harvest yields of *P. monodon* farmed in north-eastern Australia caused by gradually increasing GAV infection burdens resulting from infections being carried into ponds in PL (Callinan and Jiang 2003; Munro et al., 2011).

Extrapolating from the 0.16 ha experimental ponds to 1 ha commercial ponds typical of those used in Australia, the combined harvest yield from Ponds 2 and 3 in which the IHHNV infection burden was lower was ~3.7 tonne/ha higher than the 2 neighbouring ponds with substantially higher IHHNV infection burdens. Based on a nominal local wholesale value for cooked *P. monodon* in the 35-38 pieces/kg size class, the gross value of such improved yields estimated to be in the order of AUD$67K/pond. An increase in profitability of this magnitude should easily justify the expense of qPCR screening of individual broodstock and/or suitably-sized pools of postlarvae to ensure that only IHHNV-free or IHHNV-low seedstock are cultured. It also justifies further investment in RNAi strategies designed to reduce IHHNV loads in broodstock prior to them spawning, as well as in efforts to exclude IHHNV from domesticated breeding lines of *P. monodon* (Barman et al., 2012) and ultimately to select for IHHNV resistance/tolerance within such programs (Moss 2006).

dsRNA residuals experiment

Here we examined whether purified synthetic dsRNAs injected into tail muscle of female *Penaeus monodon* broodstock at the time they were eyestalk ablated to stimulate spawning might transfer to and be amplified in progeny larval life stages. The 2 long synthetic dsRNA molecules injected included one (dsGAV1) targeted to the 5' end of the GAV ssRNA genome and found to elicit an RNAi response capable of protecting shrimp against disease following GAV challenge (Sellars et al., 2014) and reducing loads of pre-existing GAV infections (Sellars et al., 2016). However, as GAV occurs commonly in as a low-level chronic infection in *P. monodon* sourced from North Queensland (Spann et al., 1997; Walker et al., 2001), a unique synthetic dsRNA targeting a firefly luciferous (dsLuc) gene sequence was co-injected with the dsGAV1 dsRNA.

TaqMan real-time quantitative (q)PCR tests were designed to detect each dsRNA. To ensure that each qPCR test was highly sensitive, dilution series of pDNA controls were assessed initially using and 2 or 3 different PCR primer combinations were identify which provided the most ideal amplification characteristics with the 5'-[6FAM]-BHQ1-3' labelled probe used in each test. Following PCR primer selection, the detection sensitivity of the dsGAV1 and dsLuc qPCR tests was evaluated again using cDNA prepared to serial 10-fold dilutions of synthetic dsGAV1 and dsLuc dsRNA of calculated copy number. As commonly done to effectively detect dsRNA by RT-PCR (Rimstad et al., 1990, Revill and

Wright 1997, Klepper et al., 2017), heating at 95°C to strand-separate dsRNA into ssRNA prior to cDNA synthesis was also evaluated and found to improve the confidence at which low dsRNA template amounts were detected, and to also markedly improve the linearity of detections of higher and lower dsLuc dsRNA dilutions compared to the more sigmoidal-shaped curves evident using cDNA prepared to dilutions of native dsRNA. While the dsGAV1 RT-qPCR tests reliably detected down to a single dsRNA copy with either native or heat denatured (HD) dsRNA, the dsLuc test only reached this sensitivity using HD dsRNA. These data confirmed the need to include a TNA heat denaturation step in the RT-qPCR tests to ensure that any dsRNA remaining in various tissues of injected broodstock or transferred to progeny was detected effectively.

Abdominal muscle of female *P. monodon* broodstock was co-injected with dsGAV1 and dsLuc dsRNA at a standardised dose of 0.5 µg/g shrimp weight immediately prior to each shrimp being eyestalk ablated to promote spawning. RT-qPCR analyses of TNA extracted from lymphoid organ (LO), pleopod and ovary tissues sampled from each of 3 injected females when they spawned 6 to 10 days later identified residuals of each dsRNA, indicative of their systemic transfer to and lingering presence in these tissues. However, dsRNA was detected more convincingly, and only in ovary tissue samples, when the RT-qPCR analyses employed HD TNA due to this heating step making available greater quantities of ssRNA template for cDNA synthesis. While levels of each dsRNA detected in the ovary tissue were low, this might be expected due to ovary expanding substantially in cellular mass following eyestalk ablation in preparation for spawning (Uawisetwathana et al., 2011).

Regarding the hypothesis that muscle injected anti-viral dsRNAs might be able to curtail virus from being transmitted vertically to progeny, the detection of residual dsRNA in ovary tissue at times between 6 and 10 days post-injection is promising. Promise in this strategy also comes from findings that (i) levels of GAV in ovary tissue are generally far lower than found in tissues such as LO or male spermatophores, (ii) virus detected associated with eggs is derived most commonly from the female, unless the male parent is infected at much higher loads and (iii) GAV present in male spermatophores appears to exist predominantly as mature virions in the seminal fluid surrounding sperm cells rather than as an infection of the sperm cells themselves (Cowley et al., 2002). In this regard, a single individual from the 32 PL8 derived from dsRNA-injected Female B120 tested positive using both the GAV and dsGAV1 RT-qPCR tests, suggesting it to be infected at low loads with GAV. This finding was not surprising considering that this was the only female of the 6 examined to be infected at moderate loads with GAV, and thus was the most likely to transmit infection (Cowley et al., 2002). However, the question of whether the injected dsGAV1 dsRNA reduced GAV transmission levels from this female relative to what it might have transmitted had it not received the dsRNA will now be the focus of further more targeted investigations.

Even when TNA was heat denatured prior to cDNA synthesis to maximizes RT-qPCR test detection sensitivities, neither of the co-injected dsGAV1 or dsLuc dsRNAs were detected unequivocally in any of the pools of 50 washed eggs, nauplii, protozoea or mysis larval stages, or among any of the 32 individual postlarvae (PL7 to PL12) reared independently from each of 3 injected females. This was not unexpected, as without being amplified by some mechanism, any dsRNA transferred to eggs would rapidly and massively be diluted by increases in shrimp DNA and RNA as each fertilized egg undergoes many millions of cell divisions required to grow and differentiate through these larval stages (Silas et al., 1979). However, these findings remain specific to the dsRNA dose and delivery methodology used, and further validations will be required if these are altered to improve efficacy.

In Australia, the commercial use of RNAi approaches employing injected dsRNA will required approvals from regulatory bodies such as the APVMA. However, regarding the human health risk of consuming farmed shrimp derived from female broodstock injected with purified dsRNA, our inability to detect it in spawned eggs or larval stages up to postlarvae typically stocked into grow-out ponds suggests zero risk of eating farmed shrimp 20-40 g in weight and grown in ponds for ~5 months. The findings reported here indicating that the dsRNA delivery strategy assessed is unlikely to invoke serious regulatory concerns thus provides confidence in optimizing and further evaluating its use in hatcheries to restrict virus infections from being transmitted from broodstock to seedstock.

Conclusions

In cases when GAV was reliably detected in *P. monodon* broodstock by RT-qPCR, loads were identified to reduce by 3-4 Ct values (8- to 16-fold reduction in GAV RNA copy number) several days after being injected with the dsGAV1 dsRNA targeting a ~1 kb sequence at the extreme 5'-terminus of the GAV ssRNA genome. This reduction was comparable to those evident in previous studies with GAV and similar studies with other viruses, thus confirming the potential value of using RNAi in commercial hatcheries as a practical means of reducing pathogen impacts during prawn grow-out.

The study on the grow-out impacts of high-level IHHNV infection confirmed that it is transmitted vertically from females to their progeny, as reported previously for *L. vannamei* (Lightner, 1999) and *P. monodon* (Withyachumnarnkul et al., 2006), and that infection prevalence and loads transmitted is dictated substantially by infection loads in each female. As such, the study identified IHHNV as an ideal target for using real-time qPCR screening in hatcheries to select for IHHNV-low or negative broodstock as well as injected dsRNA antivirals to reduce infection loads in broodstock prior to them spawning in circumstances when IHHNV prevalence levels are too high to accommodate broodstock culling (Sellars et al., 2011, 2016).

As IHHNV was detected by qPCR in PL20, and has been reliably detected previously in early PL stages (i.e. PL8), options also exist to incorporate a second round of qPCR-based testing at the PL stage to select batches to cultivate that pose minimal risks of developing high-level infections demonstrated here to have the ability to markedly compromise the prawn growth performance and survival, thus reducing pond harvest yields and value. Opportunity also exists to pool larval rearing batches into ponds based on their pathogen status, with a typical set up in Australia stocking four larval rearing tanks per pond. By extrapolating pond production metrics from the 0.16 ha research ponds to 1 ha commercial ponds, the impact of rearing progeny that maintained IHHNV infections at lowered prevalence and loads compared to progeny that rapidly developed high-level IHHNV infections following stocking was estimated to be AUD$67K/pond in improved gross value. These findings provide a strong impetus to undertake hatchery and farm-based R&D activities to validate the value of using qPCR-based screening/selection for IHHNV-low broodstock either in isolation or in combination with broodstock injection with IHHNV-specific dsRNA antivirals. A need also exists to incorporate such strategies in future programs to establish domesticated breeding populations of *P. monodon* SPF for viruses including IHHNV and as these programs mature, also selected for disease resistance viruses including IHHNV.

Based on data indicating that (i) dsRNA antiviral injection can reduce GAV infection loads in broodstock by 8- to 16-fold, (ii) sustained high-level IHHNV infection can compromise commercial production of *P. monodon*, (iii) IHHNV infection can reliably be detected and quantified by qPCR in hatchery stocks of PL, (iv) IHHNV occurs commonly in wild broodstock collected from several regions in northern Australia (M.J. Sellars et al., unpublished), it has become clear that IHHNV is now equally or possible more import than GAV for progressing hatchery-based RNAi strategies to avoid disease impacts occurring during grow-out. As injected dsRNAs were able to be detected unequivocally in broodstock pleopod, lymphoid organ and ovarian tissue 6-10 days after injection of female broodstock at the time they spawned, but not in their eggs of subsequent larval life stages (nauplii, protozoea and mysis) up to early-stage PL, at face value, it seems unlikely that the consumption of progeny reared from broodstock injected with dsRNA will pose any human health risk.

Implications

Data generated in this study have identified that there are production benefits to be gained in the use of PCR screening in hatcheries to identify and cull wild *P. monodon* broodstock with high-level IHHNV infection. If such broodstock screening/culling cannot be accommodated or if IHHNV prevalence is identified to be high, similar PCR screening and culling of tank batches of postlarvae (PL) with high IHHNV prevalence/loads prior to pond stocking was also identified to be an intervention point at which risks of reduced growth performance and survival resulting in production losses could be minimized. The data also allowed the value of stocking a pond with PL containing IHHNV at low prevalence and infection loads (compared to PL containing IHHNV at high prevalence, with many infected at high loads) to be estimated in monetary terms. Based on prawns having mean weight at harvest meeting the 35-38 pieces/kg size class with a typical cooked wholesale value of ~AUD$18/kg, crop value in a typically-sized commercial pond was estimated to be improved by ~AUD$67K gross/ha.

The data from this and a previous study also identified hatchery intervention points for PCR screening of broodstock (upon arrival or at the times of eye stalk ablation or female spawning) and of progeny (PL8 which would allow 8-10 days for PCR data acquisition prior to pond stocking). Data on IHHNV prevalence/infection loads generated from such screening would inform management decisions to minimize disease risks during prawn grow-out and provide opportunities for hatcheries to sell IHHNV-free of IHHNV-low seedstock at a premium price. Moreover, the demonstrated potential economic value of rearing IHHNV-free or IHHNV-low seedstock provides an impetus to progress dsRNA antiviral research to provide the industry with alternative strategies to reduce virus infection loads in broodstock. If refined sufficiently and approved for use, the injection of broodstock with such antivirals might mitigate any need for PCR screening of progeny to select for PL batches of reduced risk.

As the study demonstrated that (i) progeny spawned from broodstock injected with dsRNA antivirals are robust and suitable for commercial grow-out and that (ii) dsRNA injection can reduce GAV infection loads in broodstock 8- to 16-fold, the Australian prawn farming industry should be confident in trailing the technology in their hatcheries. However, for this to occur

1. Either further R&D funding will be needed or IP covering the RNAi technology will need to be licenced to a company prepared to provide dsRNA antivirals for commercial evaluation, and

2. APVMA will need to approve/licence/exempt to process of injecting dsRNA antivirals into broodstock that generate seedstock reared for human consumption.

The comprehensive examination of eggs and progeny life stages derived from dsRNA-injected broodstock that showed no evidence of dsRNA being transferred to progeny, with these progeny reared for a further 5-6 months before being harvested for human consumption, provides critical information needed to begin discussions with the APVMA to define the conditions under which dsRNA antivirals can be used in commercial prawn hatcheries.

Recommendations

Based on the outcomes of this project, we recommend that a small consultative working group be established to champion discussions with the APVMA to define conditions under which the dsRNA antiviral injection process can be trialled in commercial prawn hatcheries to reduce virus vertical transmission. This working group would include key CSIRO researchers, industry technical advisers and APVMA staff.

Scientific rigorous data sets that quantify the impacts to production of pathogens such as this study with IHHNV are scarce and not commonly published. Efforts within the Australian industry should look at establishing these data sets for other pathogens so as to identify the biggest pain points and thus greatest opportunities for intervention with antivirals and pathogen management strategies throughout the culture phase. Such data sets would be best obtained by scientific team(s) and farm operator(s) coming together to monitor prawn health, growth and survival on specified ponds during commercial culture.

Extension and Adoption

Outline how the project was (and will continue to be) extended and communicated to the end user, such as managers, other researchers, industry and where applicable the broader community.

If possible outline where project outputs were adopted – this may not always be possible at time of writing the final report.

The project outcomes will be reported:

1. In oral presentations at the 2018 APFA Annual Symposium, Aug 15-16, 2018, and at Aqua 2018, World Aquaculture Society Conference, Montpellier, France, Aug 25-29, 2018
2. In 2 scientific publications as outlined below in ‘Project materials developed’
3. Directly to prawn hatchery and farm managers

If APVMA approvals can be secured, we will apply for further R&D funding to trial the use of dsRNA antivirals in commercial hatcheries to assess what improvements RNAi technology can deliver to farm production security and productivity.

Based on progress made in this project on the application of dsRNA antivirals, CSIRO will continue to examine and refine aspects of the RNAi technology as part of its core strategic research.

Project materials developed

Sellars, M.J., Cowley, J.A., Musson, D., Rao, M., Menzies, M.L., Coman, G.J., Murphy, S.M. (2018) Reduced growth performance of Black Tiger prawn (*Penaeus monodon*) infected with infectious hypodermal and hematopoietic necrosis virus (to be submitted to Aquaculture)

Rao, M., Cowley, J.A., Murphy, S.M., Stratford, C.N., Sellars, M.J. (2018) Double-stranded RNA injected into female Black Tiger prawn (*Penaeus monodon*) prior to spawning does not transfer to progeny (submitted to Aquaculture)

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Supplementary Table 1: Real time PCR GAV and IHHNV data of 79 wild caught Black Tiger shrimp from Etty Bay, Qld on 25 May 2016 including standard curve and not template control anaylses

Prawn #	Ct Mean GAV	Ct Mean IHHNV
1	35.135	36.201
2	ND	ND
3	36.841	ND
4	36.544	ND
5	ND	ND
6	ND	ND
7	37.275	ND
8	36.576	ND
9	ND	ND
10	ND	ND
11	ND	24.15
12	37.012	ND
13	36.389	ND
14	35.674	ND
15	36.740	ND
16	36.631	ND
17	36.620	ND
18	ND	ND
19	36.408	ND
20	29.798	ND
21	ND	ND
22	ND	ND
23	ND	ND
24	ND	ND
25	ND	ND
26	ND	ND
27	31.399	37.02
28	ND	ND
29	ND	ND

Prawn #	Ct Mean GAV	Ct Mean IHHNV
41	ND	36.923
42	35.729	ND
43	ND	ND
44	ND	ND
45	34.673	36.727
46	ND	ND
47	36.260	34.552
48	33.972	35.824
49	ND	ND
50	35.729	33.958
51	34.312	ND
52	ND	ND
53	36.534	ND
54	36.355	ND
55	33.392	ND
56	ND	36.976
57	ND	24.687
58	ND	ND
59	36.479	ND
60	ND	ND
61	ND	36.579
62	ND	ND
63	36.956	ND
64	ND	24.978
65	37.119	ND
66	34.852	ND
67	36.138	ND
68	ND	ND
69	32.519	ND

Sample Name	Ct Mean	Ct STD	Quantity (copies)
GAV2	32.052	0.116	20
GAV3	28.674	0.065	200
GAV4	26.659	0.181	2,000
GAV5	22.360	0.066	20,000
GAV6	18.874	0.095	200,000
GAV7	15.443	0.106	2,000,000
GAV8	11.705	0.152	20,000,000
GAV9	8.055	0.172	200,000,000
NTC	ND		
NTC	ND		
NTC	ND		
IHHNV1	34.635	0.03	10
IHHNV2	32.218	0.25	100
IHHNV3	28.895	0.18	1,000
IHHNV4	25.514	0.02	10,000
IHHNV5	21.839	0.04	100,000
IHHNV6	18.057	0.09	1,000,000
IHHNV7	15.392	0.04	10,000,000
IHHNV8	11.933	0.1	100,000,000
NTC	ND		
NTC	ND		
NTC	ND		

30	ND	ND	70	ND	ND
31	ND	35.945	71	ND	ND
32	34.465	36.197	72	37.002	ND
33	36.841	ND	73	ND	ND
34	ND	ND	74	ND	ND
35	ND	ND	75	36.921	ND
36	ND	ND	76	35.429	38.508
37	ND	ND	77	35.995	ND
38	ND	23.943	78	ND	ND
39	ND	23.649	79	36.275	ND
40	ND	ND			

Supplementary Table 2: Real time PCR GAV and IHHNV data of 79 wild caught Black Tiger shrimp from Bramston Beach, Etty Bay or Position X, Qld on 4 June 2016 including standard curve anaylses

Prawn #	Location	Ct Mean GAV	Ct Mean IHHNV
1	Bramston Beach	ND	ND
2	Bramston Beach	ND	ND
3	Bramston Beach	39.59	ND
4	Bramston Beach	36.191	ND
5	Bramston Beach	33.013	24.138
6	Bramston Beach	34.578	ND
7	Bramston Beach	34.738	ND
8	Bramston Beach	36.621	ND
9	Bramston Beach	ND	ND
10	Bramston Beach	ND	ND
11	Bramston Beach	ND	ND
12	Bramston Beach	ND	ND
13	Bramston Beach	ND	ND
14	Bramston Beach	ND	ND
15	Bramston Beach	30.797	36.54
16	Bramston Beach	ND	ND
17	Bramston Beach	ND	ND
18	Bramston Beach	34.582	ND
19	Bramston Beach	ND	ND
20	Bramston Beach	ND	ND
21	Bramston Beach	37.578	ND
22	Bramston Beach	ND	ND
23	Bramston Beach	ND	ND
24	Bramston Beach	ND	ND
25	Bramston Beach	ND	ND
26	Bramston Beach	ND	ND

Prawn #	Location	Ct Mean GAV	Ct Mean IHHNV
45	Bramston Beach	ND	36.386
46	Bramston Beach	ND	ND
47	Bramston Beach	ND	22.855
48	Bramston Beach	35.678	23.66
49	Bramston Beach	ND	37.369
50	Bramston Beach	ND	ND
51	Bramston Beach	ND	22.822
52	Bramston Beach	36.735	ND
53	Bramston Beach	ND	34.266
54	Bramston Beach	36.138	ND
55	Bramston Beach	37.008	32.807
56	Bramston Beach	ND	ND
57	Bramston Beach	34.586	ND
58	Bramston Beach	36.134	ND
59	Bramston Beach	ND	ND
60	Bramston Beach	33.59	ND
61	Bramston Beach	ND	ND
62	Bramston Beach	36.621	ND
63	Bramston Beach	26.901	ND
64	Bramston Beach	ND	ND
65	Bramston Beach	ND	ND
66	Bramston Beach	36.633	ND
67	Bramston Beach	ND	ND
68	Bramston Beach	37.087	ND
69	Bramston Beach	36.283	ND
70	Bramston Beach	36.802	22.217

Sample Name	Ct Mean	Ct STD	Quantity (copies)
GAV2	31.335	0.053	20
GAV3	28.116	0.069	200
GAV4	25.012	0.046	2,000
GAV5	22.03	0.084	20,000
GAV6	18.491	0.053	200,000
GAV7	15.02	0.109	2,000,000
GAV8	12.115	0.058	20,000,000
GAV9	9.016	0.118	200,000,000
NTC	ND		
IHHNV1	34.726	0.07	10
IHHNV2	31.078	0.27	100
IHHNV3	27.699	0.04	1,000
IHHNV4	23.533	0.1	10,000
IHHNV5	20.709	0.07	100,000
IHHNV6	16.325	0.08	1,000,000
IHHNV7	14.444	0.05	10,000,000
IHHNV8	11.222	0.09	100,000,000
NTC	ND		

27	Bramston Beach	36.224	ND	71	Bramston Beach	36.905	ND
28	Bramston Beach	ND	ND	72	Etty Bay	ND	ND
29	Bramston Beach	34.461	ND	73	Etty Bay	ND	24.924
30	Bramston Beach	32.875	ND	74	Etty Bay	ND	ND
31	Bramston Beach	ND	ND	75	Etty Bay	ND	ND
32	Bramston Beach	ND	ND	76	Etty Bay	ND	ND
33	Bramston Beach	ND	ND	77	Etty Bay	ND	ND
34	Bramston Beach	36.135	ND	78	Etty Bay	ND	ND
35	Bramston Beach	ND	ND	79	Etty Bay	30.357	23.998
36	Bramston Beach	ND	ND	80	Etty Bay	ND	35.843
37	Bramston Beach	35.78	ND	81	Etty Bay	ND	22.722
38	Bramston Beach	28.499	ND	82	Etty Bay	ND	ND
39	Bramston Beach	34.952	ND	83	Etty Bay	ND	ND
40	Bramston Beach	ND	ND	84	Position X	37.12	ND
41	Bramston Beach	33.168	ND	85	Position X	ND	ND
42	Bramston Beach	ND	ND	86	Position X	32.772	ND
43	Bramston Beach	34.496	ND	87	Position X	ND	36.343
44	Bramston Beach	36.973	ND				

Supplementary Table 3: Real time PCR GAV data of 79 wild caught Black Tiger shrimp from Bramston Beach, Qld on 11 July 2016 including standard curve and not template control anaylses

Prawn #	Ct Mean GAV	Prawn #	Ct Mean GAV	Prawn #	Ct Mean GAV
1	34.94	85	ND	169	36.51
2	23.09	86	ND	170	36.40
3	25.46	87	ND	171	36.88
4	34.22	88	ND	172	35.73
5	32.91	89	ND	173	35.59
6	34.03	90	ND	174	ND
7	32.13	91	35.10	175	ND
8	36.19	92	35.63	176	ND
9	29.22	93	ND	177	36.43
10	31.63	94	ND	178	ND
11	33.91	95	ND	179	ND
12	34.23	96	ND	180	35.16
13	34.60	97	ND	181	ND
14	34.68	98	ND	182	37.67
15	26.61	99	36.61	183	ND
16	35.43	100	ND	184	ND
17	36.18	101	ND	185	36.15
18	33.28	102	35.39	186	36.88
19	31.79	103	ND	187	ND
20	35.18	104	36.82	188	36.80
21	35.62	105	ND	189	ND
22	36.12	106	36.07	190	35.57
23	35.12	107	ND	191	35.46
24	36.80	108	37.12	192	ND
25	29.15	109	ND	193	36.15
26	33.68	110	ND	194	ND
27	34.65	111	ND	195	ND
28	34.75	112	ND	196	ND
29	34.44	113	ND	197	35.80
30	33.91	114	ND	198	ND
31	34.39	115	36.42	199	ND
32	29.01	116	ND	200	ND
33	30.48	117	ND	201	37.63
34	35.20	118	ND	202	36.14
35	35.71	119	ND	203	ND
36	36.15	120	ND	204	ND
37	23.43	121	36.34	205	ND
38	35.84	122	ND	206	ND
39	36.31	123	36.99	207	36.73
40	31.57	124	ND	208	35.32
41	32.13	125	ND	209	ND
42	33.11	126	ND	210	36.01
43	34.09	127	ND	211	35.70
44	34.99	128	ND	212	36.74
45	32.19	129	ND	213	36.63
46	35.61	130	35.36	214	ND
47	ND	131	ND	215	ND
48	36.19	132	ND	216	35.77
49	36.59	133	ND	217	ND

Sample Name	Ct Mean	Ct STD	Quantity (copies)
GAV2	32.052	0.116	20
GAV3	28.674	0.065	200
GAV4	26.659	0.181	2,000
GAV5	22.360	0.066	20,000
GAV6	18.874	0.095	200,000
GAV7	15.443	0.106	2,000,000
GAV8	11.705	0.152	20,000,000
GAV9	8.055	0.172	200,000,000
NTC	ND		

50	35.58	134	ND	218	ND
51	ND	135	ND	219	36.03
52	ND	136	ND	220	36.79
53	ND	137	ND	221	ND
54	ND	138	ND	222	36.69
55	35.40	139	ND	223	ND
56	ND	140	ND	224	36.36
57	37.76	141	ND	225	ND
58	ND	142	ND	226	36.47
59	ND	143	ND	227	ND
60	ND	144	ND	228	36.72
61	ND	145	ND	229	ND
62	ND	146	ND	230	ND
63	ND	147	ND	231	35.94
64	35.07	148	ND	232	ND
65	35.86	149	ND	233	ND
66	ND	150	ND	234	37.85
67	ND	151	ND	235	ND
68	ND	152	ND	236	ND
69	ND	153	ND	237	ND
70	ND	154	ND	238	ND
71	ND	155	ND	239	36.33
72	ND	156	ND	240	ND
73	ND	157	ND	241	ND
74	ND	158	ND	242	36.82
75	37.23	159	ND	243	ND
76	ND	160	37.30	244	ND
77	ND	161	ND	245	37.24
78	ND	162	ND	246	ND
79	36.52	163	ND	247	35.26
80	ND	164	ND	248	36.25
81	ND	165	36.19	249	35.72
82	ND	166	36.56	250	ND
83	ND	167	37.15	251	35.07
84	ND	168	36.71	252	35.60