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## **Novacq™ improves resilience against viral infection and mortality in Black Tiger shrimp, *Penaeus monodon***

Melony J. Sellars<sup>1\*</sup>, Min Rao<sup>2</sup>, Nick Polymeris<sup>3</sup>, Simon Irvin<sup>3</sup>, Jeff Cowley<sup>2</sup>, Nigel Preston<sup>1</sup>, Brett Glencross<sup>1</sup>

<sup>1</sup>CSIRO Food Futures National Research Flagship, CSIRO Marine and Atmospheric Research, Dutton Park, Qld 4102, Australia

<sup>2</sup>CSIRO Food Futures National Research Flagship, CSIRO Livestock Industries, St Lucia, Qld 4067, Australia

<sup>3</sup>CSIRO Food Futures National Research Flagship, CSIRO Marine and Atmospheric Research, Bribie Island, Qld 4507, Australia

\*Corresponding author:

Melony J. Sellars

Tel: +61 7 3833 5962

Email: [Melony.Sellars@csiro.au](mailto:Melony.Sellars@csiro.au)

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### **Abstract**

The efficacy of the novel aquaculture feed ingredient Novacq™ to improve resilience against viral infection and mortality in Black Tiger shrimp, *Penaeus monodon*, was examined. Juvenile 4-6 g shrimp were fed either a control diet or treatment diet which included 10% Novacq™ for a 26 d conditioning period. Control and treatment shrimp were subsequently each divided into a no injection, saline injection or GAV challenge injection sub-treatment with each having four replicate tanks of ten shrimp. After injection, shrimp survival in all six treatments was monitored daily over a 14 d experimental bioassay period. Two of the four replicate tanks for each treatment were pleopod sampled for later GAV load quantification at day 0 and 14 of the experimental period (i.e. 'less handling'; survival comparison), whilst the other two replicate tanks were pleopod sampled for later GAV load quantification at days 0, 3, 7, 10 and 14 (i.e. 'more handling'; GAV load comparison). In the survival comparison, shrimp fed Novacq™ had significantly higher survival rates ( $P > 0.05$ ) when GAV challenged compared to shrimp not fed Novacq™. Similarly, shrimp fed Novacq™ had marginally higher survival rates when injected with saline and compared to their respective control. Shrimp that received no injection showed the same survival rates irrespective of diet. In the GAV load comparison GAV loads were generally lower in shrimp fed Novacq™ when compared to their respective GAV challenge or saline injection controls. Survival and GAV load data indicate that Novacq™ improves resilience against viral infection and mortality in Black Tiger shrimp, *Penaeus monodon*.

### **Introduction**

Novacq™ is a novel aquafeed ingredient recently discovered by CSIRO Australia (Patent #2008201886). Novacq™ is positioned to revolutionize feed formulation for the global shrimp industry conferring in excess of 30% improvement in growth for Black Tiger shrimp, *Penaeus*

*monodon* and 50% improvement in growth for Pacific White shrimp, *Litopennaeus vannamei* (Glencross et al., 2012b). Novacq™ is a bioactive feed ingredient that has been used to support a range of nutritional strategies in shrimp diets including improved growth performance and replacement of fish meal, without compromise to performance (Glencross et al., 2012a, 2012b).

Other bioactive substances like crustacean and squid meals are also routinely used in the shrimp feed industry (Williams et al., 2005). It has long been recognized that these particular ingredients confer biological properties beyond their simple protein and energy supply capacity. While some attempt has been made to identify the nature of this bioactive, its use is still largely limited to provision through the use of these marine animal ingredients (Williams et al., 2005). However, despite evidence of improved growth performance there is little to suggest that these ingredients also confer improved 'fitness' (Williams et al., 2005).

Novacq™, although a bioactive ingredient, comprises a product produced from a mixed population of marine microorganisms (microalgae and bacteria) that form a microbial biomass and as such are not reliant on wild fishery products. Anecdotal observations from earlier trials indicate that when fed to shrimp Novacq™ provides heightened fitness (Glencross et al., 2012b). This study therefore set out to quantify this fitness in terms of shrimp resilience to viral infection and mortality using an established CSIRO Bioassay Challenge System.

## **Materials and Methods**

### ***Feed manufacture***

Feed pellets were manufactured that contained either no Novacq™ (control diet) or 10% Novacq™ (treatment diet) (Table 1). Each diet was prepared fresh four days before the start of the conditioning period. After being removed from storage at -20°C, each feed ingredient was equilibrated to room temperature before being milled through a <750 µm rotor mill screen (Retsch™ ZM200 rotor mill, Retsch Pty Ltd, North Ryde, NSW, Australia), weighed and mixed in an upright planetary mixer (Hobart, Sydney, NSW, Australia). The ingredients were then mixed thoroughly with sufficient fresh water added to allow screw-pressing of the mash through a 3 mm die. The pelleted feed was then broken into 5 – 10 mm lengths, before being steamed at 95°C for 10 min prior to being oven dried at 60°C for 24 h, packed in air-tight containers and stored frozen at -20°C until used.

### ***GAV inoculum preparation***

Aliquots from the same Gill-Associated virus (GAV) inoculum preparation as used by Sellars et al. (2011) were used in this study. In brief, the GAV inoculum was prepared from *Penaeus monodon* sacrificed when they became moribund following experimental injection of an inoculum stock prepared similarly from *P. monodon* with high-level acute GAV infections. Soft cephalothorax tissues of 4 shrimp were diluted in 6 vol shrimp salt solution (SSS) (10 mM HEPES, 450 mM NaCl, 10 mM KCl, 10 mM EDTA pH 7.2-7.5) that had been 0.22 µm filter sterilized and homogenized on ice using an Ultra-Turrax blender until no granular matter was visible. Fine particulate matter in the homogenate was removed by centrifugation at 750 x g for 10 min at 4°C and then at 15000 rpm for 20 min at 4°C using a Beckman SW28 rotor. The supernatant was forced through a 0.45 µm filter and 1.0 mL aliquots of the inoculum were snap frozen on dry ice and stored at -80°C.

### ***Bioassays to define a minimum lethal dose of GAV inoculum***

As described by [Sellars et al. \(2011\)](#) three bioassays were previously performed to define a minimum lethal dose (LD) of this GAV inoculum for selectively bred *P. monodon* that would reliably result in 50% accumulated mortality of juveniles by 8 days post-injection (dpi) and 80% accumulated mortality by 12 dpi. These bioassays confirmed that the GAV inoculum diluted 1:3 in SSS provided the minimum LD reliably generating the specified accumulated mortality levels. However, as the present study was performed on unselected *P. monodon* lines for which CSIRO have anecdotal evidence that they are more susceptible to viral infection ([CSIRO unpublished information](#)), a GAV inoculum dilution of 1:30 in SSS was chosen. The decision to utilise unselected lines in this study was two-fold; firstly because selected lines are believed to have specific pathogen tolerance and secondly because selected lines have increased feed intake, reduced maintenance demands for energy, and improved protein and energy utilization efficiencies that would result in confounding complexities when interpreting bioassay results ([Glencross et al., 2012a](#)).

### ***Shrimp***

Pleopods of first generation unselected *P. monodon* collected from 11 farm ponds (10 shrimp per pond) were pre-screened for the endemic viruses GAV and Mourilyan virus (MoV) using RT-PCR ([Cowley et al., 2000, 2005](#)). Shrimp (n = 600; ~4-8 g) were collected by cast-netting 12 d later from a pond in which neither virus was detected (data not shown) ([Pacific Reef Fisheries Pty Ltd., Ayr, Queensland, Australia](#)). Shrimp were transported by road and air from Ayr, Queensland to Bribie Island, Queensland (~10 h transit) and randomly stocked into four 5-tonne circular bare-bottom tanks (150 shrimp in each) for conditioning.

### ***Diet conditioning period***

The conditioning tanks received aeration and flow-through seawater at a rate of 1.6 L min<sup>-1</sup> maintaining the water temperature at 28 ± 2°C. Tanks received alternating 12 h light and 12 h dark photoperiods and were covered with a Polygal® lid to reduce light intensity. Two tanks were randomly chosen to be fed the control diet whilst the other two tanks were fed the treatment diet which included 10% Novacq™. Shrimp were fed their respective diets for 26 d to excess at 1600 h daily and tanks were cleaned by siphoning every 2-3 d. After the 26 d diet conditioning period, tanks were drained and shrimp collected for random stocking into the bioassay trial according to conditioning treatment.

### ***Experimental bioassay***

The experimental bioassay had two diets; control or treatment, followed by three sub-treatments; no injection, saline injection or GAV challenge injection. There were four replicate tanks for each of the six treatments in total which were randomly assigned to tanks to accommodate any position-related influences in the facility. Tanks were filled to 80 L with seawater that was aerated, maintained at 28 ± 2°C and trickle fed fresh seawater at a rate of ~0.6 L min<sup>-1</sup>. The tanks had opaque white lids and were maintained in a facility providing alternating 12 h light and 12 h dark photoperiods.

After collection from corresponding conditioning tanks, 10 randomly chosen shrimp were weighed, sex determined, injection performed (described below) and tissue sampled for RNA (described below) before being stocked into their allocated tank. Shrimp were continued on their corresponding control or treatment diets fed to excess twice each day at 0930 h and 1400 h, and waste was siphoned out 3 times per week or as required to maintain water quality. The number of shrimp alive in each tank was counted and dead shrimp removed daily at approximately 1400 h. The experimental bioassay was performed over a 14 d period.

### *Injection*

In the saline injection treatments 25  $\mu$ L of SSS was injected into the muscle of the 6<sup>th</sup> abdominal segment using a 100  $\mu$ L Hamilton glass syringe on day 0 of the experimental bioassay. In the GAV challenge treatments GAV inoculum diluted 1:30 in SSS was similarly injected at  $\sim$ 5.0  $\mu$ L per 1 g shrimp weight on day 0 of the experimental bioassay. GAV inoculum volumes adjusted to accommodate variations in shrimp weights were 20  $\mu$ L (4.0-5.0 g), 25  $\mu$ L (5.0-6.0 g), 30  $\mu$ L (6.0-7.0 g), 35  $\mu$ L (7.0-8.0 g), 40  $\mu$ L (8.0-9.0 g), 45  $\mu$ L (9.0-10.0 g), 50  $\mu$ L (10.0-11.0 g), 55  $\mu$ L (11.0-12.0 g), 60  $\mu$ L (12.0-13.0 g), 65  $\mu$ L (13.0-14.0 g), and 70  $\mu$ L (14.0-15.0 g).

### *Tissue sampling for RNA*

On day 0 and 14 of the experimental bioassay, a pleopod from every shrimp stocked (day 0) or alive (day 14) was sampled for later RNA extraction and GAV load quantification using scissors alcohol sterilized between samplings, snap frozen on dry ice and stored at -80°C. Pleopods were sampled in an order so as to allow individual identification of shrimp within tanks (i.e. removal of alternating pleopods to number shrimp 1 to 10) as previous CSRIO studies have demonstrated pleopod location has no correlation with viral load when using the specified species and RT-PCR assays ([CSIRO unpublished information](#)). Two of the four replicate tanks for each treatment were designated as a survival comparison (i.e. 'less handling') and not sampled at any other time points. The other two replicate tanks for each treatment were designated as a GAV load comparison (i.e. 'more handling') and on days 3, 7, and 10 in these two tanks of each treatment pleopods were sampled similarly from all live shrimp as described.

### **Real-time PCR quantification of GAV**

Shrimp pleopods were homogenized in 600  $\mu$ L TRIzol reagent (Invitrogen) using 3 glass beads per tube and a Savant FastPrep FP120 tissue grinder and total RNA was extracted according to the manufacturer's protocol. RNA was resuspended in 15  $\mu$ L RNase-free water and before being stored at -80°C, a 1.5  $\mu$ L aliquot was examined using a NanoDrop-1000<sup>®</sup> spectrophotometer to determine the RNA concentration and relative purity. cDNA was synthesised in a 10  $\mu$ L reaction containing 500 ng total RNA, 50 ng random hexamers and 100 U SuperScript<sup>TM</sup>-III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. A TaqMan real-time quantitative (q)RT-PCR test for GAV ([de la Vega et al., 2004](#)) was performed as described except that 2  $\mu$ L cDNA (equivalent to 100 ng total RNA) was used in a 20  $\mu$ L reaction prepared using TaqMan<sup>®</sup> Universal PCR Master Mix (Applied Biosystems) and 900 nM each PCR primer, from which 3 x 5  $\mu$ L aliquots were placed into 3 wells of a 384-well PCR plate as plate replicates. PCR was performed in an ABI Prism<sup>®</sup> 9700HT Sequence Detection System (Applied Biosystems) using the default thermal cycling conditions. To quantify GAV RNA copy numbers accurately, 10-fold dilution series of synthetic GAV RNA of known copy number were amplified in the same plates to generate linear regression plots of mean cycle threshold (Ct) values vs synthetic RNA copy number. Adjusting for the presence of cDNA prepared to 25 ng total RNA in each 5  $\mu$ L reaction aliquot analyzed, infection loads were expressed as viral RNA copies per ng total RNA.

### **Statistical analysis**

Survival of shrimp from the different treatments were analyzed at day 14 using the repeated measures ANOVA test ([PROC GLM; SAS Institute Software, 1999](#)). Pairwise comparisons were made using the Least Significant Difference test ([SAS Institute Software, 1999; Kotz and Johnson, 1982](#)).

### **Results**

### **Survival comparison**

In the survival comparison, shrimp fed Novacq™ had progressively higher survival rates from day 7 onwards when GAV challenged compared to shrimp not fed Novacq™ (Figure 1). At day 14 shrimp fed Novacq™ had 3.5% survival compared to 1% survival for GAV challenged shrimp that were not fed Novacq™ ( $P > 0.05$ ). Similarly shrimp fed Novacq™ had marginally higher survival rates when injected with saline and compared to their respective control from day 3 onwards (Figure 1). At day 14 shrimp that received saline injection and that were fed Novacq™ had 9% survival compared to 8% survival for shrimp that were not fed Novacq™ ( $P > 0.05$ ). Shrimp that received no injection showed the same survival rates throughout the duration of the bioassay irrespective of diet (Figure 1).

### **GAV load comparison**

RT-PCR was used to quantify the indirect impact of Novacq™ on GAV replication levels. A representative sub-set of the GAV load comparison shrimp that were either injected with saline or GAV inoculum were chosen from each diet treatment for GAV load quantification. The process for selecting these shrimp were as follows; Initially the number of shrimp for each of the respective treatments that were alive at day 7 but not beyond, alive at day 10 but not beyond and alive at day 14 were counted (Table 2). A random sample of shrimp were then selected for GAV quantification with more emphasis on selecting shrimp that had died during this experimental period, resulting in selection of 90 pleopod samples from a total of 22 shrimp (Table 1). It is worth noting that caution must be taken when interpreting GAV load data from surviving animals in a bioassay challenge trial as you are assaying the survivors only.

In the GAV load comparison GAV loads were on average marginally lower for the individuals assayed throughout the experimental bioassay in the saline injection comparison when fed Novacq™ (Figure 2 Ai and Bi). Although less pronounced, GAV loads were also marginally lower for the individuals assayed throughout the experimental bioassay in the GAV challenge comparison when fed Novacq™ (Figure 2 Aii and Bii).

### **Discussion**

Previous studies have demonstrated that diets containing the novel aquafeed ingredient Novacq™ result in significantly improved growth rates of G8 selected and unselected lines of *Penaeus monodon* (Glencross et al., 2012a). Shrimp in this same study were observed to have an overall heightened level of 'fitness' when fed Novacq™. This paper is a first step toward quantifying the antiviral 'fitness' afforded to shrimp by feeding Novacq™, which was achieved by performing Gill-Associated virus (GAV) bioassays on unselected lines of *P. monodon* shrimp.

### **Survival comparison**

The heightened level of antiviral 'fitness' afforded to shrimp by feeding Novacq™ is evident by comparison of the survival performance of Novacq™ fed shrimp. Inclusion of Novacq™ in the diet resulted in improved shrimp survivals when exposed to the stress of saline injection only and when injection stress was combined with GAV challenge. This improved 'fitness' or ability to survive as a result of stress and viral exposure opens up an opportunity for commercial enterprises to be rearing shrimp that are more resilient to environmental extremes (stress) such as extended periods of rain or hot weather, and viral outbreaks within ponds.

### **GAV load comparison**

A heightened level of antiviral ‘fitness’ afforded to shrimp by feeding Novacq™ is also inferred from the GAV load comparison study. GAV loads of Novacq™ fed shrimp were on average lower in individual shrimp over the duration of the experimental bioassay when they received the stress of a saline injection only. It has been previously demonstrated that handling stress such as that caused by inserting a needle into *P. monodon* can result in heightened GAV loads of unselected lines (de la Vega et al., 2004). Although less pronounced, the same inferred ‘fitness’ measured as generally lower GAV loads in individuals throughout the duration of the experimental bioassay, appears amongst Novacq™ fed shrimp that received both the handling stress of injection and the GAV challenge.

The ability of numerous novel aquafeed ingredients to confer heightened ‘fitness’ in shrimp after challenge with viral pathogens have been reported in the literature. As one example, recently the herbal extract Nomex® was reported to prevent White Spot Syndrome virus infection in the Pacific White shrimp, *Litopenaeus vannamei* (Dr Dilek Yerlikaya, Soley Biotechnology Institute, USA). However, when compared to Novacq™ there are no other compounds reported in the literature that have the combined antiviral, improved growth rate and ability to provide an alternative to fish meal in the literature.

In summary, survival and GAV load data in this study confirm that Novacq™ improves resilience against viral infection and mortality in Black Tiger shrimp, *Penaeus monodon*. It is clear that Novacq™ is improving the general ‘fitness’ of shrimp and thus acting as an antiviral. Future work would benefit from further understanding how Novacq™ stimulates the immune response system in shrimp whilst also comparing the antiviral fitness in genetically selected and unselected lines. Studies are currently underway to assess the antiviral nature of Novacq™ for *L. vannamei* when challenged with WSSV. Finally, Novacq™ is unlike any other known aquafeed ingredient; providing improved shrimp growth, an alternative to fish meal and, as this study shows, resistance to viral pathogens.

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Table 1. Diet formulations and composition as analyzed (% as used).

	Control	Treatment
<i>Ingredients</i>		
Fish meal	50.00%	50.00%
Wheat gluten	5.00%	5.00%
Wheat flour	41.23%	31.23%
Lecithin (soy)	1.00%	1.00%
Fish Oil	2.30%	2.30%
Novacq		10.00%
Carophyll Pink	0.05%	0.05%
Cholesterol	0.10%	0.10%
Banox E	0.02%	0.02%
Vitamin C (Stay C)	0.10%	0.10%
Vitamin premix	0.20%	0.20%
<b>TOTAL</b>	<b>100.00%</b>	<b>100.00%</b>
<i>Diet Composition</i>		
Dry matter	95.2%	94.7%
Protein	45.0%	42.7%
Lipid	7.2%	6.8%
Ash	9.7%	16.5%
Carbohydrates	33.3%	28.7%
Gross Energy	19.5	17.6

Table 2. Numbers of shrimp selected for GAV quantification from the GAV load comparison.

	Number of shrimp alive at day:			Number of shrimp chosen for GAV quantification at day:			Total number of pleopods to quantify for GAV load at day:		
	7 but not beyond	10 but not beyond	14	7 but not beyond	10 but not beyond	14	7 but not beyond	10 but not beyond	14
Control Diet saline injection	2	2	11	2	2	2	6	8	10
Control Diet GAV challenge injection	1	4	2	1	3	2	3	12	10
Treatment Diet saline injection	1	1	17	1	1	2	3	4	10
Treatment Diet GAV challenge injection	2	8	2	2	2	2	6	8	10

## Figure Legends

**Figure 1.** Mean numbers ( $\pm$  S.D.) of *Penaeus monodon* shrimp that remained alive over the 14 d experimental bioassay for the two survival comparison tank replicates of each treatment group. Lines with different superscripts are significantly different at 14 d ( $P > 0.05$ ). Each line represents an individual shrimp.

**Figure 2.** Real-time RT-PCR quantification of Gill-Associated virus (GAV) loads in pleopods of *Penaeus monodon* from the GAV load comparison replicate tanks fed A) Control diet or B) Treatment diet with 10% Novacq<sup>TM</sup> and injected with i) saline or ii) GAV challenge injection. Each line represents an individual shrimp over time from which multiple pleopod samples have been taken.

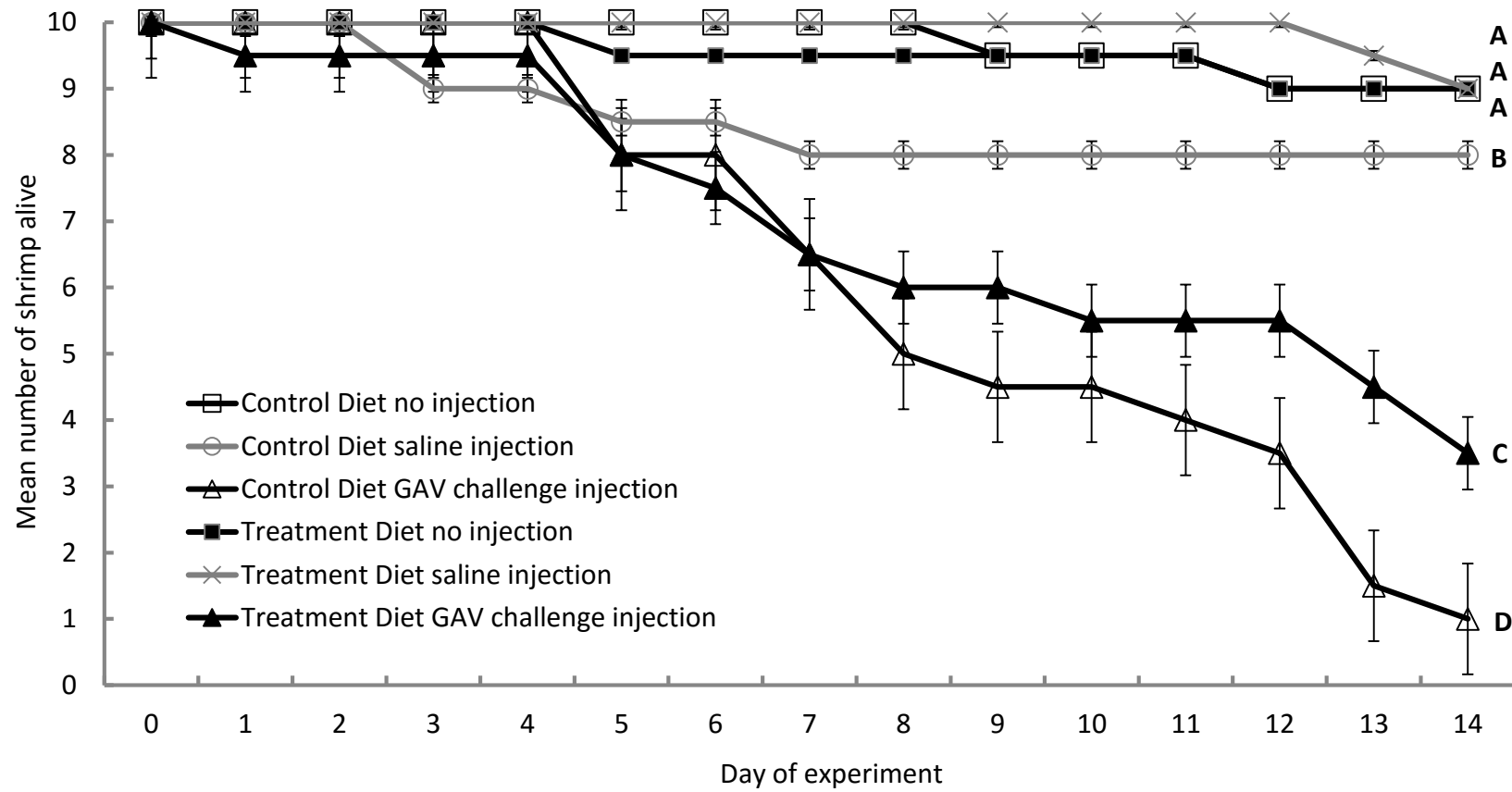
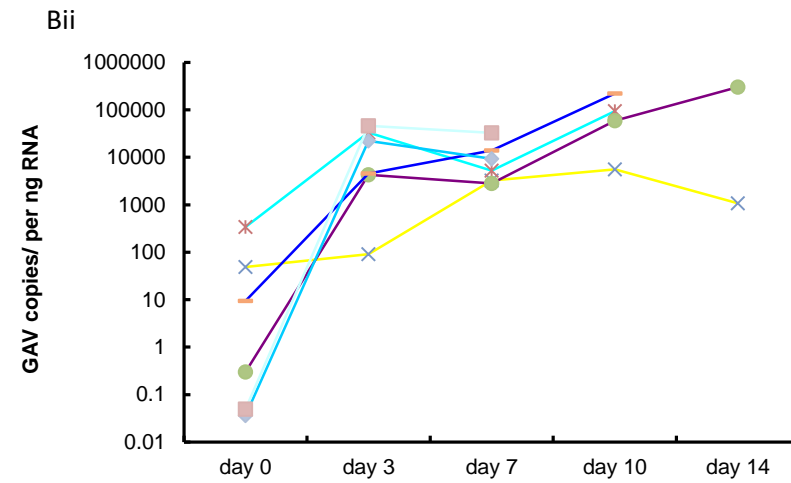
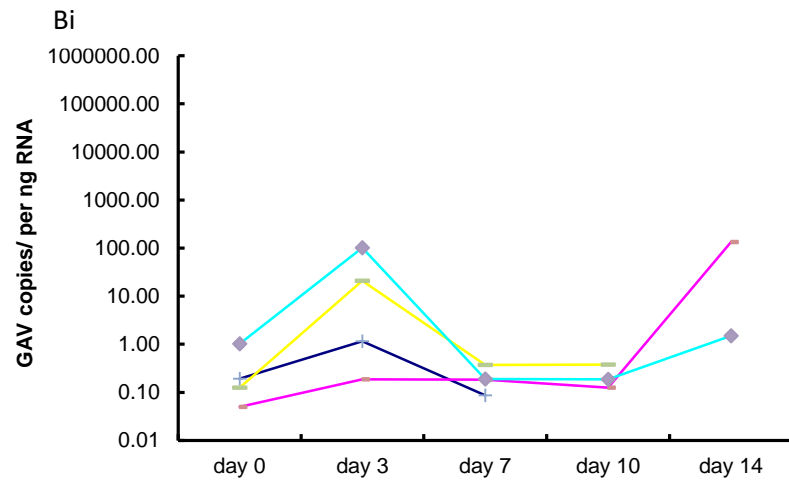
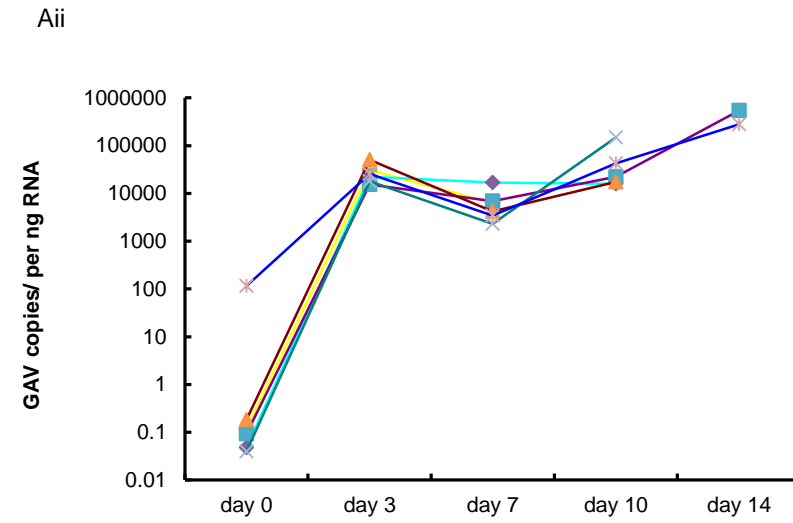
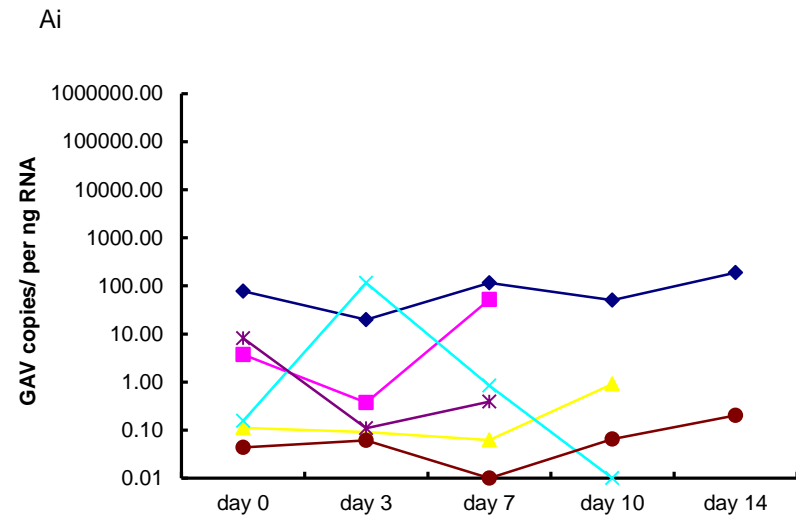


Figure 1.



**Figure 2**

