Selective breeding for tolerance to gill-associated virus in the black tiger shrimp, *Penaeus monodon*

Thesis submitted by

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Statement of the Contribution of Others

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Abstract

Shrimp farming has expanded dramatically to now be the second most valuable aquaculture industry globally. However, with the expansion of shrimp farming has come the spectre of serious diseases that some estimate wipe out up to 40% of total production. With limited tools available for managing disease, the shrimp industry has employed three strategies to lower the disease risks, including use of specific pathogen free (SPF) shrimp, breeding disease resistant/tolerant shrimp, and using best management practices to reduce stress on animals. The use of selective breeding to improve disease resistance has been in some instances an effective strategy for reducing the risk of aquatic diseases. In the context of shrimp breeding programs, sib-selection is commonly practiced where progeny are evaluated for tolerance using controlled disease challenge tests, family breeding values are estimated, and siblings from the highest ranked families are selected to breed from. However, there is no standard protocol for disease challenge tests resulting in a number of methods and designs being used. Consequently, the accuracy of disease challenge tests for shrimp selective breeding programs are poorly understood.

In Australia, the shrimp farming industry is almost entirely based on the use of wild caught *Penaeus monodon* broodstock to supply seed. Therefore, domesticated and selectively bred stocks including SPF stocks are currently unavailable. Development of domesticated and selectively bred stocks are of huge interest to the local industry with several breeding programs under development. At the time of this PhD research, the most problematic disease affecting the local industry was caused by gill-associated virus (GAV), which is a highly prevalent virus in both wild and farmed stocks that can cause significant production loss. Selective breeding techniques may be useful in managing disease associated with GAV. This research aimed to address the current knowledge gaps specifically related to establishing a reliable method for measuring GAV disease responses (through survival and viral load) and to elucidate the underlying genetic basis of GAV disease tolerance traits and how they are linked to commercial production traits.

One of the difficulties of shrimp disease challenge tests is establishing a standardised and repeatable method that allows for accurate genetic parameter estimation. Various pathogen infection methods have been used to establish disease, including feeding, injection and waterborne methods. Each method has its own advantages and disadvantages. To investigate the utility of each of these challenge methods for establishing a reliable GAV challenge protocol, groups of juvenile *P. monodon* (2 – 10 g) were challenged using either intramuscular injection of a weight-standardised dose of GAV inoculum, feeding of both fresh and frozen GAV-infected shrimp meat, and immersion in water containing the GAV inoculum. The three groups were compared based on mortality and GAV infection load measured in the survivors using a reverse transcription qPCR (RT-qPCR) assay. Results demonstrated that immersion of shrimp for 2 h in GAV contaminated water resulted in no mortalities over the 11 day challenge period and furthermore RT-qPCR identified no evidence of these shrimp becoming infected. Intramuscular injection resulted in the quickest mortality rate, with cumulative mortality surpassing 50% on day 7 post-challenge (p.c.), while feeding of GAV infected shrimp meat resulted in cumulative mortality of ~50% on day 11 p.c. Results from the RT-qPCR analysis revealed the survivors of the injection group had approximately 100-fold higher and more consistent GAV infection loads compared to the group fed infected shrimp meat. Mean GAV infection loads (after $\log_{10}$ transformation) of the injection group survivors was 7.73 ± 0.49 GAV copies μg^{-1} TNA and for the groups fed fresh and frozen infected shrimp meat, GAV infection loads were 5.71 ± 1.97 and 4.77 ± 1.90 GAV copies μg^{-1} TNA, respectively. The coefficient of variation (CV) among individual GAV infection loads of shrimp injected with GAV was much lower (CV = 0.06) compared to the survivors of the feeding groups (CV = 0.41 & 0.33). These results suggest, of the three challenge methods assessed for their suitability in establishing a standardised GAV challenge protocol, injection provided the most uniform and reliable means of infecting shrimp and consequently was the preferred method of infection to use for subsequent GAV challenge tests.

Virus detection and quantification of infection load are important measures for managing disease in shrimp farming, for understanding disease responses and potentially as a useful indirect measure of disease tolerance for selective breeding. Understanding how GAV infection loads vary between and

within commonly sampled tissues (pleopod and gill filaments) is necessary in order to generate accurate phenotypic measures. Using RT-qPCR methods to quantify GAV infection loads, two groups of juvenile *P. monodon* naturally infected with GAV were examined. Gill-associated virus infection loads were found to vary considerably within the same tissue type (both within pleopod and gill tissue) collected from the same individual shrimp by up to ~3000-fold. However, there was no significant difference in the sensitivity of either pleopod or gill tissue in either group examined ($P > 0.05$), or similarly, there was no difference in the coefficient of variation (i.e. variability) in GAV loads among individual gill filaments or pleopods in either group ($P > 0.05$). The results from this research indicate no difference between gill or pleopod tissue as more or less suitable for generating data on GAV infections. What was found to be critical was sampling of more than one gill filament or pleopod given the large within-tissue variability observed to provide more accurate data on GAV presence and relative infection loads. Consequently, a minimum of three gill filaments were used in subsequent experiments when generating data on GAV infections.

The next steps in this research were to assess whether the GAV challenge methodologies established would be sensitive enough to differentiate shrimp families based on their survivorship and/or GAV infection loads. First, a suitable dose of the GAV inoculum needed to be identified using a series of titration experiments. In these experiments different dilutions of the inoculum were injected in groups of shrimp and their mortality was tracked over a defined period (~ 14 days). The dilutions assessed across three experiments ranged from undiluted to ~ 1:80,000 dilution. Based on the results of three titration experiments a dilution of 1:3000 was chosen as this dose consistently resulted in ~50% mortality by day 14 p.c., which should allow for differentiation of families. This dilution was then applied to a small number of shrimp families to determine whether the challenge methodology and dose were sensitive enough to differentiate family-based tolerances. Following traditional designs, where families are reared in separate tanks in order to easily track pedigrees, seven shrimp families were bred and kept in separate tanks throughout their rearing and subsequent challenge tests. Shrimp from each family were challenged via injection of the GAV inoculum diluted 1:3000. Survival of the seven families was tracked over 35 days and GAV infection loads were quantified from the survivors

of each family. Overall survival at the termination of the challenge was 44%, with survival among families ranging from 22 – 72%. Using Cox proportional hazards mixed models, genetic (family) and non-genetic effects (rearing and challenge tanks) on survival were analysed. The results from these models revealed significant variability due to genetic effects (family), but also considerable variability due to separate rearing and challenge tanks. For example, survival among groups from shrimp from the same family and reared in the same tank varied from 0 – 100% between replicate challenge tanks. This level of non-genetic variability could easily mask genetic effects. Gill-associated virus infections were observed in 46% of the challenge survivors and prevalence varied between families from 0 to 100%. Mean GAV infection loads among families with GAV present ranged from 3.77×10^2 to 2.49×10^7 GAV copies μg^{-1} TNA. It is important to note that shrimp used in this experiment were also heavily infected with another endemic virus IHHNV, which may have interfered with the GAV infection response. This study provides the first evidence of family differences in GAV induced mortality, but also highlights the importance of non-genetic factors such as separate rearing and challenge tanks that can greatly impact the observed performance of shrimp during disease challenge tests.

Before GAV disease tolerance can be incorporated as a trait in a selective breeding program, knowledge of the underlying genetic basis needs to be established. Large numbers of families are needed to accurately estimate key genetic parameters such as heritability and genetic correlations between traits in order to predict genetic gains and optimise the breeding program design. Given the significant variability observed due to non-genetic factors like separate family rearing and challenge tanks from previous experiments, a new approach to shrimp disease challenge tests needed to be implemented. Therefore, in this experiment pools of families that were spawned and reared under a common environment were used to estimate genetic parameters of GAV disease tolerance in *P. monodon*. Shrimp were challenged via injection of the same GAV inoculum used previously diluted at 1:3000 and individual dosage (inoculum volume) was standardised for body weight. Mortality and genetic pedigree data were collected from 1717 shrimp made up of full (n = 72) and half-sib (maternal n = 42, paternal n = 30) families. Gill-associated virus load was measured on the challenge survivors

(n = 963) to determine its utility as an indirect measure of tolerance. Variance components were estimated for mortality using a binomial animal model (mortality as a binary trait) and Cox's proportional hazards animal model (mortality as a longitudinal trait incorporating time until death data), while GAV load was analysed using a linear animal model. Overall mortality at the end of the challenge test was 35.5%, but ranged from 0 to 71% among families with 10 or more offspring. Heritability (h^2) estimates for mortality were $h^2 = 0.11 \pm 0.03$ using the binomial model and $h^2 = 0.14$ using the Cox's model. In addition, family rankings using estimated breeding values (EBV) did not differ between the two models ($r_{EBV} = 0.99$). Heritability for viral load was $h^2 = 0.21 \pm 0.07$, however, genetic correlations and correlations of family EBVs between mortality and GAV load were weak ($r_g = 0.30 \pm 0.23$ and $r_{EBV} = 0.17^{ns}$), suggesting GAV load may not be a good indirect measure of GAV induced mortality, at least in the way the data was collected in this experiment (i.e. on survivors only). Overall, the results from this experiment demonstrate for the first time that a pooled family design can be used to estimate significant genetic variation of GAV disease tolerance among *P. monodon* families and that this trait could be improved through targeted selective breeding.

When incorporating a trait for selection it is important to understand how the trait is genetically correlated with other traits of economic importance. Therefore, this study utilised siblings of those evaluated for GAV tolerance measured using controlled challenge tests to estimate genetic parameters for three commercial production traits, GAV infection prevalence (GAV infection status), GAV infection load (GAV load) and body weight (BW), and to assess the correlation between the commercial production traits and disease tolerance traits measured under controlled challenge conditions. To do this, 1835 shrimp were sampled from two replicate commercial ponds and their pedigrees determined via genotyping and parentage analyses. The total number of full-sib families identified among those sampled was 80, which included 55 maternal half-sib and 30 paternal half-sib families. Data on GAV infection traits were determined using RT-qPCR from 913 shrimp tested. The overall prevalence of GAV infection was 45.5% and the mean GAV load of those that were positive (n = 415) was 3.11 ± 1.42 $\log_{10}$ GAV copies μg^{-1} TNA. The mean BW of shrimp sampled from the two ponds was 15.88 ± 3.68 g, but differed significantly between the two ponds and between male

and female shrimp (i.e. males were smaller). Heritability estimates for each of the three traits were assessed using mixed animal models with GAV infection status (infected versus not infected) analysed as a binary trait using a binomial model and GAV load (after $\log_{10}$ transformation) and BW analysed using linear models. Heritability estimates for each trait were; GAV infection status h^2 = 0.06 ± 0.03, GAV load h^2 = 0.21 ± 0.10 and BW h^2 = 0.38 ± 0.07. Genetic correlations (using bivariate models and Pearson's correlations of family EBVs) among the commercial production traits were all positive but ranged from low to high. Correlations between the GAV infection status and GAV load were moderate to high (r_g = 0.90 ± 0.24 and r_{EBV} = 0.36), meaning selection for low GAV load would likely lead to lower GAV infection prevalence as well. Correlations between the two GAV infection traits and BW were also positive but were weak, with GAV infection vs BW r_g = 0.36 ± 0.26 and r_{EBV} 0.10 and GAV load vs BW r_g = 0.26 ± 0.25 and r_{EBV} 0.13. Given the low correlations and high standard errors between these traits, suggests that selecting for increased body weight would unlikely have a significant impact on GAV infections. Of critical importance, however, were correlations between GAV tolerance traits measured under controlled challenge conditions and the commercial productions traits evaluated in this study. Using Pearson's correlations of family EBVs for each trait, GAV induced mortality and GAV infection load from challenged shrimp survivors were not significantly correlated with either GAV infection status or GAV load from pond reared shrimp (-0.06 $\leq$ r_{EBV} $\geq$ -0.27). These results suggest that viral infection data measured from pond reared shrimp may not be a good indicator of GAV tolerance measured as mortality under controlled challenge tests. However, there was no GAV related disease outbreak that occurred in the ponds evaluated. Correlations between the challenge test traits and body weight were also not significant (0.06 $\leq$ r_{EBV} $\geq$ 0.10., The lack of significant correlations between GAV tolerance traits measured under controlled challenge conditions and commercial productions traits would suggest that selecting for GAV tolerant shrimp would not lead to correlated responses in the commercial production traits assessed here.

In summary, this PhD research has unveiled several potential inaccuracies in the way disease tolerance has been evaluated using traditional challenge designs. It also fills several knowledge gaps

of genetic parameter estimates of GAV tolerance in *P. monodon* that are necessary to known prior to incorporating this trait in a selective breeding program. This research has shown for the first time that a communal pooled family rearing approach to shrimp disease challenge protocols can be used successfully and may improve the accuracy of genetic estimates by eliminating non-genetic effects caused by separate family tanks. Furthermore, this research has clearly demonstrated significant genetic variation exists for GAV tolerance and that selection of this trait should have little to no impact on other important traits such as body weight at harvest. Still remaining, however, is to develop a better understanding of the genetic correlation between disease tolerance measured under controlled challenge conditions and tolerance measured under field conditions when exposed to significant levels of GAV.

Table of Contents

List of Tables

List of Figures

Chapter 1. General introduction

Aquaculture is the fastest growing food producing sector globally (FAO, 2016). Aquaculture now contributes to 45% of all seafood produced and in 2014, aquaculture reached a milestone with just over half of seafood consumed by humans coming from farmed sources as opposed to wild fisheries (FAO, 2016). However, if aquaculture is to continue to grow and contribute to global food security, it will need to overcome several hurdles including that of disease and lack of genetically improved stocks, among others (e.g. improved nutrition and farming systems).

Disease is recognised as the biggest threat to sustaining the growth of aquaculture production worldwide. Disease costs the industry at least US$6 billion annually (The World Bank, 2014) and for shrimp farming, it is estimated up to 40% of production is lost to disease each year (Stentiford et al., 2012). Disease affects the aquaculture industry by causing losses through mortality, slowed or impaired growth, poor feed conversion, degradation of the product quality and costs associated with treatment and control measures (Elaswad and Dunham, 2017). Clearly, reducing the impact of disease on these losses will help increase overall production efficiency.

Less than 10% of aquaculture production is based on genetically improved stocks (Gjedrem et al., 2012). Therefore, most aquaculture production comes from wild caught, or unimproved broodstock to produce seed for farming (Olesen et al., 2015). The small fraction of genetically improved stocks stem only from a few species in which there are large-scale domestication and selective breeding programs, including Atlantic salmon (*Salmo salar*), Nile tilapia (*Oreochromis niloticus*) and Pacific white shrimp (*Penaeus vannamei*). For these species, genetic improvement through selective breeding has substantially increased farming efficiencies and thus overall production (Gjedrem et al., 2012).

1.1 Shrimp aquaculture and breeding programs

Penaeid shrimp are the second most valuable aquaculture industry globally, valued at over US$22 billion (FAO, 2016). Most shrimp farming takes place in two major regions, Asia and the Americas,

with countries such as China, Thailand, Vietnam, Indonesia and Ecuador some of the main producers (Jory, 2018). There are several species of shrimp farmed, however, the main two species are *P. vannamei* and *P. monodon* (black tiger shrimp). These two species differ in terms of their native range and level of domestication in commercial stocks. The native range of *P. vannamei* extends along the eastern Pacific coast of the Americas, from Mexico through Central and South America, whereas *P. monodon* is found throughout the Indo-West Pacific, including northern Australia (Briggs et al., 2005). In the late 1990's, *P. vannamei* was introduced to Asia for commercial aquaculture purposes and is now farmed throughout the region (Briggs et al., 2005). Prior to 2003, *P. monodon* was the dominant species farmed, but was surpassed by *P. vannamei* following its introduction into Asia (FAO Fishstat, 2016). The introduction of *P. vannamei* into Asia and subsequent rapid expansion of production was, in large part, due to the availability of domesticated-selectively bred stocks (Briggs et al., 2005). Comparatively, *P. vannamei* has been relatively easier to domesticate, particularly at commercial scale, compared to *P. monodon* due to key biological characteristics (Briggs et al., 2005). These include *P. monodon* being a larger, later sexually maturing species which likely reduces the amenability of this species when rearing through to reproductive maturity (Briggs et al., 2005). They also are more aggressive and cannibalistic than *P. vannamei*. Additionally, *P. monodon* have a closed thelycum. This means insemination of females can only be performed for a short period soon after moulting before the exoskeleton hardens (Coman and Henshall, 2010). Consequently, nearly all *P. monodon* production in Australia, at least, relies on wild caught broodstock to produce seed for stocking into production systems (Coman et al., 2007).

The use of wild stocks has several disadvantages to the shrimp farming industry, including the potential for disease introduction both through horizontal and vertical transmission, reliance on availability of wild stocks for seed supply, variability in larvae quality and performance, inability for genetic improvement and lack of control over pond stocking (Cock et al., 2009; Moss et al., 2012). For *P. vannamei*, in which large-scale domesticated and selectively bred stocks are available, the initiation of such breeding programs was largely in response to the impact of disease (Lightner, 2005). Initial breeding programs focused on domestication as a means to control the disease status of

breeding populations through pathogen screening and comprehensive biosecurity systems to produce specific pathogen free (SPF) stocks (Moss et al., 2003b). Following this, breeding programs began to investigate and incorporate commercially important traits into selection such as growth and survival (either general survival, or disease specific survival) (Moss and Moss, 2009).

Shrimp breeding programs vary in their design and unfortunately detailed information on how commercial breeding programs are conducted is often unavailable. Breeding programs can vary from mass selection on farm to family-based programs utilising Nucleus Breeding Centres (NBC). Most *P. vannamei* breeding programs nowadays are based on full- and half-sib family designs maintained within an NBC (Moss and Moss, 2009). Pedigrees are commonly maintained by spawning and rearing full-sib families in separate tanks until they are large enough to be physically tagged before being communally reared (Moss and Moss, 2009). Whilst this method allows families to be easily tracked it has several disadvantages, such as the use of separate tanks may introduce slight environmental differences between each family (Gjedrem, 2005). These slight differences in environment are called tank effects and can be a source of non-genetic variability on the expression of the observed phenotype and can potentially mask genetic variation. In salmon, for example, tank effects were estimated to account for 6.5% of the variability of tolerance to infectious pancreatic necrosis virus (Kjøglum et al., 2005). Variability of this magnitude may be substantial enough to mask genetic effects if the additive genetic component is small, such as for many complex traits like disease tolerance. The presence of additional non-genetic variance due to tank effects may reduce the ability to measure genetic differences between families and thus reduce the accuracy of selection (Gjedrem, 2005).

On top of potentially introducing tank effects, separate family rearing requires a large amount of tank infrastructure to keep large numbers of families separate (Henshall et al., 2014). Physical tagging of a large number of small shrimp to estimate genetic parameters of some traits (e.g. to assess pond performance traits) may also be impractical (Jerry et al., 2006). Alternatively, pedigrees can be established using DNA markers, which would allow families to be reared communally avoiding potential tank effects and allow tracking of larger numbers of individuals (Jerry et al., 2006; Henshall

et al., 2014). Use of DNA markers and genotyping for pedigree information reduces the constraints identified above, but also has its own limitations, such as family contributions within cohorts are not easily controlled resulting in skewing of family representation (Jerry et al., 2006). Genotyping may also be cost prohibitive, although the price of genetic resources are falling rapidly, making them more available to apply in aquaculture species. Skewed family contributions may result in some families being 'lost' throughout the production period and may exacerbate the cost of genotyping by increasing the number of individuals needed to be sampled in order to capture the majority of families and thus genetic diversity within the population (Jerry et al., 2006; Domingos et al., 2014).

1.2 Managing disease in shrimp farming

The Global Aquaculture Alliance survey of shrimp farming in 2016 identified disease to be the top challenge faced by the industry worldwide (Anderson et al., 2017). There are many viruses, bacteria and other microbial organisms that affect shrimp farming and of these the most significant are listed by the World Animal Health Organisation (the OIE). Those listed include acute hepatopancreatic necrosis disease (AHPND), infectious hypodermal and haematopoetic necrosis virus (IHHNV), Taura syndrome virus (TSV), white spot syndrome virus (WSSV) and yellow head virus (YHV) (OIE, 2018). These pathogens have all had disastrous impacts on shrimp farming. For example, white spot syndrome virus (WSSV) has cost the shrimp aquaculture industry an estimated US$8 billion across Asia and the Americas since 1992 when it was first identified, although some estimate the losses to be far greater than this, up to US$15 billion (Lightner et al., 2012). More recently, the bacterial derived disease AHPND emerged in 2010 in China and spread into other major shrimp farming countries such as Thailand and Vietnam (Boonyawiwat et al., 2017). This disease results in rapid mortality within the early phase of production up to 100% and has resulted in over US$1 billion in production losses just over a few years, up until 2012 (The World Bank, 2014). Most of these diseases are still present and continue to be major problems to the global shrimp farming.

Unlike fish or terrestrial animals, shrimp lack an adaptive immune system (Vazquez et al., 2009). This means managing disease through traditional prevention strategies like vaccines are not possible. For shrimp, the innate immune system is their major defence against pathogen entry and disease progression (Song and Li, 2014). Innate defence systems in shrimp include physical barriers (e.g. chitin based exoskeleton of prawns), as well as humoral and cellular defence systems (Bachère, 2000; Amparyup et al., 2013; Song and Li, 2014). Defence systems are activated by pattern recognition receptors (PRPs) (e.g. Toll-like receptors), that recognise and bind to generic pathogen-associated molecular patterns (PAMPs) (Wang et al., 2014). Both the humoral and cellular responses work together in shrimp innate immunity to recognise and sequester pathogen invasions (Rusaini and Owens, 2010). This results in defence responses such as clotting, antimicrobial peptide formation and activation of the prophenoloxidase (proPO) system (Flegel and Sritunyalucksana, 2011), leading to melanisation, phagocytosis, apoptosis, encapsulation and nodule formation (Flegel and Sritunyalucksana, 2011). There is also a growing body of evidence that crustaceans possess other more advanced cellular mechanisms such as gene silencing through RNA interference for antiviral immunity (see review by Escobedo-Bonilla, 2013) and a type of "immune memory" or "pathogen priming" mechanism that is stimulated from past exposure to a pathogen, although it is not clear whether this is a general or specific response (Flegel and Sritunyalucksana, 2011).

Currently, there are no commercially relevant preventative or treatment tools for shrimp viruses, or no effective antibiotic or probiotic treatment for the bacterially derived pathogens (M. Briggs personal communication, 2016). The only effective management practices currently available on a large scale for shrimp farming is to use SPF stocks, breed disease resistant/tolerant stocks and use best management practices to reduce stress on the animals (Lightner, 2005; Cock et al., 2009; Moss et al., 2012).

The development and implementation of SPF stocks has been fundamental in the shrimp farming industry as a biosecurity measure to manage the occurrence and spread of disease (Moss et al., 2012). Specific pathogen free refers to shrimp that are tested and certified free from a specified set of pathogens (Moss et al., 2003a). It is important to note that there is no universal SPF list and lists

change with new pathogens being identified and new diagnostic tools developed (Moss et al., 2003a). Specific pathogen free status also only refers to the present health status of the shrimp, which are still naïve to infection, particularly when placed in less biosecure facilities like farm ponds. There is also some evidence that SPF stocks exhibit inferior survival compared to wild seed in the presence of certain diseases (Moss et al., 2001). Furthermore, SPF stocks are not widely available on a large-scale for many shrimp species other than *P. vannamei*, including for *P. monodon*. As an additional, or alternative disease management strategy, breeding shrimp that are more resistant or tolerant to certain diseases may be an effective option.

1.3 Selective breeding for disease tolerance in shrimp

Disease tolerance and disease resistance are both terms used to describe a hosts' defence mechanisms that allow it to survive a pathogen invasion (Robinson et al., 2017). Disease resistance refers to an animals' ability to resist infection or reproduction of the pathogen, whereas tolerance is defined as the ability to limit the impact of disease following pathogen infection (Doeschl-Wilson et al., 2012). Disease resistance is typically measured as the pathogen burden, such as viral or bacterial load, whereas tolerance may be measured as the impact on performance at a given pathogen load such as survival or growth (Doeschl-Wilson et al., 2012). In practise, resistance and tolerance may be difficult to disentangle and often what is observed is the sum of both mechanisms (Robinson et al., 2017). Thus, from here on the term "disease tolerance" will be used generically to describe the response to pathogen infection.

Measuring and selecting for disease tolerance in many aquaculture species in most commonly carried out using pathogen specific challenge tests (Ødegård et al., 2011a). Under controlled conditions, challenge tests aim to minimise environmental variation and maximise the ability for genetic variation to be expressed and measured (Gitterle et al., 2006a). Factors such as individuals being equally at risk of infection, uniform infection dose and consistent time of exposure, are important to achieve results that are repeatable and allow accurate estimates of the genetic effects. Survival is typically the trait

that is measured, either at a specific time point, for example when 50% of the individuals have died, or when until mortalities cease (Fjalestad et al., 1993; Ødegård et al., 2011a).

Although challenge tests are most often used for selecting for disease tolerance, there is no universal challenge protocol applied, even within a pathogen or shrimp species. Consequently various infection methods, shrimp life stages, shrimp sizes and systems are used. These differences can affect the response to infection and lead to changes in family performances depending on the methods used. For example, significant re-ranking of family tolerances was demonstrated between infection to WSSV via individual oral inoculation and infection by waterborne virus (Gitterle et al., 2006a). Deciding on the most suitable method of exposure will depend on the pathogen and species under evaluation, and the capacity of the experimental challenge laboratory. In each case, it will likely require testing and validating various challenge protocols to ensure that the infection mechanism is reliable and repeatable.

A downside to the use of challenge tests for disease tolerance selection is that the individuals that are challenged, even if they survive, are generally excluded as breeding candidates (Robinson et al., 2017). Therefore, selection is made on unevaluated relatives using family based selection methods (sib-selection) (Cock et al., 2009). Using this strategy both the accuracy and intensity of selection is reduced as only half the genetic variation is being utilised (between family variance) and the true genetic merit of the breeding candidates is unknown (Ødegård et al., 2011a). However, more advanced methods such as marker assisted selection and genomic selection are helping to overcome this hurdle through incorporating molecular information where individuals are selected based on their individual genetic merit (Ødegård et al., 2011a).

The use of disease challenge tests to measure and select disease tolerant shrimp families will only be useful if it correlates with disease tolerance when exposed to the pathogen under commercial conditions (i.e. during grow out in farm ponds) (Ødegård et al., 2006). To date, there is no research that has comprehensively evaluated whether survival during disease challenge tests is an accurate predictor of survival in farm ponds for nearly all shrimp pathogens. Moss et al. (2005) evaluated

survival during TSV challenge tests and from a farm pond where TSV was present using phenotypic correlations, which were moderate and positive (r_p = 0.55 and 0.68). However, phenotypic correlations are the sum of both genetic and environmental components, thus, genetic correlations are required to get a better understanding of the utility of challenge tests to predict disease tolerance on farm. Studies in Atlantic salmon (*Salmo salar*) indicate a high genetic correlation (r_g = 0.71 – 0.95) between challenge and field survival following furunculosis challenge and an outbreak in the field (Gjøen et al., 1997; Ødegård et al., 2006). In oysters (*Crassostrea gigas*) genetic correlations between survival following exposure to oyster herpes virus in controlled challenge tests and in the field were slightly lower, r_g = 0.61 – 0.71, but still positive and of moderate magnitude (Kube et al., 2015). Correlations between challenge and field disease tolerance may begin to break down due to a number of reasons, such as the methods used during disease challenge tests may not always be representative of what occurs during natural disease outbreaks in the field. Also, laboratory conditions are considered 'sterile', as environmental parameters are normally highly controlled and much of the microbiota found in farming systems are removed. These factors may bring into question the transferability of challenge test results to the field. In addition, disease occurrence and progression in farming systems are uncontrolled which can also reduce the accuracy of estimated genetic variance and thus genetic correlations. Nevertheless, in general, challenge tests are likely to be more suitable for evaluating and selecting for disease tolerance as non-genetic factors are more easily controlled and the breeder is not having to rely on a disease outbreak to occur in the farming system.

1.4 Examples of selective breeding for disease tolerance in shrimp

The first reported study on breeding for disease tolerance in shrimp was in *P. vannamei* in response to the Taura Syndrome epizootic in the 1990's in Ecuador (Argue et al., 2002; Moss and Moss, 2009). On-farm mortalities of TSV were high and ranged from 40 – 90% (Lightner, 2003). Initially breeding programs started out using a mass selection approach by collecting the survivors from ponds and breeding from these individuals (Cock et al., 2009). Later, tolerance to TSV was incorporated into

already established domesticated and selectively bred (for growth) SPF *P. vannamei* stocks using a family selection approach (White et al., 2002; Cock et al., 2009; Moss et al., 2013). Initial controlled challenge tests for TSV indicated that there was high phenotypic variation for survival to TSV challenge among families, ranging from 15 – 94% (Argue et al., 2002). Heritability estimates were moderate ($h^2 = 0.19 – 0.41$) and significant genetic gains have been realised to the point where some families exhibit 100% survival following TSV challenge (Argue et al., 2002; White et al., 2002; Ødegård et al., 2011b; Moss et al., 2013). As a result, TSV is no longer considered a major threat to the industry.

In contrast to TSV, attempts at breeding WSSV tolerant stocks have proven less successful. Mortality from WSSV infection generally reach close to 100% in most cases where outbreaks occur and during specific challenge tests (Cock et al., 2009; Huang et al., 2011). Evaluations under challenge test conditions have found heritability estimates are much lower than those observed for TSV, $h^2 < 0.1$ (Gitterle et al., 2005b, 2006a, 2006b; Hayes et al., 2010; Huang et al., 2011). There does not appear to be any shrimp stocks, publically known at least, that have evolved useful WSSV tolerance and the virus remains one of the most damaging to production worldwide.

1.5 Breeding for disease tolerance in the presence of other traits

The breeding goal for most shrimp selection programs will include growth (or body weight). This is because growth is very important economically, as improving growth could result in increasing product size and thus value at harvest and/or by shortening the length of production cycles, both leading to greater profitability for the farmer (Moss and Moss, 2009). Growth is also very simple and cheap to measure phenotypically, which is commonly captured as body weight at a specific time, such as at harvest (Kenway et al., 2006). If the goal is to improve disease tolerance in addition to other commercially important traits, like growth, the breeder needs to know how these traits are associated (specifically genetically correlated). In some cases, the traits may be positively correlated, that is by selecting for one trait the other is also being indirectly improved. Alternatively, if the traits are

negatively correlated, the breeder may be inadvertently selecting against the other trait of interest. In some cases there may be no correlation between the different traits, thus selecting for one trait has no impact on the other trait and both may be improved in parallel. Even if the association is negative this does not mean both traits cannot be improved (Moss et al., 2005; Sonesson et al., 2011). For example, the Oceanic Institute in Hawaii initially developed two separate lines of *P. vannamei* as a result of growth and TSV tolerance being negatively correlated (r = -0.45), with one line that selects solely for growth and the other that selects for both growth (30%) and TSV tolerance (70%) (Argue et al., 2002). As a result improvements to both traits have been made, with a 23.4% increase in harvest weight and an 18.4% increase in survival observed following TSV challenge compared to controls after a single generation of selection (Argue et al., 2002). Similarly, Gitterle et al. (2005b) found negative genetic correlations between WSSV tolerance and harvest weight of *P. vannamei*. In contrast, positive correlations were found for survival and harvest weight under standard commercial grow out conditions (Gitterle et al., 2005a). For fish, there are also a number of studies that report positive genetic correlations between different diseases, as well as between growth and disease tolerance (reviewed in Ødegård et al., 2011a). Thus, there may in fact be individuals within shrimp populations that have positive genetic correlations for disease tolerance and growth traits, but it may be that they are hard to identify. This may be due to the structure of the shrimp industry in which suppliers of selectively bred stocks are separated from commercial growers through the use of NBC's and multiplication centres. This means that stocks are grown over a range of geographical regions, in differing systems that could introduce potential genotype by environment (G x E) effects, and further complicated by other factors like presence of pathogens and disease outbreaks. Even with these complications, it is important to evaluate how different traits of commercial relevance are associated where possible so that the most effective breeding strategies can be applied.

1.6 Gill-associated virus and breeding disease tolerant stocks in Australian *Penaeus monodon*

In Australia, at the conception of this PhD project the most problematic virus for local shrimp farmers was gill-associated virus (GAV). Gill-associated virus was first reported in 1995, which at the time was known as lymphoid organ virus (LOV) and later determined to be identical to GAV (Spann et al., 1995, 1997; Cowley et al., 2000b). Gill-associated virus is part of the yellow head complex of viruses (type 2) and thus closely related to the highly virulent yellow head virus type 1 (YHV1) (Cowley et al., 1999; Wijegoonawardane et al., 2008). Gill-associated virus contains a ~20 kb ssRNA genome and is classified within the *Okavirus* genus (Family *Roniviridae*) (Cowley et al., 2000a). Gill-associated virus commonly occurs at high prevalence in wild and farmed *P. monodon* in Australia (Cowley et al., 2000b; Walker & Winton, 2010), and pathogenesis caused by acute GAV infection can result in significant production losses (Munro et al., 2011). Vertical transmission of GAV from broodstock to progeny appears to be the primary means by which infection is perpetuated in wild populations and enters farm ponds (Cowley et al., 2002). High doses of GAV transmitted horizontally via cannibalism of moribund diseased shrimp is also likely involved in disease outbreaks (similar to YHV1) (Walker et al., 2001). Early life stages of *P. monodon* up to post-larval (PL) stage 15 (PL15) and PL30 of other penaeid species appear to have very limited susceptibility to horizontal transmission (waterborne exposure and ingestion) of YHV1 or GAV (Khongpradit et al., 1995; Lightner et al., 1998). However, in later life stages disease can be established through intramuscular injection of infected tissue homogenates or haemolymph, cannibalism of virus-infected shrimp tissue and exposure to seawater either containing homogenised tissue of infected shrimp, or cohabitation of infected shrimp (OIE, 2015). Gill-associated virus remains a significant problem to the Australian shrimp farming industry today, however, recently there have been detections and outbreaks of other diseases that were previously not known to be in Australia, such as WSSV (Queensland Department of Agriculture and Fisheries) and *P. monodon* mortality syndrome (PmMS) that is similar to that of AHPND (Landos, M., Ridley Australian Prawn and Barramundi Farmers Symposium 2016, Townsville).

Penaeus monodon is the primary shrimp species farmed in Australia and the aquaculture industry is almost entirely based on wild caught broodstock that are spawned in captivity and their subsequent post-larvae stocked into farm ponds. Some small scale domestication and breeding programs for *P. monodon* in Australia have been established (Coman and Preston, 2008). Although, these programs have introduced wild broodstock to broaden the genetic base. At present there is still a reliance on wild broodstock in Australian *P. monodon* farming to supply the majority of seed to the industry. This means the industry is burdened by potential introductions of pathogens into farm stocks and lacks the ability to undergo genetic improvement. To date, no selective breeding specific to GAV tolerance has been undertaken. The only known practice that addresses GAV directly in Australian *P. monodon* farming is screening of wild caught or domesticated broodstock prior to spawning and eliminating highly infected individuals from the spawning group (Coman et al., 2013). However, screening is not practised industry wide and is often carried out on a population level rather than screening of every individual. If GAV tolerance is found to have a genetic basis, selective breeding may be a useful strategy for managing this disease in Australian shrimp farming.

1.7 Thesis aims and structure

This PhD research was undertaken as part of the Australian Research Council Industrial Transformation Research Hub for Advanced Prawn Breeding, Project IH130200013. The overarching aim was to produce, for the first time, essential genetic information on GAV disease tolerance to determine whether this trait could be incorporated into a selective breeding program for *P. monodon*. To investigate this, the first steps involved establishing disease challenge protocols and methods specific to GAV that could be applied to large-scale genetic evaluations. In addition, information on how selection for GAV tolerance effects on-farm performance traits was also required to get a comprehensive understanding of the utility and potential implications of incorporating this trait into a selective breeding program.

Chapter 2 compared commonly used pathogen infection methods for their ability to establish uniform GAV infections and mortality in *P. monodon*. The study highlights the importance of validating alternative infection methods as they are not all guaranteed to result in infection. Those that do induce infection may generate unwanted variance all while trying to elucidate fine-scale differences in tolerance. The study examined disease progression through mortality curves, as well as GAV infection loads, to compare the different infection methods. The infection methods evaluated were intramuscular injection, feeding of infected shrimp meat and immersion in seawater containing a GAV inoculum. The methods established in this chapter are incorporated in subsequent *Chapters 4 & 5*. This study was published in the peer-reviewed journal *Aquaculture* (Noble et al., 2017) with only minor modifications in this thesis chapter for ease of reading and continuity when read together with the thesis as a whole.

Chapter 3 examined to what extent GAV infection loads vary among individual gill filaments or pleopod tissue samples collected from individual shrimp. Information on an individual's viral infection load is useful in several aspects of shrimp farming; including screening wild caught or domestic broodstock, surveillance of pathogens in farmed and wild populations, as well as relative comparison of infection severity between individuals as a proxy of comparing disease tolerance. The latter example was applied in *Chapters 4 - 6* to determine whether viral load can be used as an indirect measure of GAV tolerance and a trait for selective breeding. Therefore, it is important to understand the degree to which viral loads may vary due to sampling methods so that this can be minimised to increase the accuracy of the phenotype. This study was published in the peer-reviewed journal *Aquaculture* (Noble et al., 2018) with minor modifications to avoid repetitiveness.

Chapter 4 further fine-tuned the GAV challenge test protocol by determining a suitable dose of the inoculum used throughout the research project, and applied that dose to a small number of full-sib families to assess whether the assay was sensitive enough to differentiate families based on their tolerance. The infection dose is critical to being able to identify and measure differences in individual and family tolerances, as a dose that delivers too few viral particles may not result in infection and a dose that is too high may swamp the innate immune system and thus mask any genetic differences.

The families used in this chapter were reared using the common practice of keeping families in separate tanks. This chapter also aimed to determine whether this practice could introduce non-genetic variability due to tank effects and therefore affect the ability to measure genetic differences.

Chapter 5 uncovers for the first time the underlying genetic basis of GAV tolerance. Specifically, heritability estimates for both survival and GAV infection load following GAV challenge were estimated. This study was also unique within the literature in the fact that the shrimp families evaluated were communally reared from spawning through to challenge testing, rather than kept in separate tanks, and thus utilised DNA markers and genotyping to determine pedigree retrospectively. Shrimp breeders will be able to use the information from this study to determine whether to improve GAV tolerance using selective breeding methods.

Chapter 6 investigated how GAV tolerance measured under challenge test conditions is associated with traits measured under commercial conditions, specifically GAV infection status, GAV infection load and body weight at harvest. This chapter estimated heritability and genetic correlations for the three commercial pond traits, as well as correlation with GAV tolerance traits measured under challenge test conditions from *Chapter 5*. This information is important for breeders to fully understand how traits in their breeding goals may indirectly affect other traits that may or may not be selected.

As a whole this thesis provides a comprehensive evaluation of whether GAV tolerance is suited to a selective breeding program and how best to incorporate it.

Chapter 2. Comparison of methods for uniformly challenging the black tiger shrimp, *Penaeus monodon,* with gill-associated virus

2.1 Introduction

Disease challenge tests are essential to understanding and overcoming disease in farmed shrimp and for identifying and establishing disease-tolerant breeding lines. In addition to fulfilling Koch's postulates to associate a pathogen with a disease (Fredericks & Relman, 1996), challenge tests have been used to investigate pathogen transmission dynamics (Chou et al., 1998), species, strain and life-stage factors affecting shrimp susceptibility to disease (Spann et al., 2000; Perez et al., 2005), pathogen control strategies (Rahman et al., 2006; Sellars et al., 2011; Sanitt et al., 2014) and disease tolerance of selected shrimp breeding lines (Argue et al., 2002; White et al., 2002; Gitterle et al., 2005b).

Selective breeding for disease tolerance relies on accurately measuring genetic variation of tolerance among test animals and families. Therefore, measuring tolerance is commonly carried out in controlled tank-based disease challenge tests to limit any non-genetic variation. However, there is no standard protocol for disease challenge tests, which can vary between pathogens, the host animal (i.e. species and size/age being challenged) and laboratories. A common view is that shrimp disease challenge tests should emulate the natural pathways in which the animal becomes infected (e.g. Moss et al., 2005; Cock et al., 2009), while still having a high level of control so that each shrimp is exposed to a uniform dose of the pathogen.

To develop advanced technologies to assist in the breeding of genetically-superior lines of domesticated Black tiger shrimp (*Penaeus monodon*) in Australia, tolerance to disease caused by Gill-associated virus (GAV) is being evaluated as a trait for selection. To do this standardised challenge test protocols are needed for GAV so that differences in tolerances can be accurately measured. Intramuscular injection of a filtered inoculum has been used extensively as a means of establishing GAV infection in shrimp due to its ability to deliver a uniform dose of virus (Spann et al., 1997, 2000;

de la Vega et al., 2004; Oanh et al., 2011; Sellars et al., 2011, 2015). However, a potential disadvantage of injection is that it does not mimic natural transmission routes. As a prerequisite to undertaking challenge experiments to identify *P. monodon* breeding lines more tolerant to GAV-induced disease, (i) intramuscular injection of a filtered GAV inoculum prepared from diseased moribund *P. monodon*, (ii) immersion in seawater containing the filtered inoculum and (iii) ingestion of diced tissue pieces derived from moribund GAV-infected shrimp, were compared for their ability to reliably induce disease and to establish uniform infections in juvenile *P. monodon*.

2.2 Methods

2.2.1 Experimental animals

Penaeus monodon post larvae stage 15 (PL15) spawned from multiple wild-sourced broodstock from Joseph Bonaparte Gulf, Northern Australia, were obtained from two commercial hatcheries in Queensland. The post-larvae were transported to the CSIRO Bribie Island Research Centre (BIRC) and reared in either outdoor culture tanks (Challenge Test 1), or indoor tanks supplemented with algal mats (Challenge Test 2). Shrimp were fed commercial feed pellets (Shrimp MR Starter, Ridley Australia) and were challenged with GAV when >2 g in weight. Shrimp were observed for gross signs of sickness or disease prior to the challenge experiments and all shrimp appeared 'healthy'.

2.2.2 GAV inoculum

A GAV inoculum was prepared from *P. monodon* displaying characteristic signs of acute GAV infection (lethargy and reddening of gills/appendages). To prepare the inoculum, soft cephalothorax tissues from three shrimp were homogenized in six volumes of shrimp saline solution (SSS) (10 mM HEPES, 450 mM NaCl, 10 mM KCl, 10 mM Na_2-EDTA, pH 7.2-7.5) on ice using an Ultra-Turrax blender. The homogenate was centrifuged at 2500 × *g* for 10 min at 4 °C to remove particulate matter and centrifuged again at 15,000 x *g* for 20 min at 4 °C. The supernatant was then passed through a 5 µm membrane filter followed by a 0.45 µm filter and stored in 1 ml aliquots at -80 °C until used. The presence and concentration of GAV was confirmed and quantified using a GAV TaqMan reverse-

transcription qPCR assay (see methods below). The mean GAV concentration of the stock inoculum was 1.89×10^5 GAV copies μL^{-1} inoculum. Additionally, the inoculum was tested for presence of two commonly found viruses, infectious hypodermal and haematopoetic necrosis virus (IHHNV) and yellow-head virus type 7 (YHV7). IHHNV was not detected in the inoculum, while YHV7 was detected at very low levels with only one out of the three replicate qPCR reactions testing positive (~1.0 YHV7 copy μL^{-1} inoculum). All dilutions of the inoculum were prepared from the original stocks immediately prior to use in the challenge assays.

2.2.3 GAV-infected shrimp tissue preparation

To prepare shrimp muscle tissue for the ingestion challenge, *P. monodon* (~30 g) were injected with GAV inoculum diluted 1:10 in SSS at a dose of 5 $\mu L\ g^{-1}$ shrimp weight. On day 11 post-challenge (p.c.), the abdominal section of two moribund shrimp was diced into ~1 cm^2 sized pieces and placed in a -80 °C freezer for 1 h as a 'frozen tissue' infection source. After this period, the abdominal section of two additional moribund shrimp was diced similarly and used as a 'fresh tissue' infection source. Pleopod tissues from each of the four shrimp used for the ingestion challenge were persevered in RNAlater solution (Ambion) to confirm presence of GAV and estimate the individuals' infection load. Gill-associated virus was present in all four shrimp used and their infection load ranged from 1.53×10^6 to 3.52×10^8 GAV copies μg^{-1} total nucleic acid (TNA).

2.2.4 GAV challenge tests

Challenge tests were undertaken in an aquarium facility utilizing 100 L round-bottom tanks. Tanks were filled with 80 L filtered seawater (30 ppt salinity, 29 ± 1 °C water temperature range) and used a flow-through water system (800 mL per min) with continuous aeration. Shrimp were fed commercial feed pellets (Shrimp MR Starter, Ridley Australia) twice daily at 09:30 and 17:30 h at a rate of 10% total biomass.

Challenge Test 1 assessed intramuscular injection and immersion infection routes. For intramuscular injection, four groups of 10 shrimp (5-10 g) were injected with GAV inoculum (1.89×10^5 GAV copies μL^{-1} inoculum) at a dose of 5 $\mu L\ g^{-1}$ shrimp weight. The inoculum was injected into muscle

tissue at the 2nd abdominal segment using a 100 µL Hamilton glass syringe fitted with a 25-gauge needle. Due to the large volume of inoculum required for the immersion challenge, only two groups of 10 shrimp (5-6 g) were used. Each shrimp group was placed in 1 L seawater containing 1 mL GAV inoculum (1:1000 dilution or 1.89 x 10^5 GAV copies mL^{-1} of seawater) for 2 h with constant aeration before being returned to their 80 L tank. As negative controls, four groups of 10 shrimp were injected with SSS at 5 µL g^{-1} shrimp weight. Shrimp were observed twice daily at which times the number of surviving shrimp was recorded and moribund/dead shrimp were removed. For the injection-group the challenge test was terminated on day 7 p.c. when a minimum of 50% cumulative mortality had occurred. The immersion and control groups were terminated on day 11 p.c. Pleopod tissue from all 18 injection-group survivors and a subset of the survivors from the immersion and control groups (10 out of the 20 immersion group survivors and 16 out of the 40 control group survivors) were preserved in RNAlater solution before use.

Challenge Test 2 assessed ingestion of GAV-infected shrimp muscle tissue as an infection route. Shrimp (4.5 ± 1.6 g) stocked in 80 L tanks as above were starved for 24 h prior to being fed the GAV infected shrimp muscle tissue. Two tanks of 10 shrimp were fed fresh GAV-infected muscle tissue and two tanks of 10 shrimp were fed frozen GAV-infected muscle tissue. Each tank received 10% total biomass in shrimp meat (~4.5 g) and any uneaten meat found the following day was removed and feeding with commercial feed pellets was resumed. As controls, two tanks of 10 shrimp were continued on commercial feed pellets. Shrimp were monitored twice daily at which times numbers of surviving shrimp was recorded and moribund/dead shrimp were removed. The test was terminated on day 11 when cumulative mortality reached ~50% in both groups. Pleopod tissue from all 10 fresh-tissue group survivors, all nine frozen-tissue group survivors and a subset of 10 out of the 18 control-group survivors were preserved in RNAlater as above.

2.2.5 Viral load analysis

Total RNA was extracted from a 100 µL subsample of the GAV stock inoculum. The inoculum was mixed with 900 µL of TRIzol reagent (Life Technologies Pty. Ltd) and RNA was extracted following manufacturer's instructions. RNA was resuspended in 30 µL of RNase-free water.

Pleopod tissue samples in RNAlater were blotted dry on absorbent paper and placed into wells of a 2 mL 96 deep-well plate together with one ceramic (2.8 mm) and three glass beads (1.3 mm) and 600 µL RLT Buffer (QIAGEN). Tissue was disrupted by mechanical beating and TNA was extracted using the magnetic bead-based MagJet RNA Kit (Thermo Fisher Scientific) and a KingFisher Flex 96 robot (Thermo Fisher Scientific) according to the manufacturer's protocols, except for omitting the DNase digestion step and the use of an additional Wash Buffer 2 step. Total nucleic acid was resuspended in RNase-free water and quantified using a NanoDrop ND-8000 UV spectrophotometer. Total nucleic acid was either diluted to 66 ng/µL using RNase-free water, or used undiluted if below this concentration.

Complementary DNA (cDNA) (10 µL reaction) was synthesised using 500 ng TNA (or less for lower yielding samples), 1 x TransAmp Buffer and 0.5 µL reverse transcriptase (Bioline SensiFast cDNA Synthesis Kit) at 25 °C/10 min, 42 °C/15 min followed by 85 °C/5 min. The GAV TaqMan qPCR assay used a 6FAM-TAMRA probe and primer sequences reported previously (de la Vega et al., 2004). Reactions (20 µL) containing 1 x SensiFAST Probe Lo-ROX Kit buffer (Bioline), 2 µL cDNA (~100 ng TNA), 0.9 µM each primer and 0.25 µM TaqMan probe were dispensed (5 µL per well) into three wells of a 384-well PCR plate as technical replicates. DNA was amplified using a ViiA 7 real-time PCR System (Applied Biosystems) and default thermal cycling conditions (50 °C/2 min, 95 °C/2 min, 40 cycles of 95 °C/15 s, 60 °C/30 s). Each PCR plate included cDNA prepared to a 10-fold dilution series of synthetic GAV of known copy number to generate a standard curve to quantify GAV copy numbers in each sample. Values were adjusted based on input TNA/cDNA amount in each 5 µl reaction and expressed as GAV copies μg^{-1} TNA.

Additionally, challenge survivors were tested for pre-existing infections of IHHNV and YHV7. Total nucleic acid extracted from the pleopod tissue was used for the IHHNV PCR test described by Tang et al., (2007). Synthesised cDNA was used in a RT-qPCR test for YHV7 previously described (Cowley et al., 2015; Mohr et al., 2015). All shrimp used in the challenge experiments were confirmed free of both IHHNV and YHV7.

2.2.6 Statistics

Kaplan-Meier survival plots were produced in R version 3.3.0 (R Development Core Team 2016). The statistical significance of differences in survival rates among the test groups was assessed using the log-rank test within the 'survival' package in R.

The statistical significance of differences in GAV infection load quantified by real-time qPCR was assessed using one-way ANOVA and pairwise comparisons used the Tukey HSD post hoc test in R. Viral load data were checked for normality and homogeneity using Residual versus Fitted and Q-Q plots in R and showed that a $\log_{10}$ transformation of the data improved these assumptions. Coefficients of variation (CV) were calculated to quantify GAV RNA copy number variation among surviving shrimp sampled either at 50% mortality, or when tests were terminated.

2.3 Results

2.3.1 Injection and immersion challenge routes

In Challenge Test 1 comparing intramuscular injection and immersion GAV challenge routes, injected shrimp began to die from day 4 onwards reaching 50% cumulative mortality on day 7 p.c. (Fig. 2.1). In the shrimp groups injected with SSS only, or challenged by immersion, no mortalities occurred by day 11 when the test was terminated. Survival of injected shrimp compared to either the control or the immersion-challenged shrimp was thus significantly lower ($\chi^2_{(2)} = 44.58$; $p < 0.0001$).

From the survivors of the injection-group, GAV was detected in 100% of the shrimp and infection levels were consistently high with a mean GAV load of 7.73 ± 0.49 $\log_{10}$ GAV copies μg^{-1} TNA

(Table 2.1). In contrast, GAV was detected in only five out of 10 immersion-group shrimp at low levels, which had a mean GAV load of 1.53 ± 0.22 $\log_{10}$ GAV copies μg^{-1} TNA and in nine out of 16 control shrimp also at low levels with a mean GAV load of 2.30 ± 0.78 $\log_{10}$ GAV copies μg^{-1} TNA. As with the survival data, GAV infection loads detected among the injection-group survivors were significantly higher compared to shrimp examined from the immersion or control groups ($F_{2,41}$ = 269.8; $P < 0.001$), however, there was no difference in infection loads between the immersion and control shrimp ($P = 0.34$).

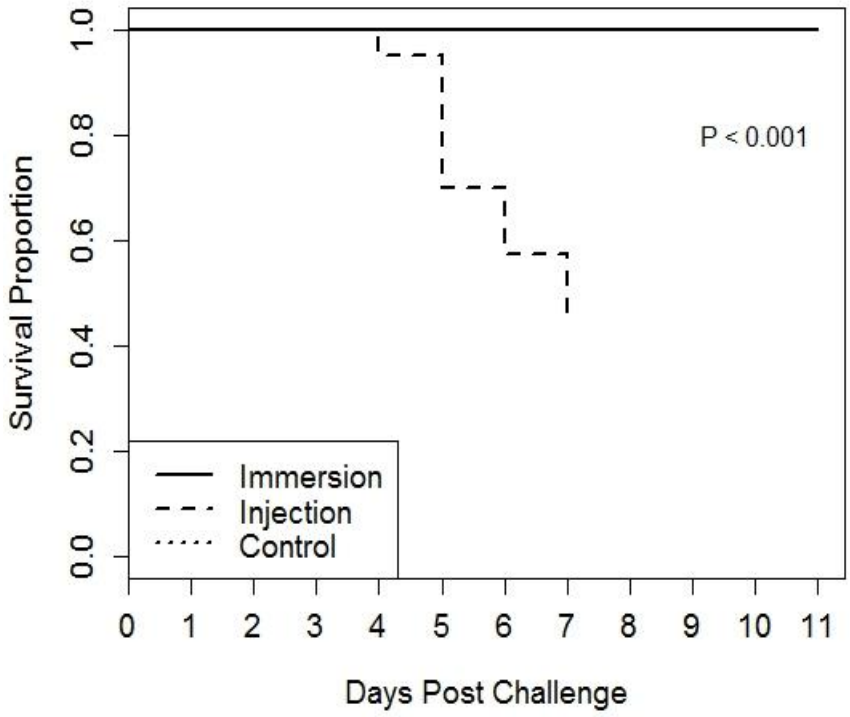

Figure 2.1. Kaplan-Meier survival curves of *Penaeus monodon* challenged via intramuscular injection of gill-associated virus (GAV) inoculum, immersion in water containing GAV inoculum and a control group injected with shrimp saline solution (SSS). Note the survival curves of the GAV immersion and SSS injected shrimp groups overlap due to both treatments resulting 100% survival.

2.3.2 Ingestion challenge route

In Challenge Test 2, examining ingestion of either fresh or frozen GAV-infected shrimp muscle tissue, shrimp began to die from day 1 onwards and 50% mortality was reached on day 11 p.c. (Fig. 2.2). A total of two mortalities occurred in the control group. Survival rates among the shrimp groups

fed either fresh or frozen muscle tissue were not significantly different, but were significantly lower than the control shrimp group maintained on commercial feed pellets ($\chi^2_{(2)} = 9.70$; $p < 0.01$).

Gill-associated virus was detected in pleopod tissue of all 10 survivors in the fresh-tissue group with a mean GAV load of 4.77 ± 1.97 $\log_{10}$ GAV copies μg^{-1} TNA and all nine survivors in the frozen-tissue group that had a mean GAV load of 5.71 ± 1.90 $\log_{10}$GAV copies μg^{-1} TNA (Table 2.1). Low-levels of GAV were also detected in 9 out of 10 control shrimp analysed with a mean GAV load of 2.09 ± 0.23 $\log_{10}$ GAV copies μg^{-1} TNA. Gill-associated virus loads in the control shrimp were significantly lower than levels detected in the survivors of ingestion challenge groups ($F_{2,26} = 12.77$; $P < 0.0014$), however, there was no difference between the two ingestion groups ($P = 0.44$).

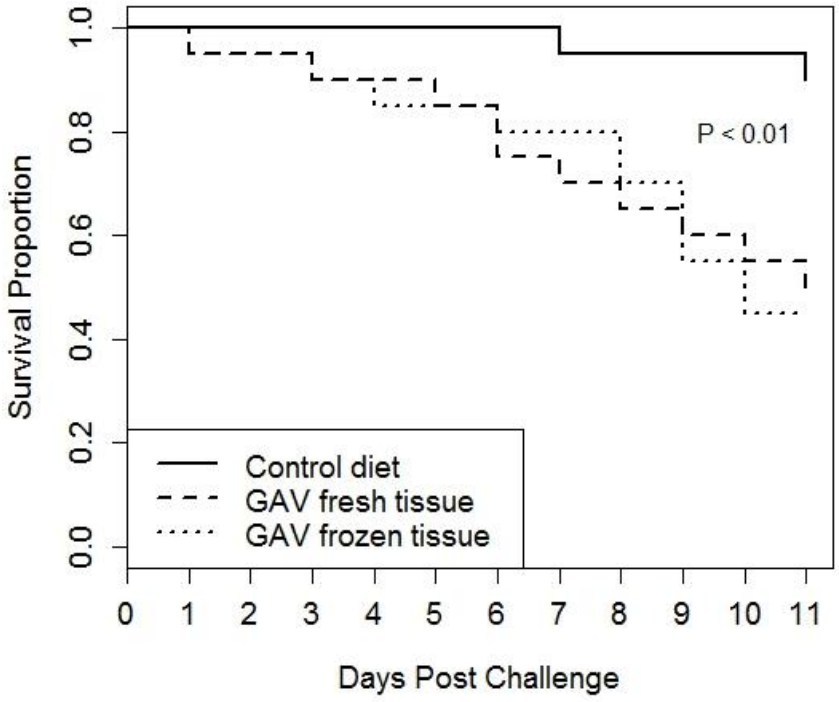

Figure 2.2. Kaplan-Meier survival curves of *Penaeus monodon* challenged via oral ingestion of fresh or frozen gill-associated virus (GAV) infected shrimp tissue and a control group fed commercial pellets.

Table 2.1. Gill-associated virus infection loads quantified by reverse-transcriptase quantitative PCR (RT-qPCR) in pleopods of *Penaeus monodon* challenge survivors once 50% mortality was reached in the group, or when the experiment was terminated on day 11 post-challenge.

GAV challenge method	Days post-challenge	No. shrimp tested	No. shrimp positive	Log_{10} GAV copies μg^{-1} TNA		
				Min	Max	Mean ± s.d.
Challenge Test 1						
GAV Injection	7	18	18	6.92	8.68	$7.73 \pm 0.49_{a}$
GAV Immersion	11	10	5	0	1.80	$0.76 \pm 0.22_{b}$
SSS Injection	11	16	9	0	4.16	$1.29 \pm 0.78_{b}$
Challenge Test 2						
GAV Fresh Tissue	11	10	10	2.79	7.99	$4.77 \pm 1.97_{a}$
GAV Frozen Tissue	11	9	9	2.83	8.02	$5.71 \pm 1.90_{a}$
Commercial Pellet	11	10	9	2.06	2.75	$2.33 \pm 0.23_{b}$

Mean values with the same subscript (a or b) were not significantly different (Tukey HSD, $P < 0.05$).

2.4 Discussion

Intramuscular injection, ingestion and immersion challenge routes were compared for their ability to establish uniform GAV infections leading to mortality of juvenile *P. monodon*. The primary reason for the tests was to identify whether natural routes of horizontal GAV transmission, such as ingestion or immersion, might be useful for challenging families of *P. monodon* to identify and establish disease-tolerant breeding lines. The data indicated that injection of a filtered GAV inoculum readily transmitted infection leading to 50% mortality by day 7 p.c. It also resulted in uniformly high GAV infection loads (mean = 7.73 ± 0.49 $\log_{10}$ GAV copies μg^{-1} TNA, CV = 0.06), as assessed by RT-qPCR analysis of TNA extracted from pleopod tissue, among survivors sampled at this time. Ingestion of diced abdominal muscle tissue of moribund GAV-diseased *P. monodon* also resulted in mortalities that accumulated more slowly (50% mortality on day 11). The rate at which mortalities accumulated using fresh muscle tissue was unaffected by freezing for 1 h in a -80 °C freezer.

However, compared to injected shrimp, GAV infection loads amounts detected in pleopods of ingestion-challenge survivors sampled on day 11 were approximately 100- to 1000-fold lower and more variable (fresh/frozen, mean = 4.77 ± 1.97/5.71 ± 1.90 $\log_{10}$ GAV copies μg^{-1} TNA, CV = 0.41/0.33). Gill-associated virus infection loads detected in the immersion group shrimp were not statistically different to the infection loads detected in the control shrimp. Therefore, no conclusive evidence of GAV infection transmission was obtained for shrimp immersed for 2 h in seawater comprising a 1:1000 dilution of the filtered inoculum.

Consistently with data obtained here, previous immersion tests to assess the potential for infection transmission through exposing shrimp to free GAV particles in water have also proved ineffective (K.M. Spann et al., unpublished data). Similarly, waterborne (cohabitation with infected shrimp via connected tanks) challenge of *P. monodon* (~20 g BW) using the more virulent YHV1 genotype from Thailand also identified transmission difficulties via this route, with only one third of the challenged shrimp succumbing to disease/mortality within 30 days p.c. and only some testing positive to infection (Hamano et al., 2015). In contrast, juvenile (7-10 g BW) *Penaeus vannamei* also challenged via cohabitation with YHV1 infected shrimp resulted in rapid morbidity and mortality soon after exposure, reaching 86% mortality on day 10 p.c. (Anantasomboon et al., 2008). Variability in infection efficiency obtained from waterborne transmission tests have also been reported for white spot syndrome virus (WSSV) (Chou et al., 1998; Prior et al., 2003; Gitterle et al., 2006a), with factors such as shrimp moult stage and cuticle damage being identified to affect transmission (Corteel et al., 2009). Waterborne exposure would seem to be a useful method for large-scale disease challenge testing needed for selective breeding, however, the variability in results obtained using this method, not only for YHV (including GAV) but also in different shrimp viruses, indicate a degree of unreliability of this method to establish uniform infections among challenged shrimp and is therefore considered not suitable for selection purposes.

In contrast to immersion, both the intramuscular injection and ingestion challenge routes transmitted GAV infection effectively, which is in agreement with earlier studies on GAV (K.M. Spann et al., unpublished data) and YHV1 (Lightner et al., 1998; Hamano et al., 2015). In addition, ingestion

survival rates were unaffected by freezing shrimp tissue for a short period at -80 °C before use, confirming that GAV infectivity is retained for at least one rapid freeze/thaw cycle. Therefore, a large batch of GAV-infected shrimp muscle tissue could be prepared and used to challenge different families of *P. monodon* at different times. However, ingestion challenge resulted in a slower and more varied GAV infection efficiency. The faster rate at which mortalities accumulated among shrimp challenged by muscle injection compared to ingestion was the likely result of several factors, including injection being immediate and delivering a higher dose of GAV directly into the shrimp muscle. Thus, injection would result in virus particles rapidly entering haemolymph and being circulated systemically throughout the shrimp and consequently infecting susceptible cells (Escobedo-Bonilla et al., 2006; da Silva et al., 2015). Conversely, oral ingestion offers a more complex and as yet undefined entry route via the digestive tract. Additionally, this route may also present multiple barriers that could potentially damage the viral particles leading to their inactivation and thus a reduced dose of virus infecting the shrimp (Escobedo-Bonilla et al., 2006). The time lag between feeding the shrimp tissue and it being ingested is also likely to have resulted in temperature-induced virus particle degradation and loss of infectivity and thus a lower and more variable GAV challenge dose compared to injection. For WSSV, similar differences in prolonged mortality were observed between shrimp challenged via ingestion and injected methods (Huang et al., 2011).

Reduced uniformity in the challenge dose via fresh/frozen shrimp tissue ingestion compared to injection challenge was supported by higher variability in GAV infection loads (CV = 0.41/0.33 compared to 0.06, respectively) detected in pleopods of survivors. Similarly, when challenging juvenile *P. vannamei* with infectious myonecrosis virus (IMNV) via ingestion of infected tissue, only a small proportion of those challenged became infected with the virus, and when accompanied with an environmental stress (lowered salinity) the number of shrimp infected increased; however, their viral load was highly variable (range 2.11×10^2 - 8.39×10^6) (da Silva et al., 2015). Increased variability of viral load in shrimp infected via natural ingestion routes may arise due to a number of reasons including differing amounts of tissue ingested by individual shrimp, heterogeneity of the infectious agent within the source material (e.g. shrimp tissue) as well as factors associated with host entry

barriers and genetic variation. To overcome non-uniformity in ingestion dose, methods involving viral inoculum delivery via a 28-gauge venocatch catheter or flexible pipette tip inserted into the oral cavity of individual shrimp has been used for WSSV (Escobedo-Bonilla et al., 2006; Gitterle et al., 2006a, 2006b). For the purposes of challenging large numbers of shrimp families to assess their disease tolerance, such methods would be arduous and impractical and most likely why feeding of infected shrimp tissues is still commonly used for this purpose (e.g. Cuellar-Anjel et al., 2012; Moss et al., 2013).

Notably, while the experimental *P. monodon* appeared overtly healthy, low-level pre-existing GAV infections were detected by RT-qPCR in approximately half of the control shrimp tested. The presence of GAV was not unexpected due to its high prevalence in wild and farmed *P. monodon* (Cowley et al., 2002; Walker et al., 2010). Moreover, the similarly low GAV infections detected in shrimp challenged by immersion and the vastly higher level infections detected in survivors challenged by injection or ingestion suggested that pre-existing GAV infections had little if any influence on the outcome of each challenge route.

2.5 Conclusion

With GAV, injection challenge has most commonly been used to assess species susceptibility and the ability of RNA-interference (RNAi) to inhibit virus replication and disease (Spann et al., 2000, 2003; de la Vega et al., 2004; Oanh et al., 2011; Sellars et al., 2011, 2015). The benefits of injection are its ability to (i) accurately and reproducibly deliver different challenge doses of virus to define a minimum lethal dose needed to generate 50% mortality within a defined time period (Sellars et al., 2011) and (ii) allows the production of a large uniform inoculum batch that can be used to challenge shrimp reproducibly at different times and result in similar mortality patterns. The disadvantage of injection it that it is an unnatural transmission route and can be laborious at large-scale.

Each of the three challenge routes trialled employed juvenile shrimp (2-9 g). It is thus possible that some challenge methodologies and outcomes might not be directly transposable to other shrimp life

stages or be impractical, such as using injection for early life stages. Both the injection and ingestion challenge routes trialled here induced GAV infection, disease and mortality in juvenile shrimp. Significantly, injection resulted in greater uniformity in GAV infection loads in survivors sampled once 50% mortality was reached. Despite being an unnatural infection route, our data indicates that injection should provide the uniformity and flexibility in GAV challenge dose needed to identify differences in disease tolerance among families of *P. monodon*.

Chapter 3. PCR testing of single tissue samples can result in misleading data on gill-associated virus infection loads in shrimp

3.1 Introduction

Disease management strategies in shrimp farming rely heavily on rapid, sensitive and accurate pathogen detection methods such as conventional and quantitative PCR methods. Various PCR tests have been developed to detect all major shrimp pathogens and those with proven analytical and diagnostic sensitivity, specificity and reliability have been endorsed by the World Organisation for Animal Health (OIE, 2017). However, beyond the analytical performance, the diagnostic accuracy of PCR tests can be impacted profoundly by pathogen-specific infection severity and tissue tropism factors. These factors can result in viral infection loads being distributed heterogeneously throughout a shrimp (e.g. Lo et al., 1997; Cowley et al., 2000, 2005; Durand and Lightner, 2002; de la Vega et al., 2004; Nunan et al., 2004; Munro and Owens, 2005; Jeswin et al., 2015). For this reason, care is required when selecting sampling methods and tissue types to maximize PCR detection sensitivity and accuracy for each pathogen (Lightner, 1996; OIE, 2017). However, in cases where non-destructive testing is required, such as screening for virus presence/infection loads in shrimp broodstock, the use of gill, pleopod, or haemolymph provide the only practical tissue sources for testing.

Gill-associated virus (GAV) replicates in shrimp tissues of mesodermal and ectodermal origin including, among others, haemocytes and cells in the lymphoid organ, gills, pleopods and connective tissues (Spann et al., 1997, 2003; Cowley et al., 2000; Tang et al., 2002; Munro and Owens, 2005). Using electron microscopy and reverse-transcriptase PCR (RT-PCR) methods, GAV infection loads have been identified to vary among different tissue types (Spann et al., 1995, 1997; de la Vega et al., 2004). However, there is potential for GAV infection loads to vary within some of these tissue types and the degree to which this may occur is not well known.

In Australia there is a growing desire to establish breeding populations of *P. monodon* that are specific pathogen free and/or disease tolerant against GAV. Both of which require accurate detection and quantification of GAV infection loads using either non-sacrificial sampling methods in the case of

broodstock, or large-scale high throughput methods for screening large numbers of shrimp. Commonly, gill or pleopod tissue has been used for RT-PCR analysis in either scenario. However, the extent to which GAV loads can vary between different gill filaments, or pleopods, sampled from the same shrimp is largely unknown. This study used RT-qPCR analyses to accurately quantify GAV infection loads in different gill filaments and pleopods sampled from individual *P. monodon* with naturally acquired infections to compare the sensitivity and variability within either tissue type. Naturally infected shrimp were used in this study, rather than artificially infected, to understand the natural distribution in GAV infection. Which is widely applicable to several shrimp farming situations such as screening of wild and domesticated broodstock or shrimp collected from farm ponds. Additionally, both lymphoid organ lobes were also sampled from a subset of adult *P. monodon* to compare the sensitivity of detection of GAV in the gill and pleopod tissue compared to the lymphoid organ, which is the recommended target tissue for GAV (OIE, 2017).

3.2 Methods

3.2.1 Sample collection

Tissues were sampled from two independent groups of *P. monodon* reared at the Bribie Island Research Centre (BIRC), Queensland, Australia. Group 1 comprised 10 juvenile shrimp (mean weight 10.9 ± 1.4 g), and Group 2 comprised 12 adult shrimp (mean weight 40.4 ± 2.3 g). Group 1 shrimp each had eight gill filaments and eight pleopods sampled and Group 2 shrimp each had 10 gill filaments, 10 pleopods and both lymphoid organ lobes sampled. Tissues were preserved in individual tubes containing RNAlater solution (Ambion) at 4°C for a minimum of two days and then at -20°C until processed as recommended in the manufacturer's instructions. Gill-associated virus infection load of each tissue sample was quantified using the same methods for total nucleic acid (TNA) extraction, cDNA synthesis and GAV qPCR assay described previously in *Chapter 2.2.5*.

3.2.2 Statistical analyses

All statistical analyses and graphics were produced in the program R (R Core Team, 2016). To evaluate how tissue type influenced GAV infection loads, the GAV RNA copy number was $\log_{10}$ transformed after adjusting zero values to one and the mean viral load was calculated for each tissue type within individual shrimp. To compare mean GAV infection loads of gill and pleopod tissue in Group 1 shrimp, a paired t-test was used. The data set was analysed either including or excluding samples that had GAV infection loads of zero. As there was no difference in the test outcome, only the analysis including all samples is reported. For Group 2 shrimp, the mean GAV infection load in gill, pleopod and lymphoid organ tissues were compared using an ANOVA and a Tukey post-hoc test was used to determine significant pairwise differences among tissues.

To assess whether gill or pleopod tissue differed in the level of variability in GAV infection loads detected among individual gill filaments and pleopods sampled from each shrimp, the coefficient of variation (CV) was used. The CV was calculated separately for each tissue type by dividing the standard deviation by the mean value for every shrimp. The overall mean CV values determined for each tissue type were then compared using a paired Wilcoxon signed rank test for both groups. For Group 1, the CV was calculated either including or excluding samples with zero GAV infection load. However, as there was no difference in the significance of the tests outcomes, only results that include all samples are reported.

3.3 Results

3.3.1 GAV infection loads

RT-qPCR was used to quantify GAV RNA amounts in pleopod, gill and lymphoid organ tissues sampled from *P. monodon* with natural-acquired GAV infections. Technical reproducibility of RT-qPCR data was assessed by testing three reaction aliquots as 'technical replicates' and assessing the standard deviation of reaction cycle threshold (Ct values). Ct standard deviations were generally low (<0.5) except, as expected, for some samples identified to contain very low GAV RNA template loads

(<610 GAV RNA copies ug-1 TNA, equivalent to <15 copies per RT-qPCR reaction), in which cases standard deviation values were more variable (range 0 - 1.5).

The RT-qPCR test data generated using gill, pleopod and lymphoid organ samples revealed that all 22 *P. monodon* examined were infected with GAV. Among the 10 juvenile shrimp in Group 1, four individuals (shrimp 2, 5, 6, 9) had between one and four of their gill filaments test PCR-negative and four individuals (shrimp 3, 6, 9, 10) had between one and six pleopods test PCR-negative (Fig. 3.1). Overall, 69/80 gill filaments and 67/80 pleopods tested PCR-positive among Group 1 juvenile shrimp (Table 3.1). Among the 12 adult shrimp in Group 2, all gill filaments, pleopods and lymphoid organ lobes were PCR-positive.

Mean GAV infection loads determined for gill and pleopod tissue across all the shrimp tested within each group identified GAV infection severity to be lower among the Group 1 juvenile shrimp than among the Group 2 adult shrimp (Table 3.1, Fig. 3.2). Among the Group 2 shrimp, GAV infection loads detected in lymphoid organ were substantially higher than detected in either gill filaments or pleopods.

Table 3.1. Number of shrimp and tissues sampled within each group of *Penaeus monodon* alongside the prevalence and mean values of gill-associated virus (GAV) infection loads quantified each tissue type using RT-qPCR (LO = lymphoid organ).

Group	No. shrimp	Tissue	No. samples per shrimp	Total no. positive detections	Mean GAV load ± s.d. (GAV copies ug^{-1} TNA)
1	10	Gill	8	69/80	$6.83 \times 10^2 \pm 4.32 \times 10^3$
		Pleopod	8	67/80	$1.65 \times 10^3 \pm 8.58 \times 10^3$
2	12	Gill	10	120/120	$1.37 \times 10^5 \pm 4.07 \times 10^5$
		Pleopod	10	120/120	$6.97 \times 10^4 \pm 2.50 \times 10^5$
		LO	2	24/24	$5.96 \times 10^7 \pm 8.67 \times 10^7$

3.3.2 Within tissue infection variability

Within Group 1, GAV infection loads detected among the eight gill filaments sampled from each of the 10 shrimp varied by 7- to 844-fold, and among the eight pleopods by 4- to 2,912-fold (Fig. 3.1). Within Group 2, GAV infection loads varied by 6- to 840-fold among the 10 gill filaments and by 4- to 2779-fold among the 10 pleopods sample from each of the 12 shrimp (Fig. 3.1). For the two lymphoid organ lobes tested from each of the Group 2 shrimp, GAV infection loads varied by up to 3.8-fold.

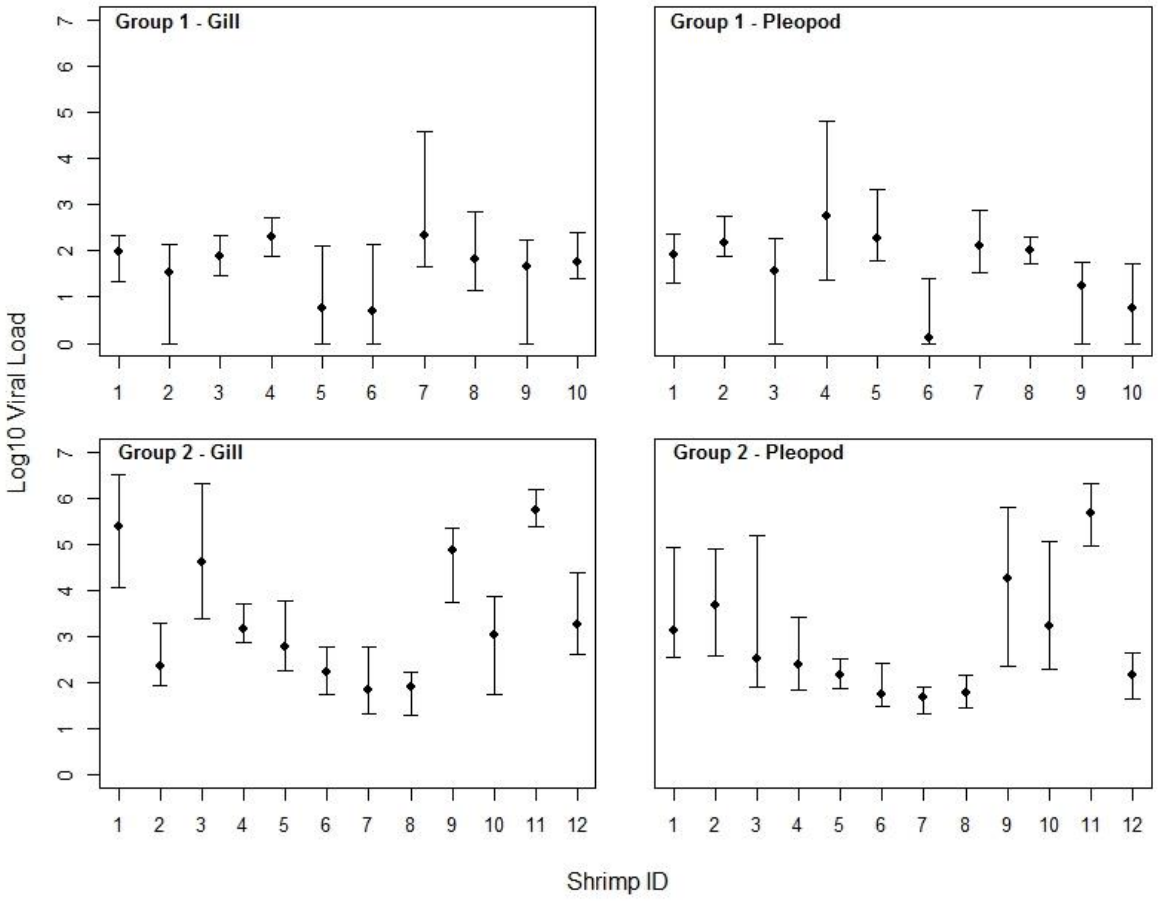

Figure 3.1. The mean and range (minimum and maximum) in gill-associated virus loads (log_{10} GAV RNA copies μg^{-1} of TNA) detected among replicate tissue samples from individual *Penaeus monodon* in Groups 1 and 2. Group 1 had 10 shrimp and 8 individual gill or pleopod samples per shrimp, whilst Group 2 had 12 shrimp and 10 individual gill or pleopod samples per shrimp.

3.3.3 Tissue effects on detection sensitivity and variability

The mean GAV infection loads were determined for gill and pleopod tissue samples from each shrimp in Group 1 and for gill, pleopod and lymphoid organ samples from each Group 2 shrimp (Fig. 3.2). No significant differences were evident between the overall mean GAV infection loads between gill and pleopod samples for Group 1 shrimp ($t_{(9)}$ = -0.19, P = 0.852). For Group 2 shrimp, lymphoid organ had significantly higher GAV infection loads than both the gill or pleopod samples ($F_{(2,22)}$ = 158.89, P < 0.001), however, there was no difference between gill and pleopod GAV infection loads (P = 0.146) .

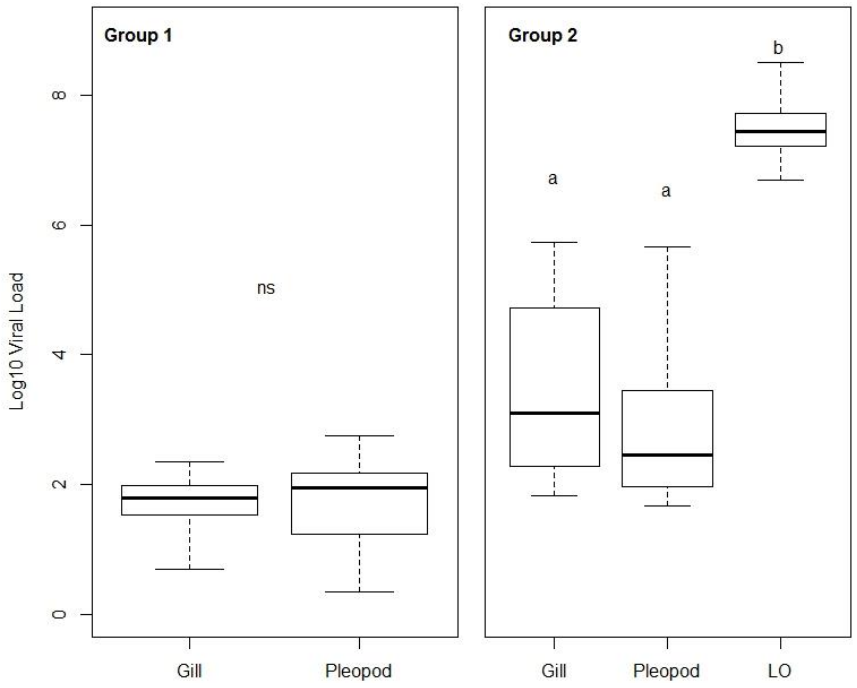

Figure 3.2. Boxplots of the mean GAV infection loads (log_{10} GAV RNA copies ug^{-1} TNA) for each tissue type from *Penaeus monodon* in groups 1 and 2. Group 1, ns = not significant; Group 2, tissues with different letters above the box plot indicate significant differences in viral loads ($p < 0.0001$). LO = lymphoid organ.

To compare the level of variability between individual gill filaments or pleopods, the mean CV for each tissue type was calculated among shrimp in each group. For both the Group 1 and the Group 2 shrimp, there was no statistical differences in GAV infection load variability between the two tissue types (Fig. 3.3; Group 1 – $T = 26$, $P = 0.922$; Group 2 – $T = 20$, $P = 0.151$).

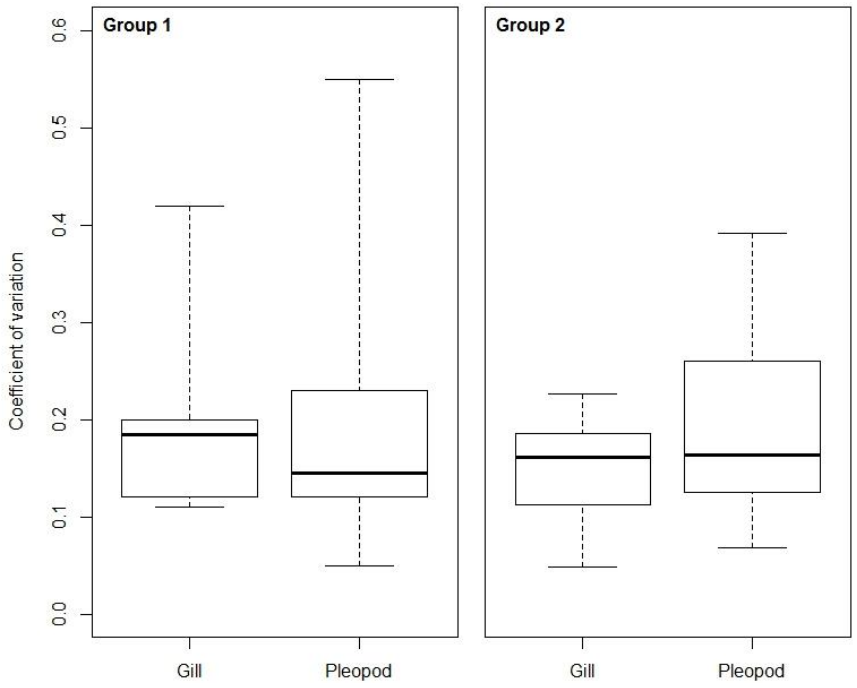

Figure 3.3. The mean coefficient of variation (CV) of GAV infection loads (log_{10} GAV RNA copies ug^{-1} TNA) observed among gill filaments and pleopods sampled from *Penaeus monodon* in groups 1 and 2. There was no significant difference in CV between gill or pleopod tissue for either group.

3.4 Discussion

Disease management in shrimp production is dependent on reliable and accurate methods for detecting and quantifying pathogen infections. This study used RT-qPCR methods to quantify and compare GAV infection loads among individual gill filaments, pleopods and lymphoid organ lobes sampled from *P. monodon* naturally infected with GAV. The precision of technical replicates was generally high (mean Ct standard deviation was 0.4), except in some samples that had very low GAV infection loads where standard deviation values were more variable (up to 1.5). Decreased precision may affect the accuracy of detecting and quantifying GAV infection and is expected as GAV RNA template numbers in each reaction approach the RT-qPCR test detection sensitivity limit. Gill-associated virus infection loads detected in individual gill filaments and/or pleopods sampled from each shrimp were found to commonly vary by >10-fold, and in some shrimp by up to ~3,000-fold. Variability of this magnitude was evident in both groups of *P. monodon* regardless of the GAV infection severity. Thus, no obvious improvement in the uniformity of GAV infection loads was

evident among either gill filaments or pleopods sampled from the adult shrimp with higher-level GAV infections (Group 2). Moreover, among the group of juvenile shrimp with generally lower-level GAV infections (Group 1), four individuals possessed gill filaments and four individuals possessed pleopods that tested qPCR-negative. Similar infection load variability, including false negatives, have been identified among pleopods sampled from individual Pacific white shrimp (*Penaeus vannamei*) infected at low-levels with White spot syndrome virus (WSSV, Francois Bentra, personal communication) and could possibly occur with other shrimp pathogens.

The findings of this study highlight the potential for pathogen infections to be missed when using single tissue samples, particularly in shrimp with low-level natural infections. It is difficult to predict the minimum required number of gill filament or pleopod samples from individuals to avoid false negatives for a number of reasons; firstly this number will change progressively based infection progression, is specific to the group of shrimp being tested and maybe be prohibitive when non-sacrificial sampling is required (e.g. broodstock screening). However, based on the juvenile shrimp in this study as an example, the minimum number of gill filament or pleopod samples that would have been required to get 100% prevalence was five and seven respectively. In the adult shrimp a single tissue sample would have been sufficient given all samples tested positive, highlighting the specificity of a minimum sample number to the groups being tested.

Misdiagnosis of infection could have disastrous implications in black tiger shrimp breeding programs where wild broodstock are commonly selected for breeding based on their testing PCR-negative for specific pathogens (specific pathogen free, SPF) (Moss et al., 2003b). In such circumstances, the data from this study indicate that testing of a single pleopod or gill filament could increase the risk of a false negative and missed infection leading to the pathogen possibly being transmitted vertically to seedstock and consequently into farm ponds or into other SPF broodstock (Cowley et al., 2002). To reduce the risk of false negative detections multiple successive PCR tests may be undertaken, ideally using pools of more than a single tissue sample, during the quarantine of wild broodstock before they are selected for use in SPF breeding programs (Moss et al., 2003a). For broodstock, it may be more suitable to carryout multiple successive PCR tests, ideally using pools of more than one tissue sample,

during the quarantine of wild broodstock before they are selected for use in SPF breeding programs (Moss et al., 2003a). Alternatively, increasing the number of shrimp tested would increase the accuracy of prevalence estimates when screening at the population level.

In addition to potential false negative detections, the large variability in GAV infection loads among individual gill filaments or pleopods in some shrimp can generate inaccurate data on infection loadings and may understate infection severity when sampling a single gill filament or pleopod. This could impact the accuracy of relative comparisons made between individual shrimp. Accurate data on an individual's infection load is critical, for example, when viral titre is used to assess and select viral resistant/tolerant breeding lines, as has been done in other aquaculture species (Sauvage et al., 2009; Purcell et al., 2010; Corbeil et al., 2013). Without accurate data on an individual's pathogen infection load, the ability to estimate genetic contributions to variation among individuals or family lines with any confidence will be severely hampered.

The GAV infection loads quantified in each of the lymphoid organ lobes from Group 2 adult shrimp were markedly higher (435- to 856-fold) and varied far less compared to those detected among either gill filaments or pleopods sampled from the same shrimp. These findings were consistent with previous analyses of GAV-infected *P. monodon* that found higher levels of GAV in the lymphoid organ (Spann et al., 1995, 1997, 2003; Cowley et al., 2000, Tang et al., 2002; de la Vega et al., 2004). Although, Munro and Owens (2005) found that haemolymph from GAV-infected *P. monodon* had the highest haemaglutination titres. Infected haemocytes circulating within haemolymph are known to be sequestered and accumulate within discrete spheroid bodies within the lymphoid organ (Rusaini and Owens, 2010) highlighting the value of testing lymphoid organ to increase RT-qPCR detection sensitivity for GAV when sacrificial sampling is possible.

Despite lymphoid organ being the optimal tissue sample for detection sensitivity for GAV, there are scenarios in shrimp farming where it is not suitable to sample this organ. This is because (i) it requires the animal to be sacrificed which is not always an option in shrimp breeding scenarios, (ii) it is difficult to sample on small animals and (iii) can be tedious to locate and dissect during high-

throughput screening. Alternative tissues like gill and pleopod are often used as they can be sampled non-destructively and efficiently under most scenarios. Within either the group of juvenile shrimp with low-level GAV infections or group of adult shrimp with higher-level infections, statistical analyses were unable to identify any significant difference between gill or pleopod tissues in sensitivity of detection, or variability of GAV infection loads. Therefore, the data suggests neither tissue is more advantageous for generating accurate GAV infection load data and the decision on which tissue type (gill or pleopod) to sample depends on which is most suited to the collection and laboratory processing. Differences in the amount of total RNA isolated per µg of tissue may exist and affect relative comparisons made between tissue types.

In summary, the data presented here on shrimp with naturally acquired GAV infections, demonstrated that infection loads can vary markedly among different gill filaments and pleopods sampled from the same shrimp. Thus testing of a single sample of these tissues can underestimate infection severity or even misdiagnose an infected individual. When sampling of lymphoid organ is not possible, the testing of pools of tissue from two or more gill filaments/pleopods is thus recommended to circumvent this variability and to generate more accurate data on GAV infection presence and severity.

Chapter 4. Impact of separate rearing and challenge tanks on measuring family tolerance to gill-associated virus using a standardised challenge dose

4.1 Introduction

Disease challenge tests are used to evaluate and select disease tolerant shrimp families in selective breeding programs. In doing so controlled challenge tests aim to limit the environmental variation and maximise the ability to measure genetic differences. However, despite challenge tests being "highly controlled", sources of variation arise unintentionally from non-genetic factors. This can include the infection methodology and dose (Noble et al., 2017), as well as confounding environmental factors including tanks effects (Kjoblum et al., 2005).

In shrimp breeding programs families are often spawned and reared in separate tanks during hatchery and nursery stages in order to easily maintain and track pedigrees (Moss et al., 2013), as well as to control family contributions. When the shrimp are large enough to be physically tagged (~1-2 g) they can then be combined in communal systems. The use of separate tanks prior to the challenge test, or even during the challenge itself, has the potential to introduce variability in survival due to tank effects and consequently reduce the ability to measure genetic differences. Although great care is taken to standardise environmental conditions, micro-environmental changes may occur unknowingly and have been found to lead to differences in performance during challenge tests in fish (e.g. Kjoglum et al., 2005), as well as for other traits such as growth in shrimp (Coman et al., 2004). If there is no replication of tanks for each family then the ability to separate tank effects from genetic (family) effects becomes completely confounded and potentially biases the estimates of genetic variation upwards (Hayes et al., 2010). Replicating family groups across replicate rearing tanks would allow for tank effects to be estimated.

A standardized challenge protocol whereby all shrimp are exposed to the pathogen equally is necessary to accurately measure genetic differences in tolerance (as in *Chapter 2*). However, the infection dose is also important to be able to compare susceptibility of individuals, or groups of

individuals (e.g. families) (Prior et al., 2003; Escobedo-Bonilla et al., 2005). A dose that delivers too few viral particles or bacteria may not result in a reliable infection and a dose that is too high may overwhelm the innate immune defence mechanisms and consequently mask any differences in tolerance such as those due to genetic factors. *In vivo* titration experiments of the infectious material (i.e. a viral inoculum) can be used to determine an suitable infection dose that reliably results in infection and mortality and at a rate that maximises the ability to observe relative differences among individuals or groups (e.g. families).

Using the injection method described in *Chapter 2,* the aims of this chapter were to (1) determine a suitable infection dose for the GAV inoculum used throughout the PhD research project, (2) apply the dose to full-sibling *Penaeus monodon* families to determine whether the assay is sensitive enough to differentiate families based on tolerance to GAV infection, and (3) assess the degree to which separate rearing and/or challenge tanks introduce variation in estimating family performance in disease tolerance.

4.2 Methods

4.2.1 In vivo titration of inoculum to determine a suitable challenge dose

Three titration experiments were performed independently to determine a suitable infection dose of the GAV inoculum (described in *Chapter 2.2.2*) that would result in a moderate rate of mortality (~50%) over a 14 day challenge period in juvenile *P. monodon*. The groups of shrimp used in the three titration experiments were obtained from a commercial hatchery in Queensland, Australia, although two different cohorts of shrimp were used in the titration experiments. Experiments 1 and 2 were carried out using shrimp from Cohort 1, whilst experiment 3 used shrimp from Cohort 2. The family composition within each of the cohorts used for titration experiments were unknown. Both cohorts were transferred to Bribie Island Research Centre (BIRC) at post larvae stage 15 (PL15) and reared in 20,000 L concrete raceways and later in 2000 L fibreglass tanks. The titration experiments were performed using juvenile shrimp with a mean body weight of 6.5 ± 0.6 g, 10.4 ± 0.8 g and 3.8 ±

0.6 g for experiments 1, 2 & 3, respectively. Experiment 1 tested four different dilutions of the inoculum (concentration 1.89×10^5 GAV copies μL^{-1}), which were undiluted and diluted 1:3, 1:9 and 1:27 in shrimp saline solution (SSS, see *Chapter 2.2.2* for details). The dilutions tested in Experiment 1 were chosen as a starting point based on the virulence of previous GAV inoculums used in challenge trials conducted by the CSIRO Aquaculture group (see Sellars *et al*., 2011, 2015). Experiment 2 tested six dilutions; 1:10, 1:30, 1:100, 1:300, 1:1000 and 1:3000 dilutions in SSS and in Experiment 3, a 3-fold serial dilution of the inoculum starting from 1:12 to 1:78,732 in SSS was tested. In all experiments, a control group was included where shrimp were injected with SSS only. In experiments 1 and 2, each inoculum dilution and the control group was tested in four replicate groups of 10 shrimp (40 shrimp total) and in experiment 3, three replicate groups of 10 shrimp (30 shrimp total) were used for each dilution and the control. The inoculum or SSS was injected into the muscle of the 2nd abdominal segment at a weight standardised dosage of 5 $\mu L\ g^{-1}$ shrimp weight using a 100 µL Hamilton glass syringe fitted with a 26-gauge needle. Shrimp survival was monitored twice daily (09:00 & 17:00), at which times data on any dead shrimp were recorded and the shrimp subsequently removed from the tank. Data obtained from the titration experiments identified that the GAV inoculum diluted at ~1:3000 resulted in accumulated mortality of approximately 50% by day 14 post challenge (p.c.) (see Table 4.1 & Fig. 4.1). Based on this mortality rate, a 1:3000 dilution was chosen as a suitable dose for subsequent challenge tests to differentiate families based on their tolerance.

4.2.2 Production of families for GAV challenge test

Broodstock used to produce the families of shrimp were caught from the coastal waters off Innisfail in North Queensland, Australia. The female broodstock used to produce the seven families in this study were screened for presence and loading of GAV prior to spawning. Gill-associated virus infection was present in all broodstock screened, however, most had low viral loads, less than 100 GAV copies ng^{-1} TNA, whilst one (Family 5 dam) had a high infection load (9.27×10^8 GAV copies ng^{-1} TNA). Females were naturally mated in communal tanks and once ready to spawn they were moved to individual spawning tanks. The nauplli were collected from the spawning tanks and transferred to individual 1,000 L rearing tanks. Approximately 6,000 post larvae stage 18 (PL18) from each family

were then transferred into three replicate outdoor 2000 L rearing tanks (2000 PL18 stocked per tank). After 30 days, the number of shrimp in each tank were culled to approximately 300 shrimp removing individuals that were much smaller or larger than the approximate mean for each tank. Shrimp were reared under these conditions until they reached challenge size of ~ 4 – 5 g. A subsample of shrimp from each family were weighed every 2 – 3 weeks to track growth rates and determine when each family was ready to be challenged.

4.2.3 Shrimp family challenge test

Shrimp were harvested from the rearing tanks once they reached challenge size and transferred to the challenge facility. From each family 60 – 100 shrimp (depending on the number of shrimp available within the size class) from at least two replicate rearing tanks were challenged with GAV (challenge group) and another 30 – 40 shrimp per family were used as controls (control group). Shrimp in the challenge group were individually weighed and injected with a weight standardised dose (5 µL g^{-1} shrimp weight) of the GAV inoculum diluted 1:3000 using the same injection methods as in the titrations. The control group shrimp were injected with SSS using the same dosage as the challenge group. After inoculation, shrimp were stocked into 80 L round-bottom tanks with 10 shrimp per tank. Shrimp from each family and each rearing tank within family were stocked into separate replicated challenge tanks so that their origin (family and rearing tank) was easily traceable. The challenge tanks were set up using flow-through filtered and UV sterilised seawater (500 mL^{-min}, 30 ppt salinity, 29 ± 1°C water temperature) with continuous aeration. An additional 10 shrimp were injected with GAV and five shrimp injected with SSS from each family to replace shrimp that died within the first 48 h.

Shrimp were monitored twice daily (09:00 & 17:00) at which times the number of surviving shrimp were recorded and moribund/dead shrimp removed. Shrimp were fed commercial pellets (Ridley Aqua-Feeds, Australia) and waste was siphoned out as required. The experiment was terminated at 35 days p.c. and gill tissue samples were collected from all challenge group survivors, as well as from a subset of the control group survivors (n = 12 – 16 per family) to determine their GAV infection loads.

Gill tissues were preserved in RNAlater solution (Ambion) and three gill filaments per individual were used to quantify individual viral loads.

4.2.4 Viral load analysis

To determine the GAV infection status within the survivors of the challenge and control groups from each family, gill tissue samples from each individual were analysed following the methods for total nucleic acid (TNA) extraction, cDNA synthesis and GAV quantitative PCR (qPCR) assay previously described in *Chapter 2.2.5.*

As specific pathogen free (SPF) stock were not available in Australia, shrimp were also tested for another common virus, infectious hypodermal and hematopoietic necrosis virus (IHHNV). Although IHHNV is not typically lethal in *P. monodon* it does cause body abnormalities and is often called 'runt deformity syndrome' (Primavera and Quinitio, 2000). IHHNV has also been found to cause viral interference with white spot syndrome virus infection (Tang et al., 2003; Bonnichon et al., 2006). To determine whether the presence of IHHNV in the shrimp families used in this study affected the response to GAV infection individual IHHNV loads of the survivors was also tested. Being a DNA virus, IHHNV loads were quantified using the TNA extracted from gill tissue samples and a recently published TaqMan qPCR assay described in Cowley et al., (2018).

4.2.5 Statistical analysis

Shrimp survival during the *in vivo* titration experiments was analysed and plotted using Kaplan-Meier survival analysis in the "survival" package of R version 3.3.0 (R Core Team, 2016). The analysis of survival during the family challenge test was also analysed using the "survival" package in R. A Logrank test was performed to determine whether there was a significant difference in survival between the control and challenge groups within each family and between control groups among families. A Cox proportional hazards mixed model (Cox mixed model) was used to test whether family origin and the use of separate rearing or challenge tanks had significant effects on survival. In the Cox mixed model, the response variable "survival" consisted of both the time until death (days post challenge) and status (whether the shrimp died during the challenge period or was censored). The

Cox mixed model allows the effects of multiple fixed and random explanatory variables to be examined. In the model, family, rearing tank and challenge tank, were treated as random with the rearing tank nested within family and challenge tank nested within rearing tank and family. A stepwise forward selection approach was used to determine the significance of each explanatory variable on survival, starting with a null model (no explanatory variables) then each explanatory variable added sequentially following the hierarchical design of the experiment (family, rearing tank then challenge tank). With each additional variable included into the model, the new model was compared against the previous model using likelihood ratio tests to assess whether the added variable better describes the data. The models used were;

Model 1 - Null

$$h(t) = h_0(t) \times \exp(1)$$

Model 2 - Family

$$h(t) = h_0(t) \times \exp(1 + Fam)$$

Model 3 - Family and rearing tank

$$h(t) = h_0(t) \times \exp(1 + Fam + Fam{:}\,RrT)$$

Model 4 - Family, rearing tank and challenge tank

$$h(t) = h_0(t) \times \exp(1 + Fam + Fam{:}\,RrT + Fam{:}\,RrT{:}\,ChT)$$

Where $h(t)$ is the hazard function or the "risk" of a death occurring at time *t*, given the explanatory variables; $h_0(t)$ is the baseline hazard function; 1 is a constant due to no fixed effects in the model; Fam is the random effects of the family; $Fam{:}\,RrT$ is the random effect of rearing tank nested within family and $Fam{:}\,RrT{:}\,ChT$ is the random effect of challenge tank nested within rearing tank and family. The Cox mixed model was analysed using the "coxme" package (Therneau, 2015) in R.

4.3 Results

4.3.1 In vivo titration of inoculum to define a suitable dose

The titration experiments performed in this study demonstrated a dose effect on survival following GAV infection. Higher infection doses (i.e. lower dilutions) resulted in quicker and higher mortality compared to lower doses (i.e. higher dilutions). Additionally, at high infection doses, like those used in Experiment 1, there was little difference in mortality rates between the different dilutions tested. All dilutions tested in Experiment 1 resulted in 50% mortality by day 7 p.c. (Table 4.1, Fig.4.1a). In Experiment 2 & 3 the mortality rates between each dilution were more variable, with the final mortality of each dilution group in Experiment 2 after 14 days ranging from 48 – 80% (Table 4.1, Fig. 4.1b). In Experiment 3, which also lasted 14 days, the end mortality of each dilution group ranged from 0 – 87% (Table 4.1, Fig. 4.1c). No mortalities were observed in the control groups from Experiments 1 and 2, whereas Experiment 3 had one mortality in the control group. Based on the survival curves from each of the titration experiments a dilution of 1:3000 was chosen as a suitable lethal dose for subsequent challenge tests due to its moderate rate of mortality that consistently resulted in approximately 50% mortality by 14 days p.c.

Table 4.1. *In vivo* titration of gill-associated virus (GAV) inoculum of *Penaeus monodon* infected via intramuscular injection and cumulative mortality at each dilution.

Experiment	Termination (days p.c.)	Dilution factor	No. of shrimp tested	No. of shrimp dead	% Mortality
1	7	Undiluted	40	22	55
		1:3	40	20	50
		1:9	40	22	55
		1:27	40	21	52
2	14	1:10	40	32	80
		1:30	40	31	78
		1:100	40	22	55
		1:300	40	24	60
		1:1000	40	23	58
		1:3000	40	19	48
3	14	1:12	30	26	87
		1:36	30	26	87
		1:108	30	23	77
		1:324	30	21	70
		1:927	30	21	70
		1:2916	30	17	57
		1:8748	30	6	20
		1:26244	30	2	7
		1:78732	30	0	0

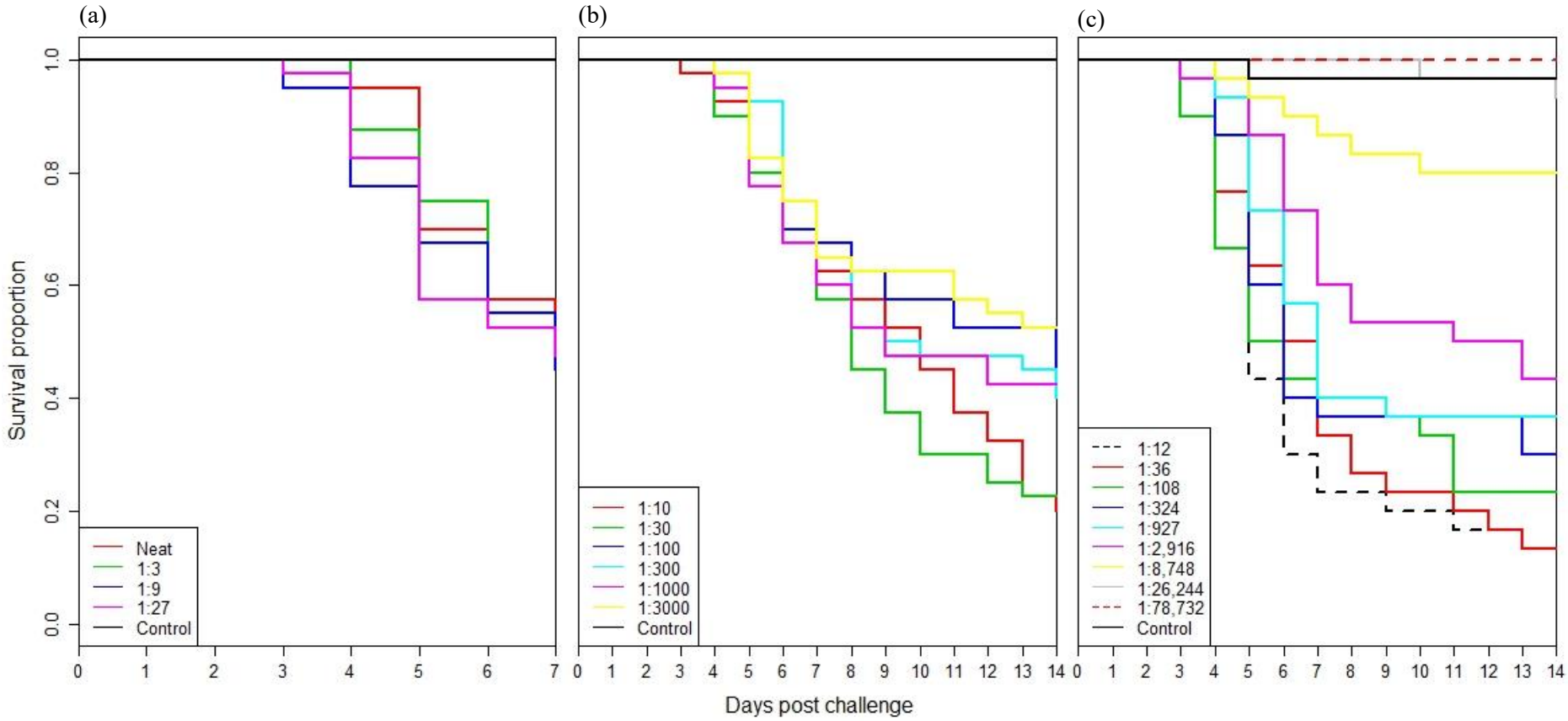

Figure 4.1. Kaplan-Meier survival curves of *Penaeus monodon* infected with gill-associated virus using different dilutions of the inoculum over three titration experiments, (a), (b) and (c).

4.3.2 Family challenge test survival

Survival in the control groups within each family were high throughout the challenge with an average survival of 92%, although survival varied from 83 – 98% (Fig. 4.2). There was no significant difference in the control groups survival when compared across the seven families ($\chi^2_{(6)} = 7.9$, $P = 0.249$), although the control group survival was always significantly higher than the challenge group within each family ($P < 0.001$). Mortalities in the challenge groups occurred from day 3 p.c. and began to plateau after day 25 in most families (Fig. 4.2). The mortality rate in Family 1 did not appear to plateau during the challenge period with deaths occurring up until day 35. At the termination of the challenge (day 35) the cumulative survival among all families was 44%, although survival varied between families from 22 – 72%.

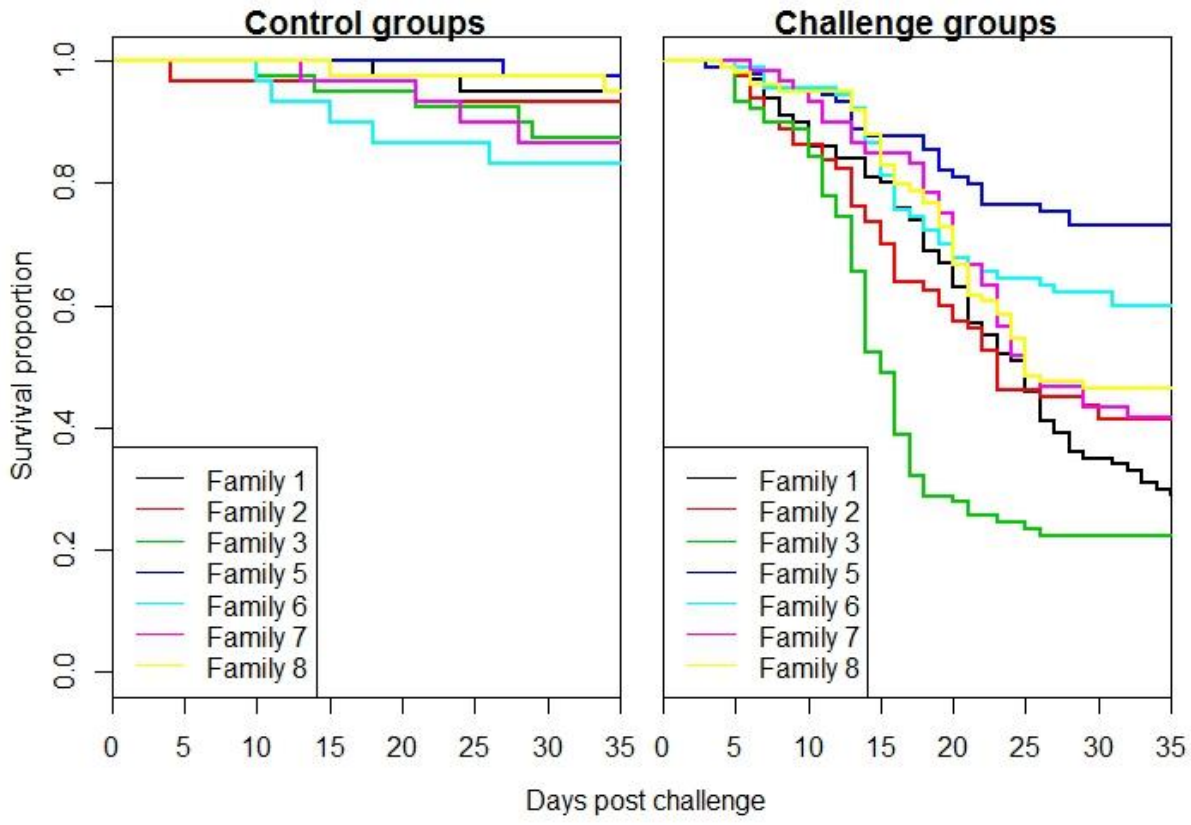

Figure 4.2. Kaplan-Meier survival curves of seven full-sib families of *Penaeus monodon* injected with shrimp saline solution (Control groups) and challenged with gill-associated virus (Challenge groups).

To determine whether survival differed in the challenge groups due to genetic (family) differences or non-genetic factors (tanks), the stepwise forward selection approach using the Cox mixed model was used. This approach resulted in the final model, model 4, best explaining the data (model 3 and 4 comparison, $\chi^2_{(1)} = 329.64$, $P < 0.001$). Model 4 included the effect of family, rearing tank nested with family and challenge tank nested with rearing tank and family. Examples of rearing tank differences are provided in Fig. 4.3 for Families 2 and 6 (see Appendix 1 for all families). Examples of challenge tank differences are provided in Fig. 4.4 showing the survival of shrimp from Family 2 rearing tank 11 and from Family 6 rearing tank 1 (see Appendix 2 for all rearing tanks and families).

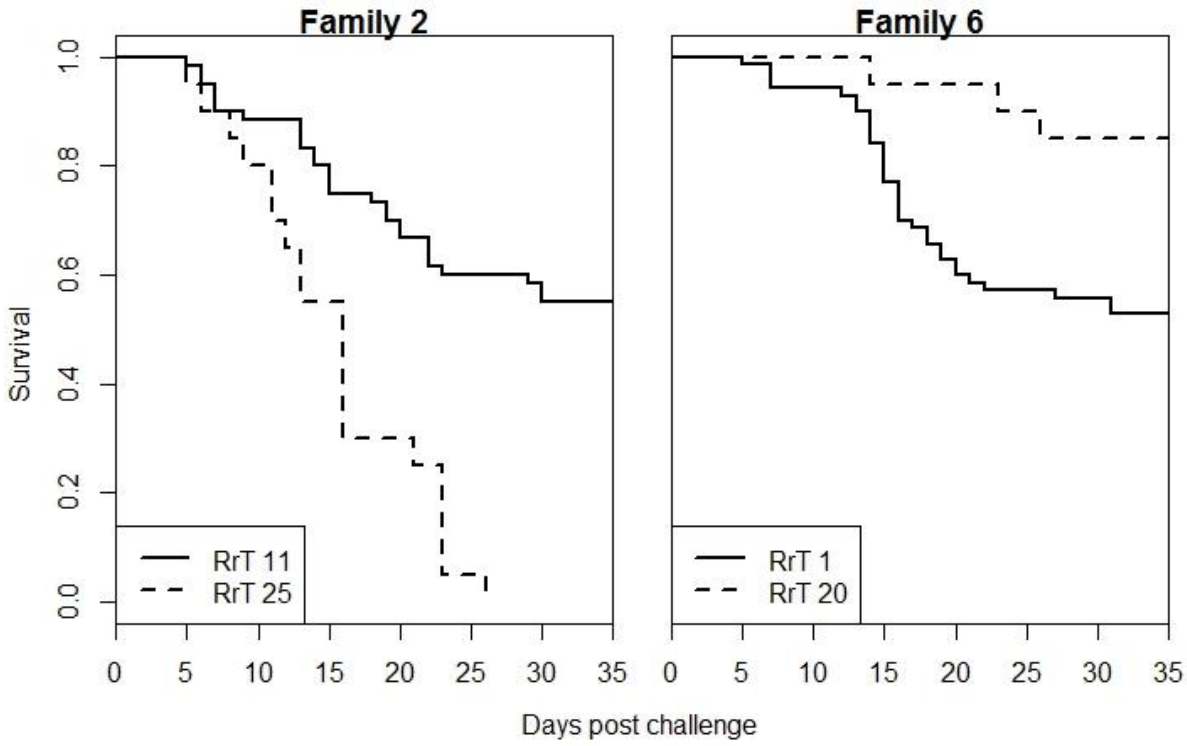

Figure 4.3. Kaplan-Meier survival curves of *Penaeus monodon* siblings reared in separate rearing tanks when challenged with gill-associated virus. Examples are from Families 2 and 6 to highlight the effects of rearing tank on survival.

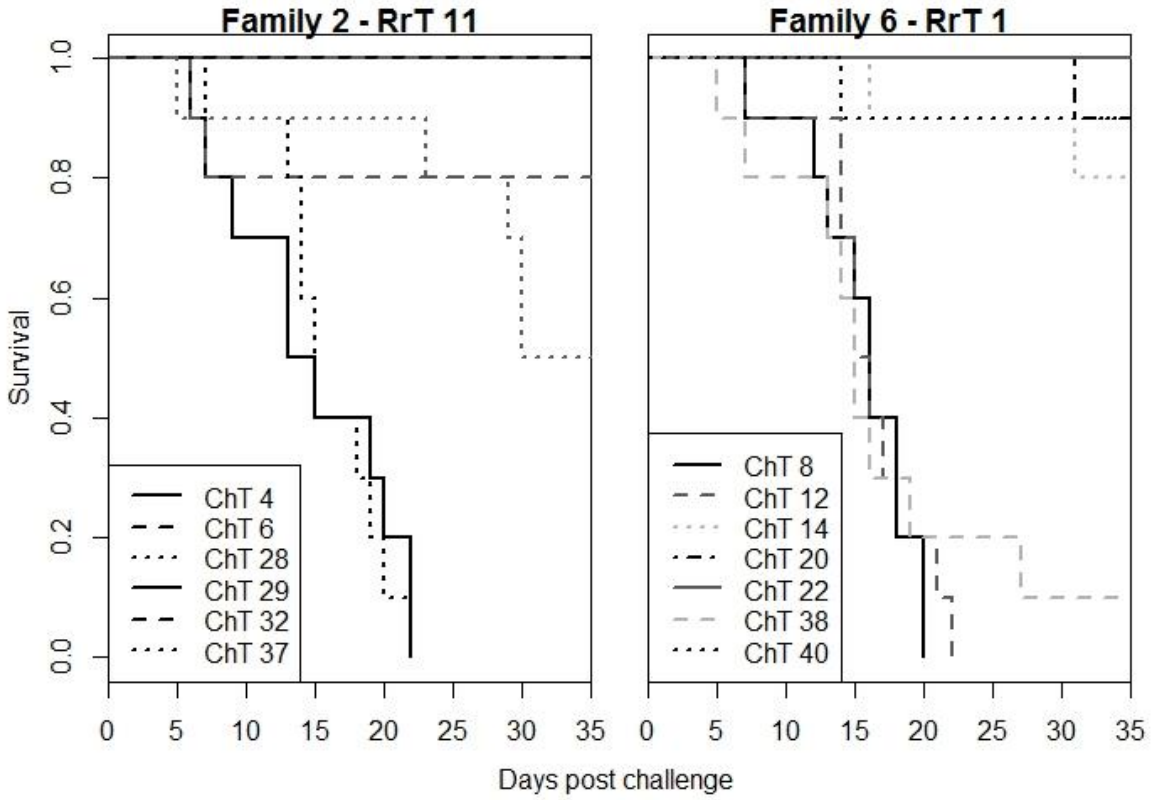

Figure 4.4. Kaplan-Meier survival curves of *Penaeus monodon* siblings from replicate challenge tanks that were reared in a single rearing tank when challenged with gill-associated virus. Examples from Family 2 – rearing tank 11 and Family 6 – rearing tank 1 are provided to highlight challenge tank effects on survival.

4.3.3 Family viral infection loads

Within the control groups, GAV was present in 4/7 families (Table 4.2). No GAV was detected in Families 1, 7 and 8, whereas prevalence of GAV in Families 2, 3, 5 & 6 ranged from 25 to 100%. The average GAV infection load observed within the families where GAV was detected ranged from 1.32×10^2 to 1.51×10^3 GAV copies μg^{-1} TNA.

Within the challenge group survivors, GAV was detected in 5/7 families (Table 4.3). Families 1 and 5 had no GAV detected in the survivors, whilst prevalence in the other five families ranged from 25 to 100%. The average GAV infection loads for each family ranged from 3.77×10^2 to 2.49×10^7 GAV copies μg^{-1} TNA.

Infectious hypodermal and haematopoetic necrosis virus was detected in all surviving shrimp tested with most having high infection loads (~10^9 copies μg^{-1} TNA). The mean IHHNV infection load for each family ranged from 1.10 x 10^5 to 1.27 x 10^{10} IHHNV copies μg^{-1} TNA in the control groups and 6.22 x 10^8 to 1.31 x 10^{10} IHHNV copies μg^{-1} TNA in the challenge groups. There was no correlation between IHHNV and GAV infection loads among the survivors across both the challenge and control groups (r = 0.08, P = 0.319).

Table 4.2. Prevalence and loading (virus copies μg^{-1} TNA) of gill-associated virus (GAV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) infection in *Penaeus monodon* survivors of control groups from the seven families that were challenged with GAV.

Family	% GAV pos.	Mean GAV load ± s.d. (copies μg^{-1} TNA)	% IHHNV pos.	Mean IHHNV load ± s.d. (copies μg^{-1} TNA)
1	0	-	100	1.10 x 10^5 ± 1.32 × 10^5
2	58	1.94 × 10^2 ± 1.96 × 10^2	100	3.68 x 10^9 ± 1.12 × 10^9
3	81	2.72 × 10^3 ± 3.79 × 10^3	100	7.59 x 10^9 ± 4.02 × 10^9
5	25	3.84 × 10^1 ± 7.62 × 10^1	100	2.26 x 10^9 ± 2.65 × 10^9
6	100	8.38 × 10^2 ± 8.36 × 10^2	100	1.27 x 10^{10} ± 4.74 × 10^9
7	0	-	100	9.69 x 10^9 ± 6.05 × 10^9
8	0	-	100	6.70 x 10^9 ± 4.34 × 10^9

Table 4.3. Prevalence and loading (virus copies μg^{-1} TNA) of gill-associated virus (GAV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) infection in *Penaeus monodon* survivors of challenge groups from the seven families that were challenged with GAV.

Family	% GAV pos.	Mean GAV load ± s.d. (copies μg^{-1} TNA)	% IHHNV pos.	Mean IHHNV load ± s.d. (copies μg^{-1} TNA)
1	0	-	100	$6.22 \times 10^{8} \pm 1.51 \times 10^{3}$
2	36	$1.45 \times 10^{8} \pm 1.45 \times 10^{8}$	100	$3.67 \times 10^{9} \pm 2.29 \times 10^{9}$
3	100	$1.22 \times 10^{3} \pm 1.22 \times 10^{3}$	100	$7.28 \times 10^{9} \pm 3.32 \times 10^{9}$
5	0	-	100	$1.97 \times 10^{9} \pm 2.24 \times 10^{9}$
6	85	$7.19 \times 10^{2} \pm 7.19 \times 10^{2}$	100	$1.31 \times 10^{10} \pm 6.24 \times 10^{9}$
7	25	$1.11 \times 10^{8} \pm 1.11 \times 10^{8}$	100	$9.93 \times 10^{9} \pm 7.50 \times 10^{9}$
8	65	$8.45 \times 10^{5} \pm 8.45 \times 10^{5}$	100	$7.14 \times 10^{9} \pm 5.48 \times 10^{9}$

4.4 Discussion

Sensitive and reliable disease challenge tests are critical to measuring disease tolerance and selecting disease tolerant families in shrimp breeding programs. Therefore, the present study aimed to optimise and test the injection based challenge methodology from *Chapter 2* to discriminate between families based on their tolerance to GAV infection. Injection based methods provide a uniform method of infection for shrimp challenge tests (Noble et al., 2017 & *Chapter 2*); however, identifying a dose that provides the sensitivity to discriminate between families is also essential. *In vivo* titration experiments employing different dilutions of the GAV inoculum injected into groups of shrimp identified that a dilution of approximately 1:3000 would result in mortality of approximately 50% over a 14 day challenge period. The moderate rate of mortality suggests the concentration of infectious GAV virions

in a 1:3000 dilution, whilst resulting in significant mortality, would allow the innate defence system of the shrimp to be expressed and thus potentially allow genetic differences in tolerance to be observed.

The 1:3000 dilution of the GAV inoculum was trialled in seven full-sibling families to determine whether this dose along with the injection challenge methodology was sensitive enough to measure differences in survival among the families. Gill-associated virus infection resulting in mortality was observed in each of the families challenged and was significantly different from the control group within each family. Therefore, there is high confidence that the mortality observed in the challenge group was a result of the infectious dose of the GAV inoculum. The Cox mixed model was used to investigate which factors had an effect on survival of the challenged shrimp. The factors investigated included both genetic (family) and non-genetic (rearing tank and challenge tank) effects. The models revealed that family did have a significant effect on survival, as were the effects of the rearing and challenge tanks. This is the first report of significant differences in family tolerances to GAV infection and suggests that the challenge dose applied was able to differentiate families based on their survival. A more comprehensive evaluation with more families would be needed to estimate whether there is significant additive genetic variation (i.e. heritability) to selectively breed for increased GAV tolerance, as has been done for other shrimp pathogens (e.g. Argue et al., 2002; Gitterle et al., 2005b, 2006b)

Non-genetic effects of the rearing tanks and challenge tanks were also found to be significant. Separate rearing tanks are often used to keep families segregated in order to easily track pedigrees. However, as evidenced in this study the use of separate rearing tanks can result in micro-environmental differences to occur during the rearing of families which in turn may cause differences in performance response for the trait under evaluation (i.e. genotype-by-environment interaction) (Sae-Lim et al., 2015). Without replication of rearing tanks within each family it would not be possible to separate the rearing tank effect from genetic effects and could bias genetic effects upwards (Kettunen & Fjalestad 2006; Hayes et al., 2010). In the case for growth in shrimp (Coman et al., 2004), rearing tank had a strong effect on growth performance due to micro-environmental

differences as well as for disease tolerance in fish (Herbinger et al., 1999). Therefore, use of separate rearing tanks, including during early rearing phases prior to physical tagging, should be carefully considered when designing experiments so that genetic effects can be accurately estimated.

The differences in survival among challenge tanks was highly variable, ranging from no survivors to all shrimp surviving, even when family and rearing tank were accounted for (Fig. 4.4). The obvious differences in survival among challenge tanks may be due to a number of reasons. The relatively low numbers of individuals per challenge tank will inherently contribute to variation and any differences are likely to be magnified. Thus replication is important so that this variation can be accounted for. Also, cannibalism of dead and moribund shrimp, which could not be easily avoided during the challenge test despite regular removal of dead shrimp, will lead to re-infection and higher doses of the pathogen to those that cannibalised within each tank. Thus permitting horizontal transmission and exposing shrimp to secondary doses of GAV whenever there was a mortality. *Penaeus monodon* are highly cannibalistic and shrimp that succumb to disease are quickly cannibalised on by other surviving shrimp (Hayes et al., 2010). More frequent monitoring of challenge tanks would help to limit cannibalism and re-infection, or alternatively use of challenge tanks that hold individual shrimp would eliminate this issue; although the use of individual tanks would substantially reduce the scale and number of shrimp and shrimp families that could be assessed.

The use of communal tanks, where multiple families are mixed together during rearing and the challenge test, would allow families to be evaluated simultaneously and reduce the differences due to non-genetic environmental effects that may be present when individual families are reared and/or challenged in separate tanks. By rearing and testing shrimp from multiple families in communal ponds (or tanks) all families are equally exposed to the same environmental conditions. However, the use of communal tanks from larval rearing through to testing will mean less control over relative contribution of each family and individual pedigrees will be unknown at the time of challenge, however, DNA markers and genotyping can be used to resolve pedigrees retrospectively (Jerry et al., 2004, 2006; Henshall et al., 2014).

Gill-associated virus infections diagnosed by RT-qPCT in the survivors of the challenged groups were either undetected or detected with low infection loads. There are several possible explanations for this; (1) the survivors were not susceptible to GAV infection in the case of shrimp with no GAV detected (2) the survivors were better able to control the virus from replicating, (3) GAV infections were present, but were missed due to the tissue sampled, (4) the presence of IHHNV prohibited GAV infection and/or establishment, or (5) they were not infected with GAV. The fifth explanation is unlikely given that each individual was intra-muscularly injected with the GAV inoculum and the challenge groups always had significantly lower survival compared to the control group, thus the deaths were due to infection with GAV. The fourth explanation has low support as there was no correlation between GAV and IHHNV infection loads, and more specifically the few survivors with high GAV infections had equally high IHHNV loads, although this viral interaction should be further investigated. The third explanation is likely as gill tissue is not the primary target tissue suggested for GAV detection and therefore GAV infections may have been missed due to this tissue not being sensitive enough. The recommended tissue for GAV detection is the lymphoid organ (OIE, 2017) and has been found to have much greater sensitivity for GAV diagnosis given its role in immune response (*Chapter 3,* Spann et al., 1995, 1997, 2003; Cowley et al., 2000; Tang et al., 2002). The lymphoid organ would have been extremely difficult to dissect from the juvenile shrimp (~4.5 g) used in this study and therefore gill tissue was used as an alternative. Lastly, many studies have shown *P. monodon* are highly susceptible to lethal doses of GAV (e.g. Spann et al., 2000; de la Vega et al., 2004; Sellars et al., 2011, 2014, 2015) and therefore it is unlikely that the survivors in this study were not susceptible. Therefore it is most likely that survivors were more tolerant and possibly resistant to the GAV infection applied, coupled with limited or no re-exposure to infection through cannibalism.

Survivors of disease challenge tests have commonly been found to have lower viral infection loads compared to those that succumbed to disease (moribund) or died during the challenge (e.g. Anantasomboon et al., 2008; Sauvage et al., 2009; Cao et al., 2010). Viral loads have also been associated with family tolerance (Cao et al., 2010; Purcell et al., 2010). Thus in this study, GAV infection load was examined as a possible additional or alternative measure of disease tolerance which

is generally measured as survival. Gill-associated virus infection loads of the survivors in this study did not correlate with the families overall survival, however, given the low prevalence and infection loads of the survivors, as well as only a small number of families were assessed, this needs further investigation.

In summary, this is the first study that has shown significant family differences in tolerance to GAV infection in *P. monodon* and therefore further investigation is warranted to determine whether there is significant additive genetic variation for selective breeding purposes. Non-genetic factors were also found to significantly impact survival and highlights the importance of understanding sources of genetic and non-genetic variation, both during and prior to disease challenge tests, so that these sources of variation are better able to be controlled or accounted for. In order to accurately estimate the additive genetic variation of GAV tolerance, large-scale challenge testing of a greater number of families need to be carried out.

Chapter 5. Genetic parameter estimates for tolerance to gill-associated virus under challenge-test conditions in the black tiger shrimp, *Penaeus monodon*

5.1 Introduction

Disease is one of the biggest threats to global shrimp aquaculture (Stentiford et al., 2012). Effective disease management strategies are critical to sustaining and expanding the industry. The use of specific pathogen free (SPF) stocks is a primary disease management strategy used widely throughout global shrimp farming (Cock et al., 2009). Whilst SPF shrimp are not selected for disease tolerance to pathogens, use of SPF shrimp has been somewhat successful in lowering the industries risk to certain diseases by minimising the introduction (and spread) of pathogens. It achieves this through the stocking of "clean" - SPF shrimp into farm ponds. Specific pathogen free shrimp are still naïve to infection and particularly in open grow-out environments disease outbreaks continue to occur. There is also evidence that SPF stocks may perform poorly in the presence of disease compared to wild stocks (Moss et al., 2001). Improving disease tolerance through selective breeding can be used in addition to the current SPF approaches to further manage the risk of disease in shrimp farming (Cock et al., 2009).

For genetic improvement of disease tolerance to be possible, the trait must have a genetic basis (i.e. heritable) and able to be accurately and reliably measured. Therefore, disease tolerance is mostly measured as mortality during laboratory challenges tests (Cock et al., 2009). This allows exposure to the pathogen under standardised and controlled conditions. Pathogen inoculation is performed through either feeding of infected tissue (e.g. Argue et al., 2002; Moss et al., 2013), individually via intra-muscular injection (e.g. Ødegård et al., 2011b), or oral delivery with a venocatch catheter (e.g. Gitterle et al., 2006a, 2006b). However, feeding methods are likely to introduce non-genetic variability in the response due to individual variability in the amount of tissue consumed by the shrimp (*Chapter 2*; Noble et al., 2017). Therefore, individual inoculation methods are likely to be

more reliable in establishing the uniform infections required for accurate and reliable measurements of tolerance, provided they accurately reflect genetic variation in tolerance under natural challenge conditions (Gitterle et al., 2006a; Noble et al., 2017).

Estimating the genetic basis of disease tolerance relies on challenge testing of large numbers of shrimp families. Traditional approaches to producing shrimp families are generally carried out by individual spawning and rearing of families in separate tanks that may then be combined once large enough to be physically tagged (1-2 g) or maintained separately during the challenge (Argue et al., 2002; Gitterle et al., 2005b; Hayes et al., 2010). This allows pedigrees to be easily tracked, as well as stocking even numbers of individuals per family in the challenge. However, separate tanks can introduce significant amounts of non-genetic variability, as shown in *Chapter 4*. Hayes et al., (2010) discussed the issue of separate rearing tanks for each family as a major limitation of their study and that tank variation could not be separated from the full-sibling effect; thus potentially biasing estimates of genetic variation upwards. A way around this would be to use a communal rearing system for all families and utilising genotyping to determine pedigrees (Jerry et al., 2006; Vandeputte and Haffray, 2014; Nolasco-Alzaga et al., 2018). This eliminates any common environmental effects due to separate rearing tanks and increases the ability to measure genetic differences.

Genetic parameters for disease tolerance have been analysed using various statistical methods and trait definitions (Ødegård et al., 2011a). Disease tolerance has traditionally been defined as a binary trait (dead/alive) at a specific time point (e.g. 50% mortality) and analysed using linear or threshold mixed models (Argue et al., 2002, Gitterle et al., 2005b). However, these types of models ignore information on when the death occurred. Alternatively, survival can be defined as a longitudinal trait where time until death data, as well as survival information of the individual is captured. Survival as a longitudinal trait can be analysed using survival analyses such as proportional hazard frailty models (Ducrocq and Casella, 1996), or by sequential threshold models utilising binary data for each test day (Ødegård et al., 2011c). Previous studies that have analysed disease tolerance data on aquatic animals using both binary and longitudinal trait definitions have mostly found genetic estimates are similar and that there is a high degree of correlation between family rankings using the different traits

(Gitterle et al., 2006b; Odegard et al., 2007; Ødegård et al., 2011b). However, survival analyses such as proportional hazards models assume that all individuals are susceptible and censored observations simply mean the death was not observed during the testing period but would occur at some point after (Ducrocq and Casella, 1996). This assumption may not always be the case where there are non-susceptible individuals within the population (Ødegård et al., 2011a). Using a cure model approach that takes into account non-susceptible individuals, which would ordinarily be censored observations in survival analyses, Odegard et al., (2011b) found substantial re-ranking of families when comparing endurance and susceptibility traits indicating that these are most likely different traits and under different genetic control.

Using various challenge protocols and statistical methods, heritability estimates for disease tolerance in shrimp have been reported mainly on two viruses, Taura Syndrome virus (TSV) and white Spot Syndrome virus (WSSV). For TSV, heritability ranges between 0.19-0.41 (Argue et al., 2002; Ødegård et al., 2011b; Moss et al., 2013) and significant improvements to TSV tolerance following selection have been observed (Argue et al., 2002; White et al., 2002). Conversely, very little additive genetic variation for tolerance to WSSV has been found (h^2 = 0.00 to 0.07) and very few reports of genetic gain for tolerance to WSSV in shrimp exist (Gitterle et al., 2005b, 2006a, 2006b; Hayes et al., 2010; Huang et al., 2011). In addition, most of the work carried out on breeding shrimp for improved disease tolerance has focused on survival as the trait evaluated and selected for. Other traits, such as viral load may be useful indirect measures of disease tolerance. As viral load in particular, is on a continuous scale, therefore more amenable to quantitative genetic analyses and further has been associated with survival in shrimp and fish disease challenge tests (Anantasomboon et al., 2008; Sauvage et al., 2009; Cao et al., 2010; Purcell et al., 2010).

In Australia, a large-scale breeding program for black tiger shrimp (*Penaeus monodon*) is under development and selection for greater tolerance to gill-associated virus (GAV) is a trait of interest. Gill-associated virus is an endemic virus that is highly prevalent in both wild and farmed stocks (Walker et al., 2001). Gill-associated virus causes significant production loss through mortality to the shrimp farming industry in Australia (Callinan et al., 2003; Munro et al., 2011). Given the high

prevalence of GAV and its impact of farm productivity and profitability, selective breeding for tolerance may be a useful strategy for managing GAV related disease. There are no reports on whether there is significant additive genetic variation for GAV tolerance and therefore it is unknown whether genetic selection to improve tolerance may be possible for the shrimp aquaculture industry. The aim of this study was to use for the first time in shrimp a communal rearing approach to challenge test families of *P. monodon* with GAV to evaluate disease tolerance. Genetic parameters for GAV induced mortality were estimated using two statistical approaches, defining mortality as either a binary trait (either dead or alive at the end of the challenge period) or a longitudinal trait (incorporating survival time and censoring). Additionally, GAV infection load of survivors post challenge was also collected to determine whether this trait could be used as an indirect measure of GAV disease tolerance measured as mortality.

5.2 Methods

5.2.1 Experimental animals

Penaeus monodon used in the study were sourced from a commercial hatchery in north Queensland, Australia. The broodstock were captured from wild stocks in Joseph Bonaparte Gulf, Australia and transported to the commercial hatchery where they underwent a combination of natural and artificial matings. Female broodstock were transferred into communal spawning tanks where nauplii were collected and transferred into two communal larval rearing tanks (20,000 L). Nauplii that were stocked into the two larval rearing tanks were spawned within four days of each other and reared in these tanks until post larvae stage 15 (PL15). At post larvae stage 6 (PL6), three pools of larvae (represented as pools of 300 mg) from each larval rearing tank were screened for the following pathogens GAV, Yellow head virus type 7 (YHV7), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Hepatopancreatic parvovirus (HPV) and pirA toxin gene for *Penaeus monodon* mortality syndrome (PmMS), using PCR and qPCR methods at the James Cook University AquaPath Laboratory, Australia. Low level (< 100 viral copies) of infections were detected for GAV

and YHV7, whilst no other pathogens were detected. Approximately 10,000 PL15 were transported to the CSIRO Bribie Island Research Centre (BIRC) and stocked into a single lined 200 m^2 pond using standard commercial rearing conditions (stocking density of 40 per m^2 and fed a commercial diet, Ridley Aqua Feed Australia). Following 67 days post stocking in the nursery pond, juvenile shrimp (5 -12 g) were collected by cast net from the pond and transferred into challenge facility tanks (2,000 L).

5.2.2 Challenge test

The GAV inoculum used for challenging shrimp was previously described (*Chapter 2.1.2.* and Noble et al., 2017) and *in vivo* titration experiments performed to define a suitable infection dose (*Chapter 4*). From the titration experiments, the inoculum diluted at 1:3000 was chosen as it resulted in a consistent moderate rate of mortality reaching 50% cumulative mortality by 14 days post challenge. A single 1 ml aliquot of the GAV inoculum was thawed and further aliquoted into single use (50 µl) tubes prior to being diluted and used for the challenge test. This ensured all shrimp were inoculated with inoculum from the same vial that had undergone the same number of freeze thaw cycles.

The GAV challenge test was carried out at the Bribie Island Research Centre, Australia, in 12 x 2000 L round-bottom tanks. Tanks were filled with ~1800 L filtered seawater on a flow-through water system (8 L per min) with water parameters maintained as 30 ppt salinity, 29.5 ± 0.6 °C and 5.8 ± 0.5 mg/L dissolved oxygen. Shrimp (n = 200) were stocked into each of the 12 experimental tanks and an additional 200 shrimp were caught and stocked into a spare 2000 L tank to replace any individuals that died following transportation, or were not in the required size range when weighed using an electronic scale (see below). Shrimp were allowed to acclimate in the challenge test tanks for 6-12 days (depending on experimental block) prior to being challenged and were fed a commercial diet (Ridley Aqua Feed, Australia) twice daily at 0800 and 1700 h at a rate of 5% total biomass initially and adjusted thereafter following any mortalities that occurred.

The challenge experimental design followed a randomised block design whereby the 12 tanks were separated into three experimental blocks of four tanks. Each block consisted of three GAV challenge treatment tanks and one control treatment tank. The three blocks were inoculated on separate days

using an individual aliquot of the inoculum diluted 1:3000. Shrimp were caught from their experimental tank and held in 2 x 100 L tubs with abundant aeration whilst shrimp were being inoculated. To inoculate shrimp, each individual was weighed (individuals within the size range of 5-12 g were kept) and injected with a weight standardised dose (5 μL g^{-1}) of the GAV inoculum diluted 1:3000 (challenge treatment) or shrimp saline solution (SSS, methods given in *Chapter 2..2.2*) (control treatment) into muscle tissue at the 2nd abdominal segment using a 100 μL Hamilton glass syringe fitted with a 26-gauge needle. Shrimp were given a unique eye tag (2.5 mm bird leg band; Bird Bands Pty Ltd) and a pleopod tip sampled and stored in RNAlater solution (Ambion) for total nucleic acid (TNA) extraction and subsequent genotyping. The eye tag was linked to the genotype to track individual mortality times. Shrimp were then returned to their experimental tank.

Shrimp were monitored three times per day (08:00, 12:30 & 17:00) at which times moribund/dead shrimp were removed and eye tags collected and recorded. The total challenge period for each block differed due to circumstances beyond the control of the study. The challenge period for Blocks 1, 2 and 3 were 19, 16 and 15 days respectively. At the termination of the challenge all survivors from each tank were recorded and gill tissue sampled and stored in RNAlater prior to reverse transcriptase quantitative PCR (RT-qPCR) analysis of GAV infection load.

5.2.3 Viral load quantification

Pre-challenge pleopod tip samples and gill tissue samples collected from survivors underwent the same TNA extraction protocol. Methods regarding TNA extraction and GAV load quantification were previously described in *Chapter 2*. The only adjustment was three gill filaments were used to quantify GAV infection load in the survivors and TNA extracted from pleopod samples for genotyping were diluted in RNase-free water to 50 ng/μL.

In addition to the survivors of the challenge test, a subset of the control shrimp (n = 94) were tested for the presence and loading of GAV, IHHNV and YHV7 and a subset of the challenged survivors (n = 94) were tested for presence and loading of IHHNV and YHV7. A sample size of 94 from the population size sampled from (n = 2600) provides a >95% confidence of detecting the pathogen

(Lightner, 1996). Total nucleic acid extracted from gill tissue samples as above were used for the IHHNV qPCR assay described by Cowley et al., 2018. For YHV7, synthesized cDNA as above was used in a qPCR test described by Cowley et al., (2015) and Mohr et al., (2015). To determine whether pre-existing infections may have affected the response of the challenge test, GAV infection loads were compared between the subset of control shrimp and a subset of challenge survivors.

5.2.4 Genotyping and pedigree analysis

Tissue samples (gill, pleopod, or muscle) were collected from broodstock, either prior to or following spawning, and preserved in RNALater solution until TNA isolation (as above). The broodstock were genotyped using a single nucleotide polymorphism (SNP) genotype by sequencing (GBS) approach (DArTseq; Sansaloni et al., 2011), whilst the offspring (challenged shrimp) were genotyped using a targeted 4K DArTcap custom SNP panel (4,194 SNPs) developed from the DArTSeq dataset (Guppy et al., 2018). All genotyping was performed at Diversity Arrays Technology Laboratory, Canberra, Australia. Data integrity was undertaken using a custom pipeline (github.com/esteinig/dartqc; described by Guppy et al., 2018), resulting in 2,305 high quality informative SNPs. The overall average SNP call rate per individual was 93.6% and the average minor allele frequency per SNP was 0.244 ± 0.003.

Family and pedigree relationships of the challenged shrimp were constructed using parent-progeny assignments in CERVUS version 3.0.7 (Kalinowski et al., 2007). For offspring with missing parents, either the broodstock was not sampled or had missing genotype information due to poor quality DNA, Colony version V2.0.6.4 (Jones and Wang, 2010) was used to assign the offspring to genetic groups based on the clustering of their genotypic fingerprints and an arbitrary parent ID was given.

5.2.5 Statistical analysis

Shrimp that were not confidently (> 95%) assigned to a family or with missing mortality data were removed from the data set prior to statistical analysis; the final data set included 1717 records. As the challenge duration of each experimental block differed slightly, mortality data was standardised to 15 days post challenge. Kaplan-Meier survival plots generated for each tank within each block and

differences in the survival curves were tested using a log-rank test. Kaplan-Meier survival analyses were performed using the survival package version 3.3 in R (R Core team, 2016).

Quantitative genetic analyses were performed to estimate variance components and breeding values for GAV challenge mortality and GAV infection load of the challenge survivors. Two trait definitions were used to analyse mortality which were, (1) as a binary trait with the response recorded as either dead or alive (1/0) at day 15 post challenge and (2) as a longitudinal trait that consisted of both the number of days until the mortality occurred, and whether or not the individual died or was censored (alive at day 15). For the GAV infection load trait measured on survivors, a normal linear model was used. Univariate models were used initially to obtain variance components, heritability estimates and estimated breeding values for each trait. A bivariate model with mortality as a binary trait and GAV infection load, similar to the univariate models, was performed to obtain covariance components to estimate genetic and phenotypic correlations between the two traits. The longitudinal trait using the Cox model could not be incorporated into bivariate models. Based on the family structure of the dataset which consisted of both full- and half-sib families, animal models were fitted which take into account all pairwise relationships based on pedigree information (Falconer and Mackay, 1996). ASReml-R V3 (VSNi) (Butler et al., 2009) was used to perform the univariate binomial and linear mixed models, ASReml V4 (Gilmour et al., 2015) was used to perform the bivariate analysis and Survival kit V6_12 (Meszaros et al., 2013) was used to fit Cox's proportional hazards model when mortality was defined as a longitudinal trait.

The univariate models for each trait were;

Binomial animal model for mortality as a binary trait (Binomial):

$$\log\left(\frac{p_{ijkl}}{1-p_{ijkl}}\right) = \mu + \beta_{BW}BW_i + block_k + tank_l + a$$

Where p_{ijkl} is the probability of the shrimp dying during the challenge period; μ is the overall mean; BW_i is the fixed covariate of body weight (BW) and β_{BW} is the regression coefficient associated with BW; $block_k$ and $tank_l$ are the random effects of the challenge block (3 levels) and challenge tank

nested within block (9 levels); and **a** is the random animal genetic effects assumed **a** ~ *MVN* $(0, \boldsymbol{A}\sigma^2_{animal})$, where **A** is the numerator relationship matrix using pedigree based analysis.

Cox's proportional hazards frailty model for mortality as a longitudinal trait (Cox):

$$\log h_{ijl}(t) = \log h_0(t) + (\beta_{BW} BW_i + tank_l + a)$$

Where $h_{ijk}(t)$ is the hazard function for the ith individual at time t, $h_0(t)$ is the unspecified baseline hazard function, BW_i; β_{BW}; $tank_l$ and **a** are as in the binomial model. The current version of Survival Kit is unable to fit nested variables, thus only tank was included in the model which captures variance due to block.

Linear animal model for GAV infection load of the challenge survivors (GAV load):

$$GAV_{ijkl} = \mu + \beta_{BW} BW_i + block_k + tank_l + a$$

Where GAV_{ijkl} is the survivors GAV infection load (GAV copies μg^{-1} TNA) for the ith individual and all other terms are the same as in the above models.

The significance of the fixed covariate of body weight was tested for each model using Wald-F statistics (within the two programs). Heritability of each trait was estimated using variance components extracted from corresponding univariate models fitted above. For mortality using the binomial model heritability was calculated as $h^2 = \frac{\sigma^2_a}{\sigma^2_a + \sigma^2_{block} + \sigma^2_{tank} + \pi^2/3}$, where σ^2_a, σ^2_{block} and σ^2_{tank} were the variance attributed to additive genetic, experimental block and tank effects respectively, and $\pi^2/3$ or 3.29 is the fixed residual variance for binomial models (Falconer and Mackay, 1996). For the longitudinal mortality trait using Cox's proportional hazards model, heritability was estimated on the log-hazard scale where $h^2_{logt} = \frac{\sigma^2_a}{\sigma^2_a + \sigma^2_{tank} + \pi^2/6}$ and $\pi^2/6$ is the fixed residual variance for survival analysis (Ducrocq and Casella, 1996). For GAV infection load of the survivors using a linear mixed model, heritability was calculated as $h^2 = \frac{\sigma^2_a}{\sigma^2_a + \sigma^2_{block} + \sigma^2_{tank} + \sigma^2_\varepsilon}$, where σ^2_ε is the random residual error variance. Genetic correlations (r_g) were calculated from the bivariate analysis of mortality (binary

trait) and GAV infection load as $r_g = \frac{cov(A_1A_2)}{\sqrt{(\sigma^2_{A_1} \times \sigma^2_{A_2})}}$, where $cov(A_1A_2)$ is the additive genetic covariance and $\sigma^2_{A_1}$ and $\sigma^2_{A_2}$ are the additive genetic variances for trait 1 and 2 respectively. Standard errors for heritability estimates obtained from univariate models were calculated following the equation of Van Vleck (2000), however, for the Cox model standard error could not be estimated from the output given by Survival Kit V6_12.

To assess the agreement between the two statistical models and trait definitions of mortality following GAV challenge, the correlation between sire (used as a proxy for family) EBVs was estimated using Pearson's correlation coefficient (r_{EBV}). Similarly, to evaluate the accuracy and repeatability of the challenge and each model to predict family performance, the data was subset by experimental block (n = 3) and variance components as well as breeding values were estimated for each block independently using both mortality models. The correlation between sire EBVs from each experimental block was estimated using Pearson's correlation coefficients and is closely related to the accuracy of selection (r_τ) (Gitterle et al., 2006b; Hayes et al., 2010). Correlations of sire EBVs were also estimated separately for days 5 and 10 p.c. to assess whether predicted family performance rankings change during the challenge period.

5.3 Results

5.3.1 Viral screening

From the subset of control shrimp that were screened for GAV, as representative of pre-existing GAV infection levels within the population, 79% tested positive with a mean infection load (on log10 scale) of 1.89 ± 1.04 GAV copies μg^{-1} TNA. All (100%) challenge survivors were GAV positive and had a mean infection load of 6.02 ± 1.33 GAV copies μg^{-1} TNA. Gill-associated virus levels detected in the challenge survivors were approximately 10,000 fold higher than the pre-existing GAV levels detected in the control shrimp. Infectious hypodermal hematopoietic necrosis virus was detected in 66% of control shrimp with a mean infection load (on log10 scale) of 1.76 ± 1.17 IHHNV copies μg^{-1} TNA

and in 100% of the challenged survivors tested (n = 94), which had a mean infection load of 2.15 ± 0.64 IHHNV copies μg^{-1} TNA. Infectious hypodermal hematopoietic necrosis virus levels were significantly higher in the challenge survivors compared with the control shrimp ($t = 4.29$, $P < 0.0001$), but were significantly lower than the GAV levels in the challenge group ($t = -26.70$, $P < 0.0001$). Individual GAV and IHHNV loads detected in shrimp were not correlated in either the control or subset of challenged group survivors ($r = 0.13$, $r = -0.02$, respectively; $P > 0.05$). Yellow head virus type 7 was not detected in either the control or challenge shrimp tested. Given the high survival and relative lower GAV infection prevalence and load of shrimp in the control groups, it was determined that any pre-existing infection of GAV or IHHNV would have had a minor impact on the outcome of the challenge test. Further, it must be noted that in Australia at the time of this study there were no available pathogen free stocks for these viruses and shrimp appeared healthy (high survival and growth rate in pond) prior to challenge testing. Ideally, disease challenge tests would be performed on individuals that are certified pathogen free.

5.3.2 Pedigree assignment

The final data set for genetic evaluation included 1717 individuals that were assigned to a full-sib family group (95% confidence) and had reliable mortality data recorded (Table 5.1). The total number of full-sib families identified in the challenge test shrimp was 72, from 48 dams and 56 sires. Within the 72 full-sib families, there were 42 maternal half-sib families (from 18 unique dams) and 30 paternal half-sib families (from 14 unique sires). The number of individuals within each full-sib family was highly variable, ranging from 1 – 176, and the average number of individuals per family was 23.8 ± 33.7.

5.3.3 Overview of challenge test

Mortality of challenge treatment tanks, standardized to 15 days p.c., within each block ranged from 35.0 – 35.9 %, with the overall mortality across blocks and tanks being 35.5% (Table 5.1). The mean days to death within each block ranged from 6.5 ± 3.4 in Block 1 to 8.5 ± 3.8 in Block 2, with the overall mean days to death 7.6 ± 3.8 (Table 5.1). Based on Kaplan-Meier survival analysis (log rank

test), there was no significant difference in survival curves between the three blocks ($\chi^2 = 0.1$, $P = 0.952$). A significant difference was found between tanks within Block 3 ($\chi^2 = 8.4$, $P < 0.05$), but not between tanks within the other two blocks (Block 1, $\chi^2 = 2.9$, $P = 0.231$; Block 2, $\chi^2 = 0.3$, $P = 0.856$) (Fig. 5.1). Survival in the control tanks at the termination of the challenge for each block was high; 96%, 95% and 97% for Blocks 1, 2 and 3, respectively. Kaplan-Meier survival curves were plotted for the overall cumulative mortality, as well as for the best and worst performing families with at least 10 offspring challenged (Fig. 5.2). A log rank test of family survival using those with 10 or more offspring challenged (number of families n = 37, number of individuals n = 1617) indicated significant variation in survival following GAV infection among families ($\chi^2 = 136$, $P < 0.001$). The percent mortality within each family (with 10 or more offspring) combined across blocks and tanks ranged from 0% to 71%. Using the most abundant shrimp family (number of offspring challenged = 176), Kaplan-Meier survival curves were plotted for the progeny from the same family within each challenge tank across the three experimental blocks (Fig. 5.3.). A log rank test of the single family's survival within the replicate blocks and tanks revealed no significant differences among the three experimental blocks ($\chi^2 = 1.8$, $P = 0.414$) or among challenge tanks ($\chi^2 = 5.7$, $P = 0.680$).

Table 5.1. Overview of *Penaeus monodon* gill-associated virus challenge data standardized to 15 days post challenge including; the number of shrimp used in the analyses, the number of shrimp that died during the challenge period, percent mortality and the mean number of days to death for the shrimp that died within the challenge period.

	Total number of shrimp	Number of dead shrimp	Mortality (%)	Mean days to death ± s.d.
Block 1	574	201	35.0	7.5 ± 3.4
Block 2	579	208	35.9	9.5 ± 3.8
Block 3	564	201	35.6	8.8 ± 3.9
Total	1717	610	35.5	8.6 ± 3.8

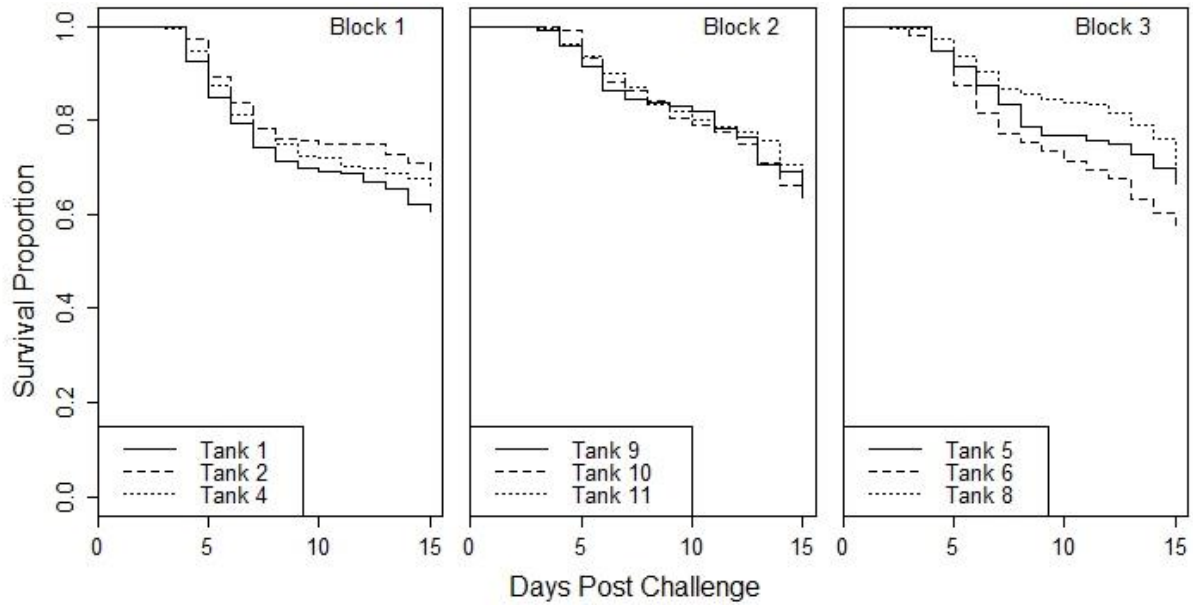

Figure 5.1. Kaplan-Meier survival curves of *Penaeus monodon* challenged with gill-associated virus. Each line represents the survival curve of each tank within the three challenge blocks standardised to 15 days post challenge.

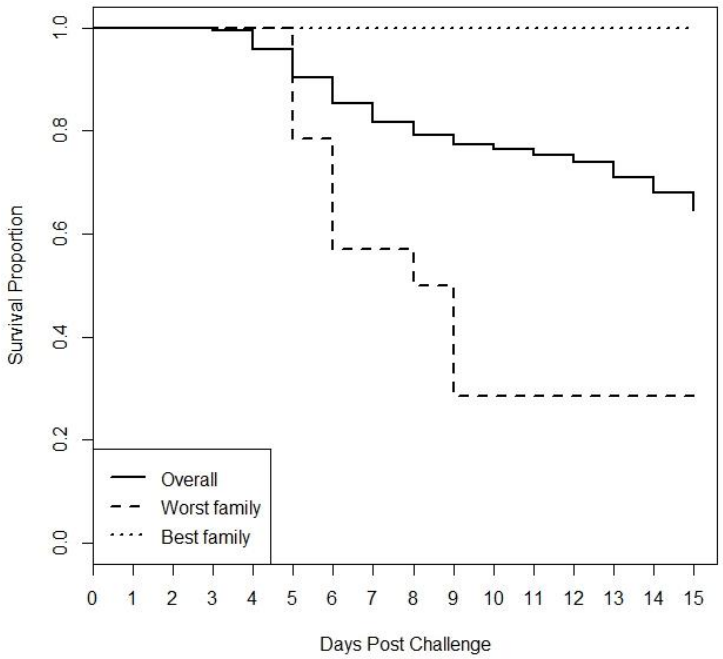

Figure 5.2. Kaplan-Meier survival curves of *Penaeus monodon* challenge with gill-associated virus, including the overall survival of all individuals (n = 1717) and survival of the worst (n = 14, 29% survival) and best performing families (n = 14, 100% survival) from full-sib families that had 10 or more offspring challenged (n = 37).

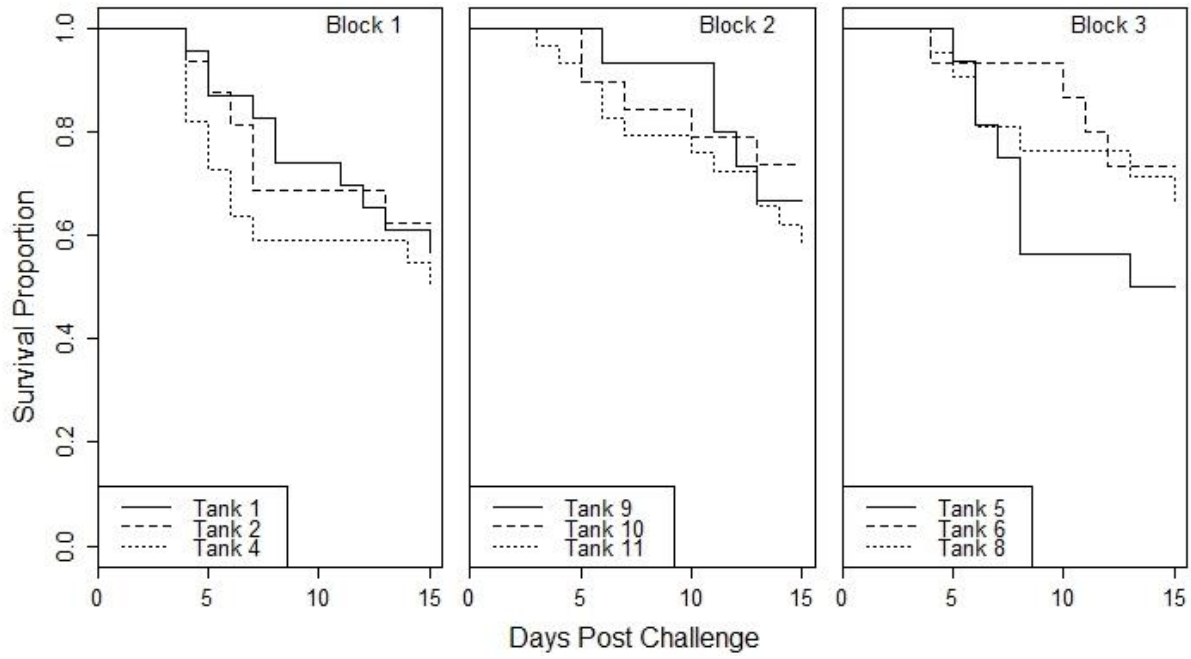

Figure 5.3. Kaplan-Meier survival curves of offspring (n = 176) from a single *Penaeus monodon* family (Fam 01) challenged with gill-associated virus in each replicate challenge tank (n = 9) within the three experimental blocks.

5.3.4 Genetic parameters

Variance components and heritability for mortality were estimated using either a binomial or Cox model. Heritability of mortality was low using both models with the Cox model resulting in a slightly higher estimate ($h^2 = 0.14$) compared with the binomial model ($h^2 = 0.11 \pm 0.03$) (Table 5.2). Variance due to block or tank were small and non-significant (based on z-ratios) in both models. Shrimp body weight had a significant negative effect on mortality in both models (Table 5.2), meaning smaller shrimp had a higher risk of mortality despite receiving a weight standardised challenge dose of GAV. Sire EBVs from the binomial model are provided in Fig. 5.4 to illustrate the spread of predicted family performance. Pearson's correlation between sire EBVs from each model was almost at unity, $r_{EBV} = 0.99$, meaning there was no re-ranking of family performances due to the model used.

Table 5.2. Estimated additive genetic variance (σ_a^2), variance due to block (σ_{block}^2) and tank (σ_{tank}^2), the random residual error variance σ_ε^2, heritability and the effect of body weight (BW) covariate for both mortality models (Binomial and Cox) and GAV infection load of *Penaeus monodon* challenged with gill-associated virus.

Model	σ_a^2 ± SE	σ_{block}^2 ± SE	σ_{tank}^2 ± SE	σ_ε^2 ± SE	h^2 ± SE	BW ± SE
Mortality Binomial	0.40 ± 0.13	0.01 ± 0.02	0.00 ± 0.00	$\pi^2/3$	0.11 ± 0.03	-0.07 ± 0.03*
Mortality Cox	0.27	NA	0.03	$\pi^2/6$	0.14	-0.07 ± 0.03**
GAV Load	0.40 ± 0.14	0.19 ± 0.20	0.01 ± 0.01	1.30 ± 0.11	0.21 ± 0.07	ns

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

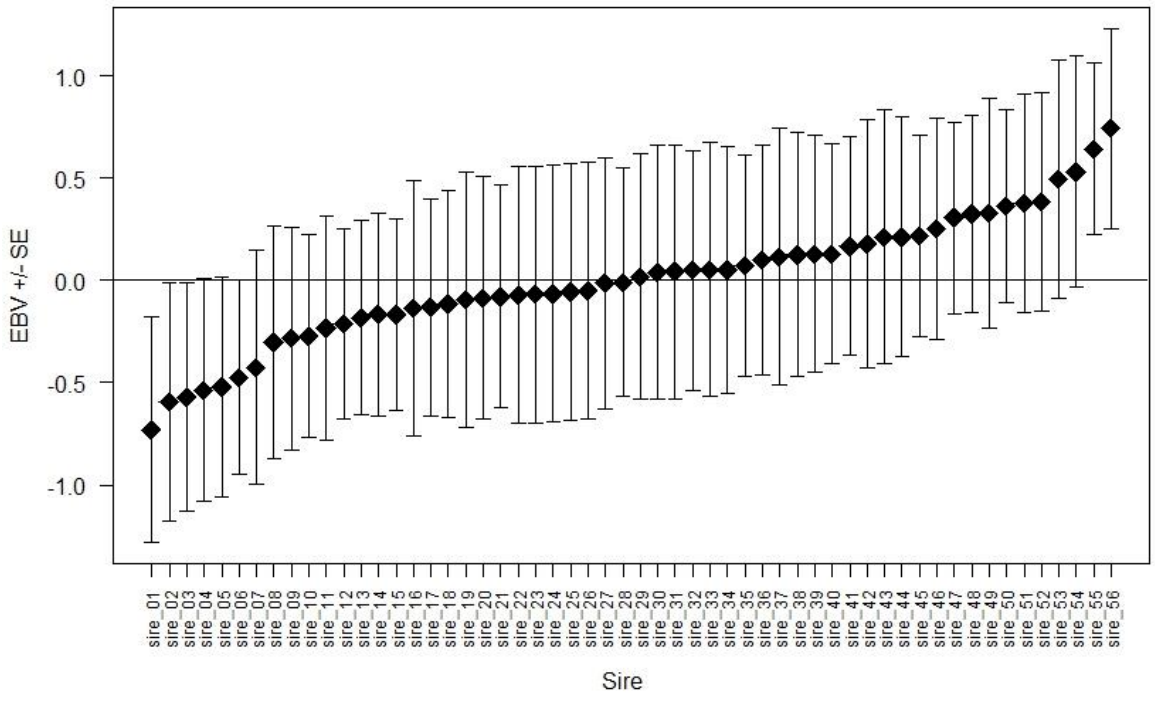

Figure 5.4. *Penaeus monodon* sire estimated breeding values (EBV ± SE) for mortality following challenge with gill-associated virus using a binomial animal model.

Using the binomial model, variance components and heritability were estimated for each day of the challenge test to assess whether variance components were stable over the challenge period. This was not performed for the Cox model, however, as time until death is accounted for in the model. Estimates did not vary greatly across the challenge period, particularly from day 7 to day 15 where heritability ranged between 0.11 and 0.14 (Table 5.3). Using sire breeding values estimated independently for days 5, 10 and 15, Pearson's correlation coefficients were calculated to assess whether the challenge duration affected prediction of family performance (Table 5.4). The correlation coefficients between each of the days compared were moderate to high, with the highest correlation 0.85 between days 10 and 15.

Table 5.3. Variance components and heritability estimates using the binomial model for gill-associated virus (GAV) induced mortality defined as a binary trait on each day of the challenge period up to day 15. Days 1 and 2 were not estimated due very few deaths occurring during this time which were also most likely due to handling stress and not related to GAV infection.

Days p.c.	σ_a^2 ± SE	σ_{block}^2 ± SE	σ_{tank}^2 ± SE	h^2 ± SE	No. shrimp alive	No. shrimp dead
3	0.28 ± 1.73	0.41 ± 1.01	0.09 ± 0.14	0.07 ± 0.39	1696	9
4	0.75 ± 0.45	0.00 ± 0.00	0.01 ± 0.02	0.18 ± 0.09	1633	72
5	0.29 ± 0.19	0.00 ± 0.03	0.01 ± 0.01	0.08 ± 0.05	1532	173
6	0.36 ± 0.17	0.00 ± 0.02	0.01 ± 0.01	0.10 ± 0.04	1444	261
7	0.43 ± 0.17	0.01 ± 0.03	0.01 ± 0.01	0.11 ± 0.04	1380	325
8	0.44 ± 0.17	0.00 ± 0.02	0.01 ± 0.01	0.12 ± 0.04	1340	365
9	0.56 ± 0.18	0.00 ± 0.02	0.01 ± 0.01	0.14 ± 0.04	1308	397
10	0.50 ± 0.17	0.00 ± 0.00	0.01 ± 0.01	0.13 ± 0.04	1290	415
11	0.44 ± 0.15	0.00 ± 0.00	0.01 ± 0.01	0.12 ± 0.04	1266	439
12	0.41 ± 0.15	0.00 ± 0.00	0.01 ± 0.01	0.11 ± 0.04	1242	463
13	0.44 ± 0.15	0.00 ± 0.00	0.01 ± 0.01	0.12 ± 0.03	1191	514
14	0.40 ± 0.14	0.00 ± 0.00	0.01 ± 0.01	0.11 ± 0.03	1142	563
15	0.42 ± 0.13	0.01 ± 0.02	0.00 ± 0.00	0.11 ± 0.03	1080	625

Table 5.4. Pearson's correlation coefficients between sire estimated breeding values for gill-associated virus mortality in *Penaeus monodon* on days 5, 10 and 15 post challenge.

Days Post Challenge	Day 5	Day 10	Day 15
Day 5	-	0.75***	0.60***
Day 10		-	0.85***

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Pearson's correlation coefficients of sire breeding values estimated independently for each block (n = 3) were used to assess the repeatability of the challenge and effect of models applied. All models resulted in similar correlations between each block and all were significantly different from zero (Table 5.5). The highest correlations were observed between Block 1 and 2 using the Cox model with a correlation of 0.61. Block 2 and 3 had the lowest correlation, ranging between 0.30-0.32 depending on the model used.

Table 5.5. Pearson's correlation coefficients between sire estimated breeding values for gill-associated virus induced mortality estimated independently for each of the three experimental blocks and using two different statistical models.

Model	Block 1 v 2	Block 1 v 3	Block 2 v 3
Binomial	0.53***	0.37*	0.38**
Cox	0.58***	0.38*	0.35*

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Gill-associated virus infection was quantified by RT-qPCR from the challenge survivors to assess whether this trait could be used as an indirect measure of GAV induced disease. All survivors were found to be GAV positive with individual infection loads ranging from 7.20 $x10^2$ to 8.03 $x10^8$ $\log_{10}$ GAV copies µg TNA. Mean GAV infection loads among full-sib families with 10 or more survivors (n = 30) varied from 4.91 ± 1.34 to 7.32 ± 1.22 $\log_{10}$ GAV copies µg TNA (Fig. 5.5). Heritability of

GAV infection load was moderate, $h^2 = 0.23 \pm 0.08$ (Table 5.2) and a weak positive genetic correlation was found between mortality (binary trait) and GAV infection load, $r_g = 0.30 \pm 0.23$ and $r_{EBV} = 0.17^{ns}$ (Fig. 5.6). A positive correlation between mortality and GAV infection load indicates shrimp families with lower risk of mortality were associated with lower GAV infection loads.

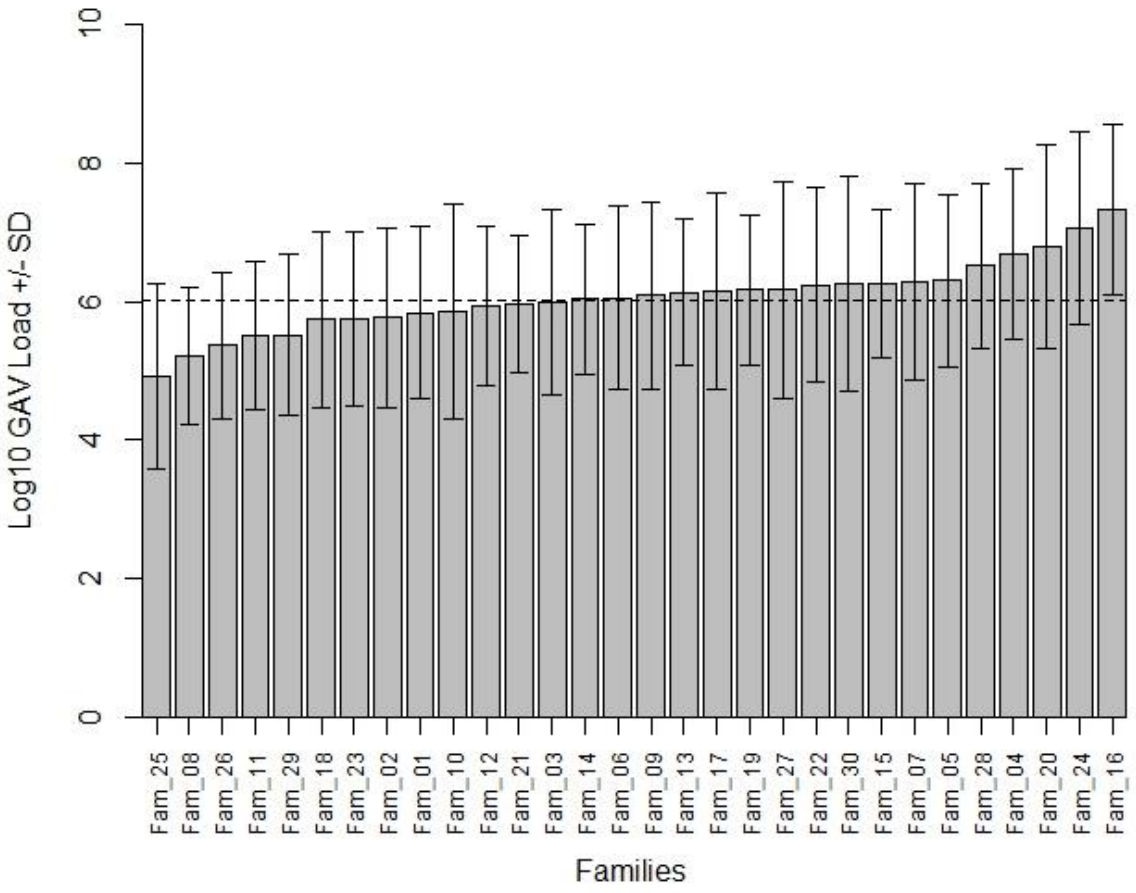

Figure 5.5. Mean (± s.d.) gill-associated virus (GAV) infection load (log_{10} GAV copies μg^{-1} TNA) of *Penaeus monodon* challenge test survivors from 30 full-sib families (with at least 10 offspring). The dashed line represents the overall mean GAV infection load among families (6.02 ± 1.33 log_{10} GAV copies μg^{-1} TNA) and family ID is based on family prevalence among survivors (i.e. Fam_01 is the most abundant family).

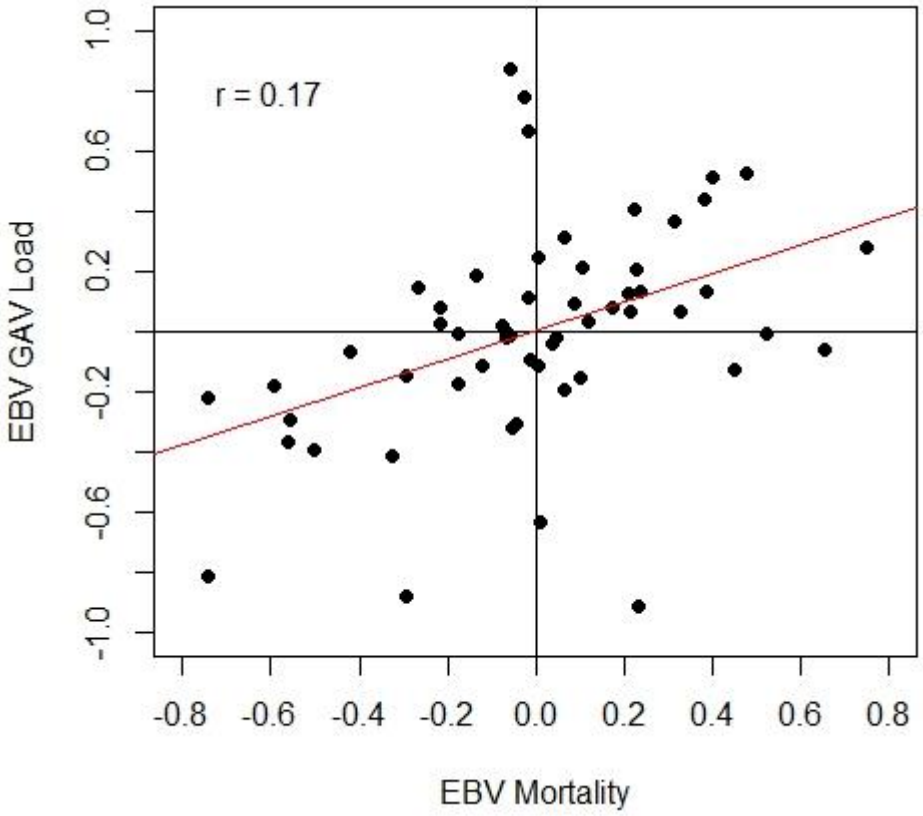

Figure 5.6. Scatter plot of *Penaeus monodon* sire estimated breeding values (EBV) for traits mortality (binomial model) and gill-associated virus (GAV) infection load following challenge GAV challenge testing.

5.4 Discussion

Disease is the biggest issue facing shrimp farming globally. Therefore, efforts to reduce production losses associated with disease is paramount to a sustainable and profitable industry. Selective breeding for improved disease tolerance is one strategy that may help to combat such losses through increased survival (Odegard et al., 2011a). However, selective breeding for a trait, like disease tolerance, is only possible if it is heritable. This study provides the first estimates of heritability for GAV tolerance using a unique challenge design where by families of shrimp were communally reared from spawning through to challenge testing.

Heritability of GAV tolerance, measured as mortality under controlled challenge conditions, ranged from 0.11 to 0.14. Heritability estimates for GAV tolerance are slightly lower than reports for TSV tolerance in *P. vannamei*, which ranges between 0.19 and 0.41 (Argue et al., 2002; Ødegård et al., 2011b; Moss et al., 2013), but were higher than those reported for WSSV, which are typically less than 0.1 or not significantly different form 0 (Gitterle et al., 2005b, 2006a, 2006b; Hayes et al., 2010). Two different statistical approaches were used to evaluate GAV-induced mortality. These were as a binary trait (binomial model) of whether or not the individual survived or died, or as a longitudinal trait that accounts for both time until death and censored observations (Cox model). The Cox model resulted in a slightly higher heritability estimate and suggests the added information on time until death and censoring information slightly improved the ability to partition genetic effects from random environmental effects compared to the binary trait. The two models were compared by correlating predicted family performance by the way of EBVs, which was close to unity, $r_{EBV} = 0.99$. Therefore, the different models had an insignificant effect on predicted family performances. This is consistent with the findings of Ødegård et al., (2011b) in which correlations between binary and longitudinal models for susceptibility to TSV were above 0.98. Thus, in our study time until death did not appear to be a major factor in contributing to genetic variation for GAV tolerance. This may be because the overall mortality at day 15 was 35.5% and therefore of greater effect was whether the individual survived or died rather than when the individual died. Time of death may be more important for data sets with very high mortality such as for WSSV (Ødegård et al., 2011b). For WSSV, mortality often reaches 100% for all families, consequently larger differences in family rankings were observed between binary (at the point of 50% mortality) and longitudinal models, $r \approx 0.89$ (Gitterle et al., 2006b). In the case for GAV, there is high concordance between the two models used and little advantage between statistical approaches that use binary or longitudinal traits. The use of a binary trait is operationally simpler, allows for multi-variate analyses to be performed and given the high genetic correlations between challenge days, challenge tests could be performed for a standardised time duration that is convenient from an operational point of view. Of greater concern is to incorporate more families with sufficient progeny numbers to ensure accurate measurement of the trait, mortality.

Body weight was found to have a significant effect on mortality in both mortality models. The risk of mortality increased with decreasing weight, meaning that smaller shrimp were more susceptible to GAV. This might suggest selecting larger shrimp may also increase tolerance to GAV. However, this requires further investigation and a genetic correlation between the two traits will determine whether GAV tolerance and body weight are linked genetically. Correlations between growth and tolerance for other shrimp diseases tend to be unfavourable, with larger shrimp often more susceptible to the disease (Lotz, 1997; Argue et al., 2002; Gitterle et al., 2005b; Moss et al., 2013); thus careful management of both traits need to be considered for a selective breeding program.

The reliability of the challenge and statistical methods are critical for the success of genetic improvement of any trait in a selective breeding program. To assess the accuracy and repeatability of the challenge methods and statistical models at predicting family performance, family breeding values were independently estimated for each of the three experimental blocks. The blocks represent independent GAV challenge tests on the same group of families and thus allows us to evaluate the accuracy of selection among these groups. Ideally assessing the accuracy of selection using challenge tests would be carried out by assessing the accuracy of predicting field performance following an outbreak of the disease (Ødegård et al., 2006). In the absence on this data, the correlation of family EBVs between replicated blocks is a good alternative. In this study, correlations of family EBVs ranged between 0.35-0.58. These correlations are lower than those found for WSSV tolerance (Gitterle et al., 2005b, 2006b). A reason for lower correlations between blocks in this study could be due to uneven and substantially lower numbers of individuals within each family across the blocks which will likely affect the accuracy of EBVs. Families could not be stocked evenly across blocks as pedigree was realised from genotyping after the challenge. However, when EBV correlations were performed between blocks using only those families with at least 10 individuals represented in each block, the correlation coefficients differed marginally (and did not necessarily improve the correlation) to when all families were included. Further, there were only minor differences in the block correlations between the two statistical models used to analyse mortality. In other studies, significant improvements in the accuracy of selection were found for models that incorporated

survival as a longitudinal trait (Gitterle et al., 2006b; Ødegård et al., 2007), or when more advanced genomic relationship information is used (e.g Correa et al., 2017). For example, Gitterle et al., (2006b) found a 12% increase in selection accuracy when the Cox proportional hazard model was applied compared to a binary linear model at 50% mortality.

As survival is quite a complex trait that is influenced by numerous (many unknown) factors and can be difficult and expensive to measure, alternative measurements that are relatively easy to measure would be beneficial. Additionally, disease may not always manifest entirely through survivorship, rather through other traits such as direct impact on growth, or saleability of the product. Alternative measures that may be used to indirectly select for disease tolerance will only be useful if they are easy to measure, heritable, highly correlated with the disease response and preferably not adversely genetically correlated with other production traits (Moss et al., 2005). Viral load is one measure that is easily and reliably measured on a continuous (quantitative) scale. Gill-associated virus infection load in this study was found to be moderately heritable ($h^2 = 0.23 \pm 0.08$) based on samples from survivors only. However, the genetic correlation ($r_g = 0.30 \pm 0.23$) and correlation of sire EBVs ($r_{EBV} = 0.17$) between GAV load and mortality (binary trait) were weak with a high standard error and were not significant. If the correlation was stronger, the positive relationship between the two traits would indicate shrimp families that had a higher risk of mortality were correlated with higher GAV infection loads. The limitation of this study was that GAV load was only measured on the survivors of the challenge test and loading of those that died was unknown. It is presumed that the shrimp that died during the challenge would have had higher GAV loads than those that survived, as higher viral loads have been shown to be correlated with increased or early mortality in several different aquaculture species (Jorgensen et al., 2008; Cao et al., 2010; Degremont, 2011; Huang et al., 2011; Oden et al., 2011). If samples were collected from dead/moribund shrimp that had died recently, the correlation between mortality and GAV load may have been stronger. In future studies, more frequent observations and removal of dead/moribund shrimp should be performed so that viral load data can be collected on these shrimp also.

A difficulty during the challenge was controlling cannibalism. *Penaeus monodon* is highly cannibalistic with moribund or dead shrimp cannibalised very quickly, thus exposing the survivors to repeated and higher GAV exposure. This may affect the accuracy of genetic estimates for both mortality and GAV load as the challenge dosage would no longer be standardised. Shrimp were checked three times daily (08:00, 12:30 and 17:00) at which times, particularly in the morning, it was obvious that shrimp had been cannibalised. More frequent removal of moribund and dead shrimp would reduce cannibalism and potentially increase the accuracy of genetic estimates obtained; although, in a communal challenge environment, such as that used in this study, it would be assumed that the probability of a shrimp cannibalising moribund or dead shrimp would be random among families and therefore genetic estimates are unlikely to be biased upwards. Additionally, another approach to reduce effects of cannibalism could be to shorten the duration of the experiment and in doing so limit the number of deaths recorded later that may be due to secondary exposure to GAV via cannibalism. However, the correlation between family EBVs estimated on day 15 with earlier days (day 5 and 10) suggest a shorter challenge duration would have had only a small impact on family rankings, particularly if the challenge was terminated on day 10 (r_{EBV} = 0.85). Thus, later deaths that occurred in the challenge that may have been due to re-exposure through cannibalism likely only had a small impact on the genetic estimates attained.

Finally, use of disease challenge tests to evaluate and select families that are more tolerant will only be of value if tolerance selected for under challenged conditions results in improved tolerance under field exposure (Robinson et al., 2017). There are currently no studies that have estimated genetic correlations to investigate this in shrimp. To test this, families of shrimp would need to be split prior to stocking into farm ponds with a subset undergoing disease challenge testing whilst the others evaluated for pond survival particularly in the presence of the pathogen. Using this methodology with *P. vannamei*, Moss et al., (2005) found phenotypic correlations of 0.55 and 0.68 for mean family survival during TSV challenge tests and during grow out on a commercial farm with TSV present; however, they did not estimate genetic correlations of the trait in the two environments. Given that TSV is no longer considered a major threat to the shrimp farming industry following the development

of resistant/tolerant lines (Cock et al., 2009; Moss et al., 2012), it is assumed the use of disease challenge tests do provide improvement in the field in the case of TSV. In other aquaculture species, such as salmon (*Salmo salar*) and Pacific oysters (*Crassostrea gigas*), genetic correlations between challenge and field survival are high > ~0.70 (Ødegård et al., 2006; Wetten et al., 2007; Degremont et al., 2015). This level of genetic correlation suggests only a slight genotype by environment interaction between challenge and field performance, however, in general the top performing families should perform well in both environments and thus selection of these families under challenge should improve tolerance in the field. The significant heritability found for GAV tolerance under challenge conditions suggests that positive improvement could be made via selection, the next step in investigating the utility of selection would be to validate family performance against that in commercial ponds with GAV present using genetic correlations.

In conclusion, this was the first study to our knowledge that utilised a communal mixed family design for large-scale disease tolerance testing under experimental challenge in shrimp. The results using this experimental design indicate that GAV tolerance measured as mortality can be improved using controlled challenge testing and selective breeding. This was evident by the considerable variation in family mortality and significant heritability estimates. The results from GAV infection loads measured in survivors suggests this approach would not be useful as an indirect measure of mortality given the low genetic correlation between the two traits. Future studies should aim to capture data from susceptible (i.e. dead) shrimp which may provide a better assessment of whether GAV load could be used to indirectly select for more GAV tolerant shrimp. Given that GAV tolerance is heritable under controlled challenge conditions, it is important to understand how GAV is associated with other commercially important traits, as well as to validate family performance in the challenge with disease tolerance in the field before selection of this trait.

Chapter 6. Correlations between gill-associated virus tolerance traits during controlled challenge testing and pond performance traits in the Black tiger shrimp, *Penaeus monodon*

6.1 Introduction

In shrimp aquaculture, the main traits typically selected for are growth, survival and tolerance to specific pathogens (Gjedrem and Baranski, 2009; Moss and Moss, 2009). This is because these traits have direct impacts on harvest yields and drive profitability for the farmer. A selective breeding program may focus on improving one or multiple traits. In either case, it is important for the breeder to know how traits are associated (specifically genetically correlated) so that selection can be optimised and commercially important traits not adversely impacted. Few studies have investigated the correlation between specific disease tolerance and growth traits in shrimp. In shrimp this relationship typically appears to be unfavourable. For example, in *Penaeus vannamei* the genetic correlation (r_g) between WSSV tolerance (measured under experimental challenge) and harvest weight (under commercial conditions) was -0.55 and -0.64 for the two lines evaluated (Gitterle et al., 2005b). Similarly, for TSV tolerance and growth, the genetic correlation was -0.46 (Argue et al., 2002). In the latter example, two separate breeding lines were established to improve both traits individually (Argue et al., 2002). In addition to understanding the correlation between specific disease tolerance and growth, an understanding of how disease tolerance measured under controlled challenge conditions relates to performance during exposure to the pathogen on-farm is critical (Robinson et al., 2017).

Improving disease tolerance to specific pathogens would ideally be carried out by assessing and selecting individuals under commercial culture conditions (Ødegård et al., 2006). However, in practise this is difficult, as on-farm disease outbreaks may be sporadic, involve multiple pathogens and environmental conditions that often trigger the onset of disease and may not be standardised between ponds, across production seasons, or among generations (Robinson et al., 2017). For robust

genetic analysis, and to be able to measure fine-scale genetic differences, these factors must be standardised. This is why controlled challenge tests are commonly used to evaluate disease tolerance (Cock et al., 2009). However, making use of data that can be easily collected from commercially reared animals where thousands of individuals can be phenotyped relatively efficiently is an attractive option for large-scale disease evaluation and selection (Robinson et al., 2017). For example, a "gill score" methodology based on categorising the extent of damage caused by the parasite *Neoparamoeba* sp. to the gills of Atlantic salmon (*Salmo salar*) is readily collected from commercially reared fish and used as an indirect measure of amoebic gill disease tolerance in selective breeding programs (Taylor et al., 2009a, 2009b; Robledo et al 2018). In a similar fashion, viral infection load from commercially reared animals may also be a useful indirect measure of disease tolerance.

Viral load has been shown to have a clear relationship with mortality in several aquatic species (Jorgensen et al., 2008; Cao et al., 2010; Degremont et al., 2011; Huang et al., 2011; Oden et al., 2011). Natural disease outbreaks relating to GAV have also been associated with increased GAV prevalence and infection loads during commercial culture (Munro et al., 2011); although it is unknown on an individual level how these measures correlate with tolerance and whether they have an additive genetic basis. Measuring viral infection loads from commercially reared shrimp can be easily incorporated into large-scale collection of other important phenotypes, such as body weight, by collecting tissue samples and using qPCR to quantify the number of viral copies. In fact, hepatopancreatic parvo-like virus (HPV) infection load was recently investigated for its utility as a trait to estimate HPV resistance in farmed banana shrimp, *Fennerpenaeus merguiensis* (Knibb et al., 2015; Phuthaworn et al., 2016). Infection load of HPV was found to be moderately heritable ($h^2 \approx 0.40$); however, both studies likely overestimated the additive genetic component associated with viral load, as the study design incorporated only full-sib families and they did not account for potential maternal effects in the form of confounding vertical transmission of the virus from parent to offspring. Additionally, it is unknown whether HPV load is genetically correlated with disease traits like survival, or whether they are genetically separate traits. Viral load is thought to reflect an animals

ability to limit the pathogen burden and is referred to as host resistance, whereas an animals ability to maintain performance without necessarily reducing pathogen burden is referred to as host tolerance (Ayres and Schneider, 2008). Therefore, survival following challenge with a pathogen could be due to both or either of these defence mechanisms, but the two are generally found to be weakly genetically correlated (Kause and Odegard, 2012). Despite viral load from GAV or HPV infection being heritable, there is thus still a lack of understanding on the genetic correlation between viral load and survival in shrimp, as well as with other commercially important traits. Understanding how viral load is related to survival during disease outbreaks, or controlled challenge testing, will help inform decisions on the most suitable traits for selection.

In *Chapter 5*, GAV tolerance measured under controlled challenge conditions was shown to be heritable ($h^2 = 0.11 – 0.14$) and thus potentially suited to a selective breeding program for *Penaeus monodon*. Gill-associated virus infection load was also found to be heritable, but weakly correlated with mortality. The weak correlation between GAV infection load and mortality in the challenge test may have been due to how GAV load was measured (i.e. from the survivors only). However, before incorporating any trait for selection it is critical to understand how it is associated with other commercially important traits, and to understand how disease tolerance measured under controlled challenge conditions are associated with disease related traits under commercial conditions. Therefore, the aim of this chapter was to assess the correlation between GAV disease traits (mortality and viral load), measured using controlled challenge tests (*Chapter 5*), with commercial production traits, body weight and GAV infection, measured from siblings reared under commercial conditions.

6.2 Methods

This study used siblings from the same spawning cohort as those used for trials in *Chapter 5*. In this study, post-larvae (PL) were stocked into two commercial ponds in North Queensland (Seafarms) at the same time as the PL were sent to the Bribie Island Research Centre (BIRC) for GAV challenge testing. Therefore, the methods regarding the production of PL, including broodstock information and

hatchery conditions, were exactly the same as that described in *Chapter 5.2.1*. Additionally, methods used for genotyping and DNA parentage analysis were also the same as those described previously in *Chapter 5.2.4.*

6.2.1 Commercial farm grow-out

The post-larvae were stocked into two replicate earthen ponds, Pond 149 (1.14 ha) and Pond 150 (1.21 ha), at 45 /m^2. Water quality parameters were provided by the farm and showed that mean dissolved oxygen concentrations were 7.32 ± 2.83 mg/L and water temperatures were 29.5 ± 2.7 °C throughout the production period. Shrimp were sampled from the farm ponds at the time of harvest, after 105 days of culture. In total, 940 shrimp (10 genotyping plates) from each pond were collected by cast netting and anaesthetized in an ice slurry bath. Sampling of individual shrimp consisted of collecting gill filament tissue samples for DNA and viral load analysis, sex and body weight using an electronic scale (nearest 0.1 g).

Gill-associated virus infection load quantification followed the same methods as described in *Chapter 2.2.5*. In addition to measuring GAV load, a subsample (n = 94 that provides >95% confidence of detection (Lightner, 1996) of the shrimp collected from each pond were tested for the presence and loading of two other viruses, Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) and Yellow head virus type 7 (YHV7), using the same methods as described in *Chapter 5.2.3*. Infectious hypodermal and hematopoietic necrosis virus was not detected in Pond 149, but was detected in 4/94 (4%) shrimp tested from Pond 150 at extremely low levels (<40 copies μg^{-1} TNA). Yellow head virus type 7 was not detected in any of the shrimp tested. Due to the low prevalence and loading of IHHNV it was decided that it would have minor impact on the phenotypes measured and was not tested on the remaining samples.

6.2.2 Statistical analysis

Statistical analysis of phenotypic data was performed in R v3.3.0 (R Core team, 2016). Differences in the relative family contribution of full-sib families within each pond were assessed using Pearson's two-sided chi-square tests with results considered significant at $P < 0.05$.

Genetic analyses were performed for pond traits using the software ASReml-R 3.0 (VSNi) (Butler et al., 2009). For each trait recorded on the pond reared animals, restricted maximum likelihood methods were used to estimate variance components and breeding values. For GAV infection traits two statistical approaches were applied; (1) defining GAV infection as a binary trait of infection status using a binomial animal model (1 = infected, 0 = not infected), and (2) using a linear animal model of $\log_{10}$ GAV infection load from GAV infected shrimp only. For body weight, a linear animal model was also used to estimate variance components and breeding vales.

The general form of the univariate models for the three traits were:

$$y = \mu + sex + pond + animal + \varepsilon$$

Where y is the vector of observations (for GAV infection status the vector is 1 for infected and 0 for not infected; for GAV infection load the vector is individual infection loads after $\log_{10}$ transformation; and for body weight the vector is individual shrimp body weights), μ is the overall mean of observations, sex and pond were fitted as fixed effects, animal is the random animal genetic effect and ε represents the unknown random residual effects. For GAV infection status, the binomial residual variance is fixed as $\pi^2/3 = 3.29$ (Falconer and Mackay, 1996). To determine if common full-sib family effects (i.e. common environment effect) due to possible vertical transmission of virus from parents to offspring were significant, likelihood ratio tests were performed by comparing the univariate models with similar models for each trait where the full-sib family identity was added as a random effect. For all traits, the effect of common full-sib family were not significant and so were removed from the final models. Bivariate animal models, similar to univariate models above, were used to estimate covariance components and genetic correlations among the three traits.

Heritability was calculated as $h^2 = \frac{\sigma_a^2}{\sigma_A^2 + \sigma_\varepsilon^2}$, where σ_A^2 and σ_ε^2 were the variances due to additive genetic and residual error effects, respectively. Genetic correlations (r_g) were calculated from the bivariate analyses as $r_g = \frac{cov(A_1 A_2)}{\sqrt{(\sigma_{A_1}^2 \times \sigma_{A_2}^2)}}$, where $cov(A_1 A_2)$ is the additive genetic covariance and $\sigma_{A_1}^2$ and $\sigma_{A_2}^2$ are the additive genetic variance component for trait 1 and 2 respectively. In addition to

bivariate analyses, Pearson's correlations between sire EBVs (as a proxy of family) for each trait using univariate models were also used to approximate genetic correlations and the ranking of family performance among traits. Similarly, Pearson's correlations were also used to assess the correlation between GAV challenge traits measured under controlled challenge conditions in *Chapter 5* with pond production traits in the current chapter (GAV infection load and body weight), by correlating sire EBV for each trait. Pearson's correlations of sire EBV were used as approximates of the true genetic correlation, but tend to underestimate them (Astles et al., 2006; Sae-Lim et al., 2015). This is because family based EBV, such as sire EBV used in this study, are estimates of family mean performance relative to a population mean with each EBV associated with error. The accuracy of family EBV can be further affected when the number of animals per family are low (Astles et al., 2006).

6.3 Results

6.3.1 Pedigree assignment

Using DNA parentage assignment, 911 and 924 (total = 1835) shrimp from ponds 149 and 150, respectively, were assigned to full-sib families. The total number of full-sib families identified was 80, from 47 dams and 61 sires. Within the 80 full-sib families, there were 55 maternal half-sib families (from 21 unique dams) and 30 paternal half-sib families (from 12 unique sires). The number of individuals within each full-sib family was highly variable, ranging from 1 – 230 (Fig. 6.1), with the average number of individuals per family 22.7 ± 33.5. Therefore, the number of full-sib families with 10 or more individuals was 41, although all individuals were included in genetic analyses. Using the 41 most abundant families, there were a small number of families (7/41) with significant differences in the relative contributions of each family within the two ponds ($\chi^2_{(79)} = 108.86$, $P < 0.05$) (Fig. 6.1).

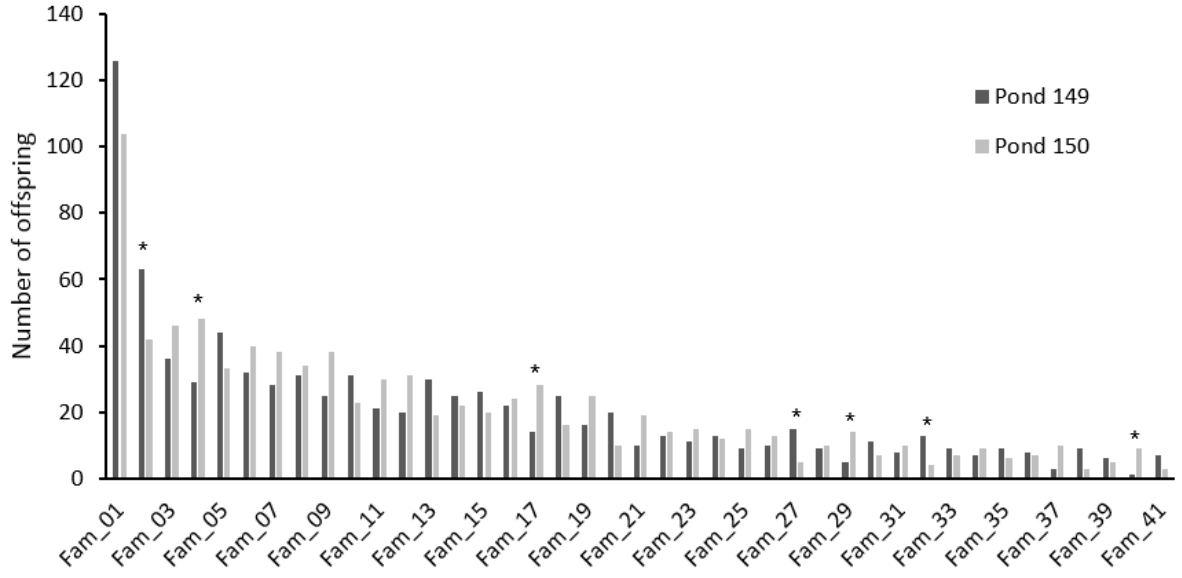

Figure 6.1. The number of offspring from the 41 most abundant full-sib *Penaeus monodon* families (with 10 or more offspring sampled) identified in each pond, 149 and 150. Significant differences in relative family contribution between the two ponds is indicated by * ($P < 0.05$).

6.3.2 Overview of phenotypic data

Summary statistics of phenotypic data related to the traits measured on shrimp reared in two commercial farm ponds are given in Table 6.1. Prevalence of GAV infection in the farmed shrimp was less than 50% and infection severity of positive shrimp was low to moderate (overall mean GAV infection load = 3.11 ± 1.42 $\log_{10}$ GAV copies μg^{-1} TNA). There was a significant difference in shrimp weight between the two ponds ($t_{(1748.3)} = 10.64$, $P < 0.001$), but there was no difference in the proportion of each sex (m: $\chi^2_{(1)} = 0.05$, $P = 0.821$; f: $\chi^2_{(1)} = 0.05$, $P = 0.821$), prevalence of GAV ($\chi^2_{(1)} = 0.26$, $P = 0.612$), or GAV infection loads of GAV infected shrimp ($t_{(411.8)} = -0.89$, $P = 0.374$). The observed mean (± s.d.) GAV infection loads, on a $\log_{10}$ scale, of 25 full-sib families (that had at least five offspring tested and were GAV positive) ranged from 2.04 ± 0.60 to 4.08 ± 1.29 (GAV copies μg^{-1} TNA) (Fig. 6.2), and for body weight the observed means from 41 full-sib families (that had at least 10 offspring measured) ranged from 12.8 ± 3.2 g to 19.4 ± 3.7 g (Fig. 6.3).

Table 6.1. Phenotypic data (observed mean ± s.d.) of *Penaeus monodon* sampled from two replicate commercial ponds, included is the percentage of each sex, prevalence of gill-associated virus (GAV) (GAV prevalence), GAV infection load (log_{10} GAV copies μg^{-1} TNA) of infected shrimp (GAV load) and body weight (g).

Phenotype	n	Pond 149	Pond 150	Overall
sex (m/f)	1790	50.1/49.9	49.3/50.7	50.4/49.6
GAV prevalence (%)	913	44.5	46.3	45.5
GAV load	415	3.05 ± 1.43	3.18 ± 1.41	3.11 ± 1.42
Body weight (g)	1790	16.81 ± 3.72	15.01 ± 3.42	15.88 ± 3.68

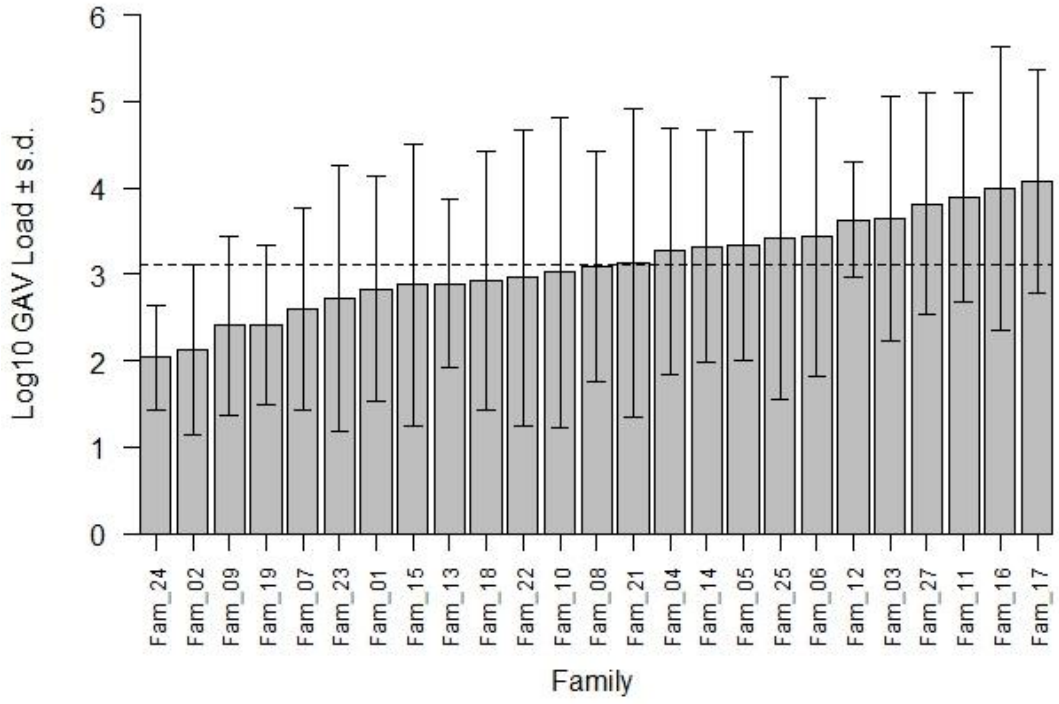

Figure 6.2. Mean (± s.d.) GAV infection load (log_{10} GAV copies μg^{-1} TNA) of GAV infected *Penaeus monodon* from the 25 full-sib families (with at least 5 offspring) that were screened for GAV and reared under commercial farm conditions. The dashed line represents the overall mean GAV infection load of infected shrimp (3.11 ± 1.42 log_{10} GAV copies μg^{-1} TNA) and family ID is based on the level of contribution (i.e. Fam_01 is the most abundant family within the cohort).

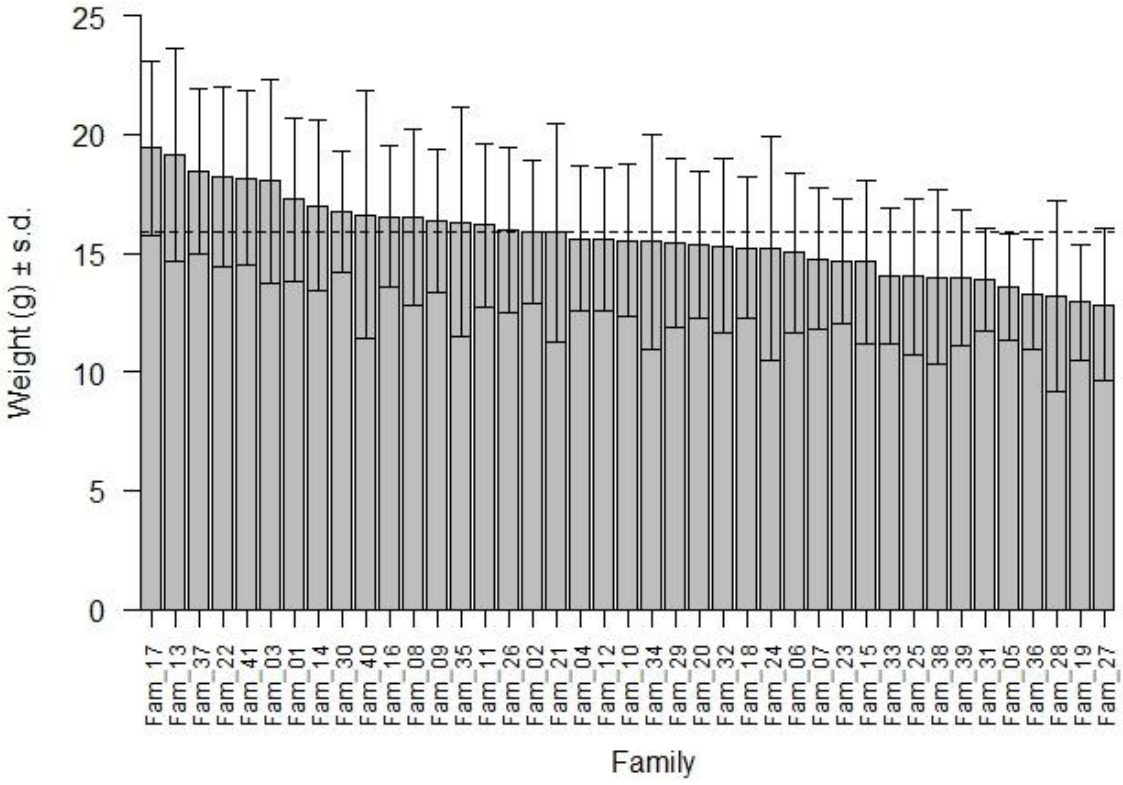

Figure 6.3. Mean (± s.d.) body weight of the 41 most abundant *Penaeus monodon* full-sib families (with at least 10 offspring) reared under commercial farm conditions. The dashed line represents the overall mean body weight (15.88 ± 3.68 g) and family ID based on the level of contribution (i.e. Fam_01 is the most abundant family within the cohort).

6.3.3 Genetic parameters of pond traits

When GAV infection was modeled as a binary trait of infection status (infected or not infected), or as a linear trait of infection loads from infected shrimp, heritability differed markedly. Heritability of GAV infection status was $h^2 = 0.06 \pm 0.03$, whilst for infection load $h^2 = 0.21 \pm 0.10$ (Table 6.2). Sex had a significant effect on GAV infection status, with males having a lower infection prevalence (41.8%) compared with females (50.2%). The estimated heritability for body weight was 0.38 ± 0.07 and both sex and pond had significant effects on shrimp body weight (Table 6.2). Males on average were slightly lighter (~ 0.5 g) compared with females and shrimp from Pond 150 were almost 2 g lighter on average than shrimp from Pond 149.

Table 6.2. Estimates of additive genetic variance (σ_a^2), random residual error variance (σ_ε^2), heritability and regression coefficients for sex and pond that were included as fixed effects for pond related traits measured under commercial farm conditions. GAV binary = GAV infection status as a binary trait, GAV load = infection load of GAV infected shrimp and body weight (g).

Model	σ_a^2 ± SE	σ_ε^2 ± SE	h^2 ± SE	Sex	Pond
GAV binary	0.23 ± 0.13	Fixed ($\pi^2/3$)	0.06 ± 0.03	Male: -0.37 ± 0.14**	ns
GAV load	2.34 ± 1.15	8.67 ± 1.01	0.21 ± 0.10	ns	ns
Body weight	4.89 ± 1.17	7.94 ± 0.69	0.38 ± 0.07	Male: -0.48 ± 0.15**	Pond 150: -1.95 ± 0.15***

ns = not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

6.3.4 Genetic correlations among ponds traits

Bivariate analyses were used to estimate genetic correlations between the three traits measured from pond reared shrimp. Gill-associated virus infection status and GAV load were highly correlated (r_g = 0.90 ± 0.24), whilst the sire EBV correlation (r_{EBV} = 0.36) was much lower (Table 6.3). Weak positive genetic correlations were found between GAV infection status and body weight (r_g = 0.36 ± 0.26) and between GAV infection load and body weight (r_g = 0.26 ± 0.25), whilst the sire EBV correlations were not significant and close to zero (r_{EBV} = 0.10 and r_{EBV} = 0.13, respectively).

Table 6.3. Genetic correlations (below diagonal) from bivariate analyses and Pearson's correlation coefficients (above diagonal) of sire estimated breeding values from univariate models for the three pond traits measured on *Penaeus monodon* reared under commercial conditions. The traits measured were; GAV binary = GAV infection status as a binary trait, GAV load = infection load of GAV infected shrimp and body weight (g).

Trait	GAV binary	GAV load	Body weight
GAV binary	-	0.36*	0.10^{ns}
GAV load	0.90 ± 0.24	-	0.13^{ns}
Body weight	0.36 ± 0.26	0.26 ± 0.25	-

6.3.5 Genetic correlations between pond traits and GAV tolerance measured under controlled challenge test conditions (Chapter 5)

Using Pearson's correlations of sire EBVs as a proxy of genetic correlations, the correlations between GAV infection traits (infection status and infection load) from the pond reared shrimp and GAV challenge traits (mortality and infection load of survivors) were all negative, but not significantly different from zero (Fig. 6.4). A negative association between sire EBVs for mortality, or GAV infection measured during controlled challenge testing and sire EBVs for GAV infection in the ponds, indicates families that were more susceptible in the challenge had lower GAV infections in the ponds. For body weight measured on pond reared shrimp, the correlation with GAV challenge traits, mortality and infection load, were also not significant and close to zero.

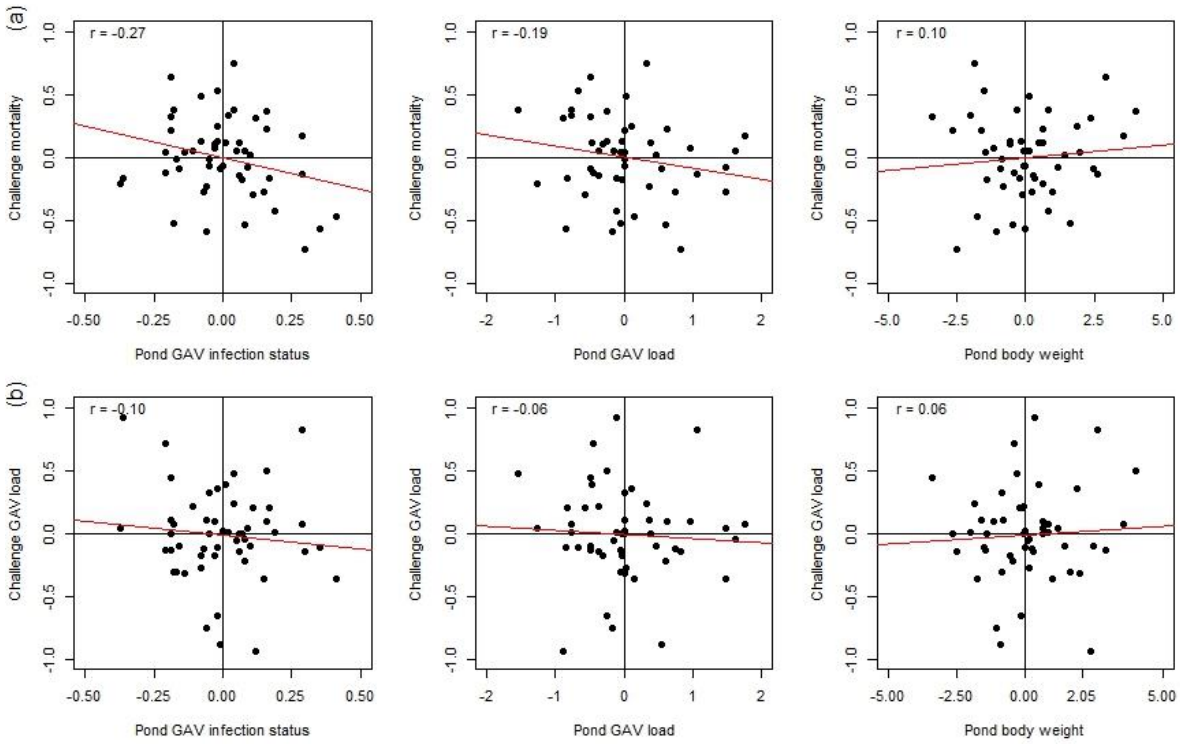

Figure 6.4. Scatter plots of *Penaeus monodon* sire estimated breeding values (EBV) for traits measured under controlled challenge conditions, (a) challenge mortality on the top row and (b) challenge GAV load of survivors on the bottom row against sire EBVs for traits measured under commercial conditions; pond GAV infection status, pond GAV load of infected shrimp and pond body weight.

6.4 Discussion

In shrimp breeding programs, disease tolerance is commonly assessed by challenging shrimp with a specific pathogen under controlled conditions and measuring survival. However, selecting shrimp families to breed from under these conditions will only be useful if it correlates with improved survival in commercial ponds when exposed to the pathogen. To evaluate disease tolerance in a farm pond is difficult, as it relies on the pathogen being present and ideally, from an experimental point of view, disease to occur. Furthermore, commercial farming systems are highly complex with many uncontrolled environmental factors that also play a significant role in pond survival making it difficult to get reproducible data (Robinson et al., 2017). Therefore, GAV infection, measured using a RT-

qPCR assay, from commercially reared shrimp was assessed for its utility as a tool for selecting GAV tolerant families to breed from by correlating pond GAV load with GAV challenge mortality data.

Heritability estimates of GAV infection status and infection load traits under commercial conditions were low to moderate depending on the trait. Heritability of infection status was lower than infection load, $h^2 = 0.06$ and $h^2 = 0.21$, respectively, indicating very little genetic variation for infection status, but significant genetic variation for infection load. Therefore, despite commercial culture conditions being highly variable and difficult to replicate, the environmental effects on GAV infection load within and between the two ponds were not large enough to mask genetic effects. The difference in genetic variance between infection status and infection load might also suggest that the mechanisms for controlling viral replication once infected are under stronger genetic control compared to whether infection occurs in the first place. Although, there was a positive genetic correlation between the two traits ($r_g = 0.90$, $r_{EBV} = 0.36$). This relationship is not surprising as pathogen burden is said to reflect the host's ability to resist pathogen infection, which is probably represented by both viral load and whether or not infection was established. In oysters (*Crassostrea gigas*) a strong positive correlation ($r = 0.95$) was also found between herpesvirus (OsHV-1) prevalence and infection load of animals deployed in the field (Degremont, 2011). Therefore, in general selection of families with lower viral infection load is also likely to lead to lower GAV prevalence. The heritability estimate of GAV infection load obtained here was lower than that reported by Knibb et al. (2015) and Phutheaworn et al. (2016) for HPV load in *F. merguiensis*; although, heritability estimates in these studies may have been inflated due to potential non-genetic and vertical transmission effects that could not be accounted for given the study designs. In this study on GAV, a number of half-sib families were included allowing for possible non-genetic effects, such as those common to full-sib families due to potential vertical transmission of GAV by dam and/or sire to offspring, to be tested and were found to be not significant. However, given the presence of a number of full-sib families (other than those within half-sib families), as well as complexities and unknowns surrounding GAV vertical transmission (Cowley et al., 2002), estimates of additive genetic variances, particularly for GAV infection traits, may still be inflated due to non-genetic factors. Future studies with sufficient data on

parental GAV infection loads may help to determine whether vertical transmission significantly impacts GAV infection in their offspring at harvest.

The present study is the first to investigate the genetic association between pathogen infection data from commercially cultured shrimp and specific disease challenge mortality data. Genetic correlations between commercial GAV infection and GAV induced mortality under controlled challenge tests were negative, but not significantly different from zero. Similarly, in *Chapter 5* the correlation between challenge mortality and infection load of survivors was also weak. These results combined suggest that the two traits (GAV induced mortality and viral infection load) may be weakly genetically correlated and that GAV infection load likely reflects disease resistance only, whereas survival likely includes both resistant and tolerant traits, but does not distinguish between the two. This is in line with the results on other animal species (Kause et al., 2012), in that resistance and tolerance are generally weakly correlated (Kause and Odegard, 2012). For example, Odegard et al. (2011b) used a cure survival model on data from *P. vannamei* challenged with TSV to distinguish susceptibility from endurance (in which the authors suggest are comparable to resistance and tolerance respectively); by using probabilities to partition the survivors into non-susceptible and susceptible but still alive. The authors found that susceptibility and endurance to TSV challenge were not genetically correlated ($r_g = 0.22 \pm 0.25$). Although the correlation between GAV induced mortality under challenge and GAV infection under commercial conditions were not significant, the negative relationship, if stronger, would suggest that families with low mortality had siblings with higher infection prevalence and loading in commercial ponds. This may mean these families, at a group level, were better able to tolerate GAV infection rather than resist infection *per se*. In most shrimp diseases, as with other aquaculture species, disease outcome is usually correlated with increased pathogen burden (both prevalence and load) such as in GAV outbreaks in *P. monodon* farm ponds (Munro et al., 2011) and between field mortality and prevalence or infection load of OsHV-1 in oysters (*Crassostrea gigas*) (Degremont, 2011). However, it is unknown whether there is a genetic component to these associations and the underlying cause of disease is likely due to the sum of both animals with low resistance and low tolerance. Although disease resistance and tolerance may be

weakly genetically linked, they are both important factors in reducing the impact of disease. Therefore, perhaps the breeding goal for reducing disease impact needs to be re-defined by combining measures of pathogen load with measures of disease outcome, such as mortality (Moss et al., 2005).

Similar to *Chapter 5,* the limitations of this study were that there was no information (GAV infection data) on the shrimp that died during the culture period, or on family survival (although this is influenced by many environmental factors other than the pathogen). Those that died could have been highly infected with GAV (or may have died from unknown reasons), but this data may have provided more information on the relationship between pond GAV infection and mortality during challenge. In commercial culture it is virtually impossible to sample dead shrimp, as they are not easily identified or able to be collected and further are quickly cannibalized. Disease outbreaks due to GAV did not occur during the commercial production period which was evident by only moderate infection prevalence and low infection load in the pond reared shrimp sampled. Therefore, it is unlikely that any shrimp that died during culture died due to high GAV infection; otherwise this would likely induce horizontal transmission and potentially cause a disease outbreak.

The heritability estimate for body weight was moderate ($h^2 = 0.38 \pm 0.07$) and within the range of those previously reported for *P. monodon* (Kenway et al., 2006; Macbeth et al., 2007; Coman et al., 2010; Krishna et al., 2011). Genetic correlations and correlations of sire EBVs between body weight and GAV infection traits under commercial conditions were weak, although all were positive (Table 6.3). A positive correlation between body weight and GAV infection traits suggest families that were heavier at harvest also had a slightly higher incidence of GAV infection and loading. This is in contrast to a previous study in *F. merguiensis* that found a negative genetic correlation between HPV load and body weight (Phuthaworn et al., 2016). When correlating body weight with GAV challenge traits mortality and infection load (from *Chapter 5*), sire EBV correlations were also slightly positive, although not significant and close to 0. Therefore, selecting families with high GAV tolerance (low mortality) is not likely to result in an unfavorable correlated response in body weight. The correlation observed for GAV tolerance traits and body weight are slightly lower (and not significant) compared to what has been found for other shrimp viruses such as TSV and WSSV. For both of these viruses,

genetic correlations between disease tolerance and growth traits were negative (Argue et al., 2002; Gitterle et al., 2005b).

In summary, GAV infection, particularly infection load of GAV positive shrimp under commercial culture was found to have a significant genetic basis suggesting it could be used for selecting shrimp with lower infection loads (higher GAV resistance). However, GAV infection traits measured in pond reared shrimp were not strongly correlated with GAV induced mortality measured under controlled challenge conditions. Thus, as measured in the current study, GAV infection measured on pond reared shrimp at the end of the harvest might not be a good predictor on its own of GAV induced disease and mortality. In addition, GAV infection traits from pond reared shrimp and GAV induced mortality under challenge were not strongly correlated with body weight. Body weight is an important economic trait in most shrimp breeding programs and inclusion of GAV resistance/tolerance traits would likely have little impact on body weight based on the present dataset, although this relationship would need to be closely monitored.

Chapter 7. General discussion

7.1 Significance and major outcomes

Aquaculture plays an important role in food security and recently hit an important milestone, with almost half of all seafood consumed by humans now farmed (FAO, 2016). Shrimp are an important aquaculture sector, being the fastest growing industry within aquaculture and the second most valuable, with over 5 million metric tonnes of shrimp produced in 2016 (FAO, 2016). However, it is estimated that 40% of farmed shrimp is lost to disease each year (Stentiford et al., 2012). Therefore, shrimp farming critically depends on effective disease management strategies if it is to continue to grow and meet future demands for seafood products.

In shrimp farming, disease is managed mostly through maintaining optimal environmental conditions and biosecurity procedures, such as the stocking of specific pathogen free (SPF) post larvae (Cock et al., 2009). Even with these practices in place, disease remains a big problem in shrimp farming. Selective breeding for improved disease tolerance offers an additional long term management strategy that can be implemented alongside existing practises, such as use of SPF stocks, as well as incorporated into breeding programs that select for other important traits such as growth. By growing shrimp that have been genetically improved via selective breeding to be more tolerant or resistant to pathogen infections, thereby reducing instances of disease, it is possible to reduce production losses and increase production efficiency of shrimp farms. However, within the scientific literature at least, selective breeding for improved disease tolerance has focused mainly on two pathogens, Taura syndrome virus (TSV) and white spot syndrome virus (WSSV), with contrasting outcomes. In addition, there have been numerous different disease challenge protocols used to evaluate disease tolerance in shrimp and it is unclear how these may affect the accuracy of genetic breeding value estimates. Disease tolerance in shrimp has almost always been evaluated through survivorship only and the utility of other measures like viral load, until now, have not been thoroughly investigated. The research presented here makes a significant contribution to shrimp breeding by addressing several

knowledge gaps on the use of disease challenge tests to evaluate disease tolerance and presents new ideas on ways to improve the accuracy of these tests. In addition, this thesis provides the essential knowledge on the genetic basis of GAV tolerance to determine whether it could be incorporated as a trait for selection in a *P. monodon* selective breeding programs. The major outcomes of this research are presented below.

Understanding the significance of genetic and non-genetic factors that affect shrimp disease responses is pivotal to investigating the genetic basis of disease tolerance and how to effectively incorporate it as a trait for selection. To fully maximise the ability to measure and exploit genetic effects, non-genetic factors need to be well understood and controlled. *Chapters 2-4* focused on understanding non-genetic factors that can play a role in the measured GAV-induced disease response in *P. monodon*. *Chapters 5 & 6* applied this knowledge to elucidate the underlying genetic basis of GAV tolerance and its link with other commercial production traits.

There is no standard protocol for shrimp disease challenge tests resulting in numerous methods being used, such as different infection methods for example. It is unclear the degree to which various infection methods can lead to stochastic infections (e.g. varying doses and timing of infection) and consequently result in unreliable and inaccurate genetic estimates. *Chapter 2* examined three commonly used disease challenge infection methods (injection, ingestion and immersion) for their utility in establishing a reliable and standardised GAV challenge test. The results from this chapter revealed that the response to GAV infection varied greatly depending on the method used, with injection and ingestion inducing disease and mortality, whereas immersion did not. Furthermore, injection resulted in greater uniformity in GAV infection loads of survivors compared with ingestion. The results from this chapter highlighted the need to assess the effectiveness of different infection methods for specific pathogens as well as the need for individual infection methods such as injection. Individual methods provide the ability to deliver a standardised infection dose to all individuals being challenged, as well as the flexibility and control to vary the dose as required, for example to identify genetic differences. However, it is still unclear whether the artificial nature of injection, which bypasses some natural host entry pathways, alters the disease response to what may occur naturally.

Another individual infection method has been established that utilises oral infection routes using a catheter to deliver a controlled dosage of a viral inoculum (e.g. Gitterle et al., 2006a, 2006b). This method is highly laborious and difficult to perform at large-scale (N. Robinson personal communication, 2018). In any case the critical component to a disease challenge test is to ensure that individuals under evaluations are exposed to the same challenge conditions so that any differences between groups can be identified and assigned accurately to genetic differences, or that differences are not overshadowed by uncontrolled non-genetic factors.

In *Chapter 3*, variability of GAV infection loads within and between commonly used tissues for GAV testing was investigated and provides invaluable information to researchers and industry when screening and testing for shrimp pathogens. This was particularly important for outcomes sought by the work outlined in this thesis as viral load, measured via RT-qPCR, was investigated as a potential indirect measure for GAV disease tolerance and thus a trait for selection. Therefore, it is important that the measurement is reliable and accurate so that relative differences between individuals, or groups of individuals can be identified. From this study, it was revealed that pathogen infections (both prevalence and loading) can vary greatly among different samples collected of the same tissue type within an individual shrimp. The outcomes of this research revealed the importance of collecting multiple samples when possible, which have already led to changes to industry practises in Australia. Now multiple pleopod tips are collected from broodstock for initial pathogen screening as opposed to a single tip that was previously used. Subsequent chapters in this thesis have also utilised multiple tissue samples when generating GAV infection load data.

Studies that have investigated disease tolerance in shrimp have generally been associated with traditional family based breeding programs. These programs are based on using separate spawning and rearing tanks for families so that pedigrees can be easily tracked and family contributions controlled. However, to enable accurate measurements of genetic effects individuals should be exposed to identical environmental and challenge conditions. This is not the case if families are reared (even up until they can be physically tagged) and/or challenged in separate tanks, thus separate environments. Using a small number of full-sibling families, *Chapter 4* exposed the degree to which

disease responses can vary due to separate environments or "tank effects". This had not been characterised previously and demonstrates the need for standardised communal rearing systems that should provide more accurate genetic estimates. Despite the substantial tank effects, Chapter 4 also provided the first evidence of family differences in GAV susceptibility.

Chapters 2-4 increased our understanding of the potential pitfalls in current disease challenge protocols and methods used to measure disease responses, whilst also eluding to family/genetic differences in GAV susceptibility. Subsequently, the thesis moved to apply this knowledge in *Chapter 5* to establish a novel protocol for shrimp disease challenge tests that subjects all individuals to the same environmental and challenge conditions to more accurately identify genetic differences in disease response. In doing so, this thesis also provides the first genetic estimates of GAV-induced disease tolerance in *P. monodon*. The results of this chapter demonstrated that large-scale communal rearing and challenge systems can be used successfully for shrimp disease challenge tests to genetically evaluate tolerance traits. They also revealed that GAV-induced disease tolerance/resistance traits in *P. monodon* are under low-moderate additive genetic control. Heritability estimates of mortality and GAV infection load were h^2 = 0.11-14 and h^2 = 0.23, respectively. However, the results also indicated the two traits may be weakly genetically related, although the limitation to this experiment was that GAV infection load was only recorded on survivors. Acquiring infection loads from dead shrimp may reveal a stronger and more accurate estimate of the relationship between mortality and GAV infection load and should be investigated in future studies. Additionally, the cohort of shrimp used to estimate genetic parameters of GAV disease tolerance had pre-existing GAV infections, albeit, at low infection severity. However, it is unknown whether the pre-existing GAV infections affected individual and family disease responses. The development of SPF *P. monodon* stocks are being pursued in Australia currently, but were not available at the time of this work. Ideally, SPF stocks would be used for disease challenge testing in the future. Another important aspect of this experiment was the unavoidable cannibalism during the challenge test that likely caused secondary exposure to GAV. Future studies should endeavour to limit cannibalism by more frequent monitoring and removal of dead shrimp. Nevertheless, the level of additive genetic variability found

for both traits (mortality and GAV infection load) does indicate that improvement of survival or reduction in viral load should transpire with targeted selection.

Finally, selective breeding programs aiming to improve disease tolerance would ideally evaluate performance under commercial conditions to ensure that the outcome is relevant to farming conditions. However, this would be inefficient as it would rely on natural outbreaks of the disease to occur and may also be hindered by uncontrolled non-genetic factors. For these reasons, controlled laboratory based disease challenge tests are used instead. It is important therefore to assess whether selection under controlled challenge conditions will translate to improved performance under commercial conditions. In addition, understanding how disease tolerance is associated with other commercially important traits is also critical to a well-designed breeding program. In *Chapter 6*, GAV infection load and body weight were recorded on siblings of those challenged in *Chapter 5*. This allowed correlations between disease tolerance under challenge conditions and traits recorded under commercial conditions to be estimated. Overall, the data collected showed that GAV-induced disease tolerance recorded under challenge conditions was not correlated with GAV infection or body weight in the commercial ponds. Thus, it is still unknown whether selection for GAV tolerance using challenge tests will lead to improvement of survival during GAV disease outbreaks on-farm. Ideally survival data during commercial grow out would have been collected to better understand the correlation between GAV induced mortality in the challenge and survival on farm, but this data was unavailable; although, given the prevalence and loading of GAV infection found in the commercially reared shrimp it is likely that GAV infections progressed to a level resulting in a disease and mortality. Accordingly, it is unlikely that a strong correlation between GAV-induced mortality during challenge and survival during commercial grow out would have been found. Future studies need to address this issue to ensure that the families being selected under challenge conditions will lead to positive improvements on-farm. The results from *Chapter 6* did indicate that selection for GAV tolerance will have little to no adverse impact on body weight. Therefore, these two traits could be incorporated into a selective breeding program and be selected simultaneously.

The research outlined in this thesis as a whole provides a comprehensive assessment of the utility of disease challenge tests for genetic evaluations of disease tolerance and provides new information to consider when designing and establishing challenge tests for shrimp breeding programs – particularly those targeting GAV. This study provides the Australian shrimp farming industry with the essential information needed to determine whether GAV tolerance could be incorporated into future breeding programs.

7.2 Future direction of research for breeding disease tolerant shrimp

The series of studies reported in this thesis have already made several advances in our knowledge on the use of disease challenge tests for selective breeding for improved disease tolerance in shrimp and how best to undertake them. However, there are still several important knowledge gaps that should be addressed in the future so that the full potential for genetic improvement of disease tolerance can be exploited. Critically, there is lack of data that demonstrates the genetic correlation between disease tolerance under controlled challenge tests and disease tolerance under commercial culture to understand the efficiency of diseases challenge tests for selection of this trait. In fish, there have been strong genetic correlations between the outcomes of disease outbreaks in the field and that under challenge (Gjoen et al., 1997; Ødegård et al., 2006). However, for shrimp there are no genetic correlations of this type. The reasons behind this are likely due to the complexities involved in obtaining the necessary data including; relying on a natural disease outbreak to occur on farm, lack of feedback of data from commercial farms, and prohibitive costs involved in genotyping large numbers of individuals pre- and post-disease occurrence to generate survival data. Natural GAV disease outbreaks in farms are usually triggered by environmental stressors that cause shrimp to go into an acute viral infection and subsequent disease state (de la Vega et al., 2004). Understanding the environmental triggers that play a role in GAV disease outbreaks in farm ponds may allow for more targeted sampling of shrimp in commercial ponds, or could be used as a tool to induce disease. Advances in DNA genotyping techniques have meant that the cost of developing and applying

genotyping assays has fallen dramatically in recent years. Furthermore, advances in statistical methods have meant that even further cost savings can be achieved for genotyping large numbers of individuals through DNA pooling (Henshall et al., 2014). DNA pooling can be used to determine family contributions and could therefore be used to estimate family survival during commercial grow out relatively cheaply. Future studies correlating disease tolerance between challenge and commercial conditions may employ these ideas to generate field data more efficiently and the evidence required to prove the efficacy of disease challenge tests.

Shrimp genetic improvement programs that include disease tolerance as a trait for selection, are based on evaluating siblings of the breeding candidates to rank families. Subsequently the breeding candidates are then selected from the top performing families. Selection using this method only utilises half of the available genetic variation (i.e. between family variation) and limits the genetic gains possible by reducing the accuracy and intensity of selection (Falconer and Mackay, 1996). However, advances in genetic technologies have resulted in techniques that can improve both selection accuracy and intensity through marker assisted selection (MAS) and genomic selection (GS) (Gjedrem and Rye, 2016). Marker assisted selection encompasses information from a few gene variants that have been proven to be linked to quantitative trait loci (QTL's) (e.g. Robinson et al., 2014). This means, these few gene variants have a significant effect on the expressed phenotype for the trait under evaluation. Genomic selection on the other hand, uses information from thousands of gene markers spread across the genome, so that all QTL's, even those with minor effects are captured, to estimate genomic breeding values, as well as genomic relationships (Goddard and Hayes, 2007). Genomic selection, unlike MAS, does not rely on proven gene association with the phenotype rather that the gene markers (SNPs) are sufficiently dense across the genome so that most QTLs will be in high linkage disequilibrium with at least one marker (Khatkar, 2017). Using MAS or GS allows breeding candidates themselves can be evaluated using genetic information only (i.e. without the need to record the phenotype of the actual individual breeding candidate). This will be particularly useful for disease tolerance traits, which the breeding candidate, even if they survive the challenge test will be compromised (i.e. infected with the pathogen and/or of poor quality following the stress event).

Therefore, MAS or GS allow for breeding candidates to be selected based on their genetic merit thus resulting in more accurate selection, greater selection intensities and consequently greater genetic gains. Future research should use these tools to determine whether QTLs exist for GAV tolerance or to establish genomic selection methodologies for tolerance traits.

Finally, selective breeding for disease tolerance should not be seen as the panacea for the disease problems in shrimp farming. Breeding tolerant shrimp stocks to one specific pathogen does not infer they are tolerant to other pathogens (Moss et al., 2005) and new pathogens are likely to emerge in the future. Therefore, selective breeding should be used in conjunction with other health management strategies which might include; maintaining low stress environmental conditions, implementing strict biosecurity, use of genetically superior stocks (e.g. domesticated to farm environments and selectively breed for important traits like fast growth), use of SPF stocks, and use of other health/immune related tools, for example immunostimulants. Thus, selective breeding for improved GAV disease tolerance appears viable as an effective long term management strategy, but should form part of the overall health management program for the *P. monodon* industry.

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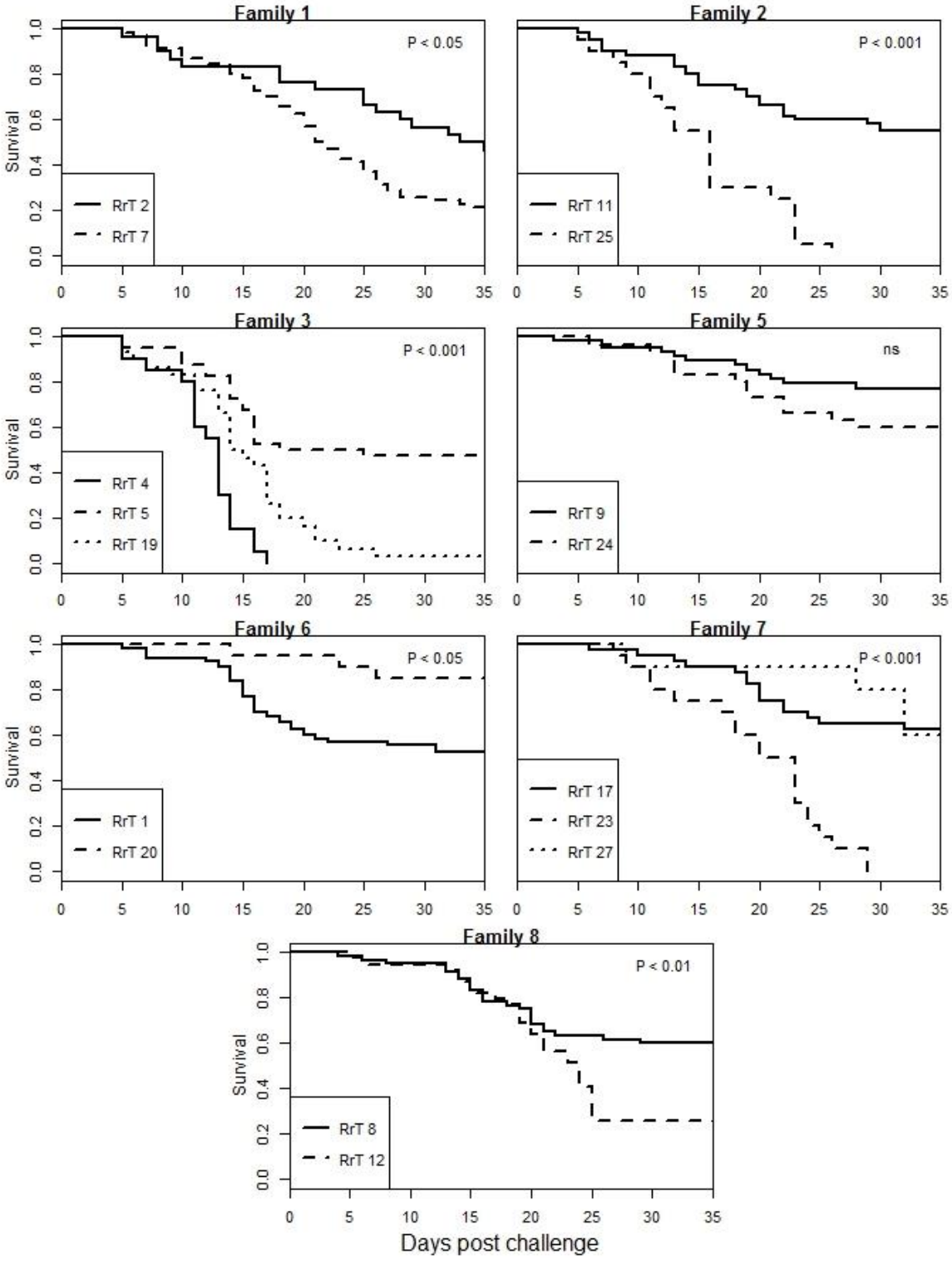
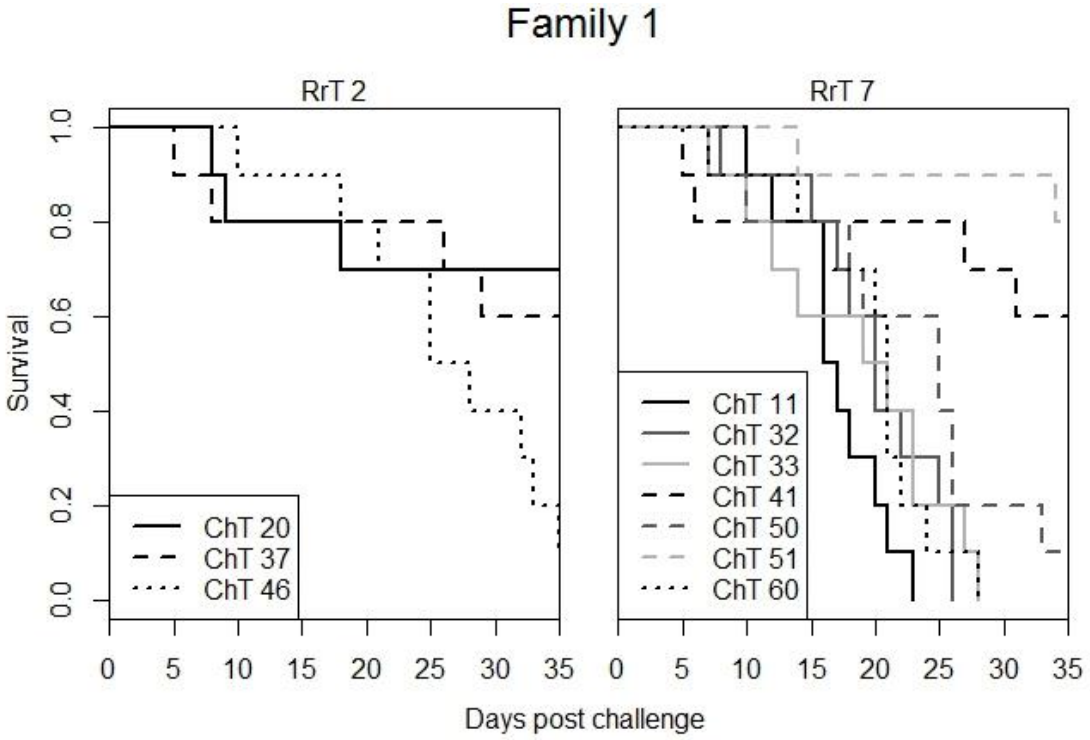
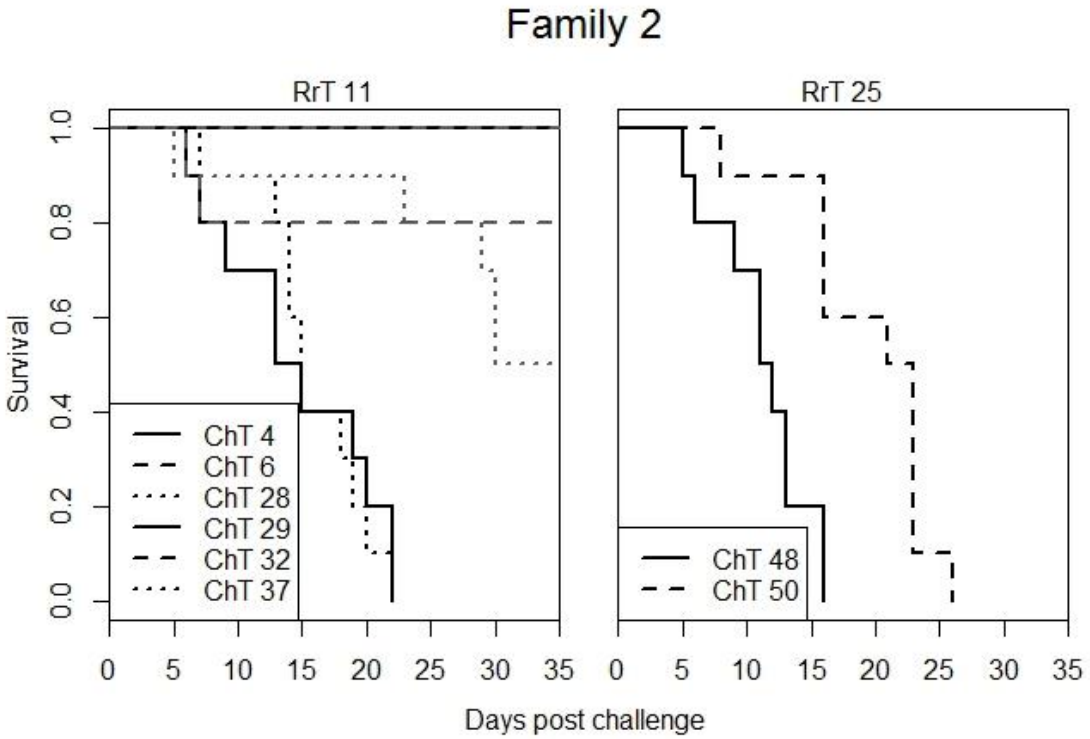
Appendices

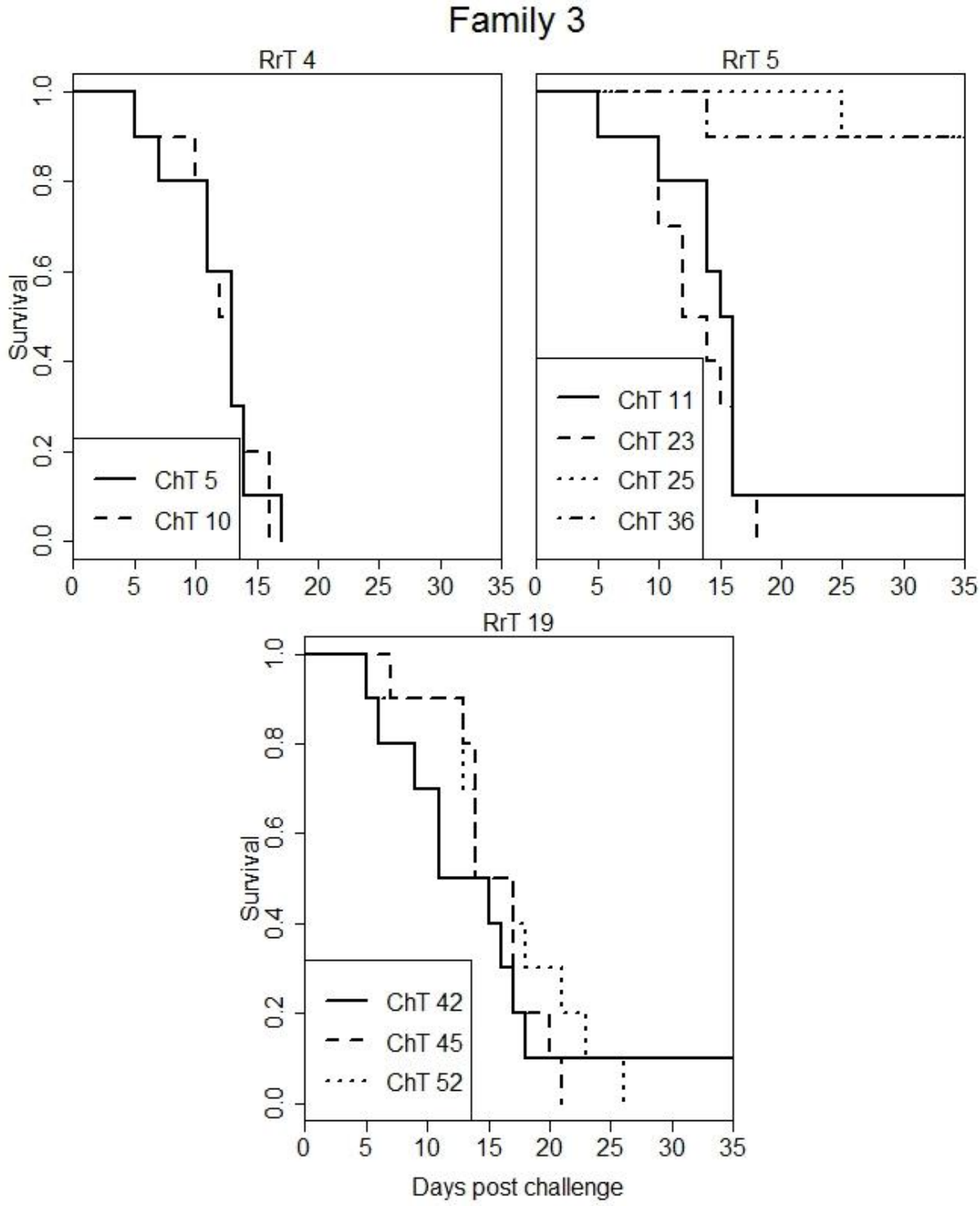
Appendix 1. Kaplan-Meier survival curves of progeny from seven full-sib families reared in replicate rearing tanks following challenge with gill-associated virus. Significance of rearing tank effects on the survival distributions using a log-rank test are provided in each of the family plots.

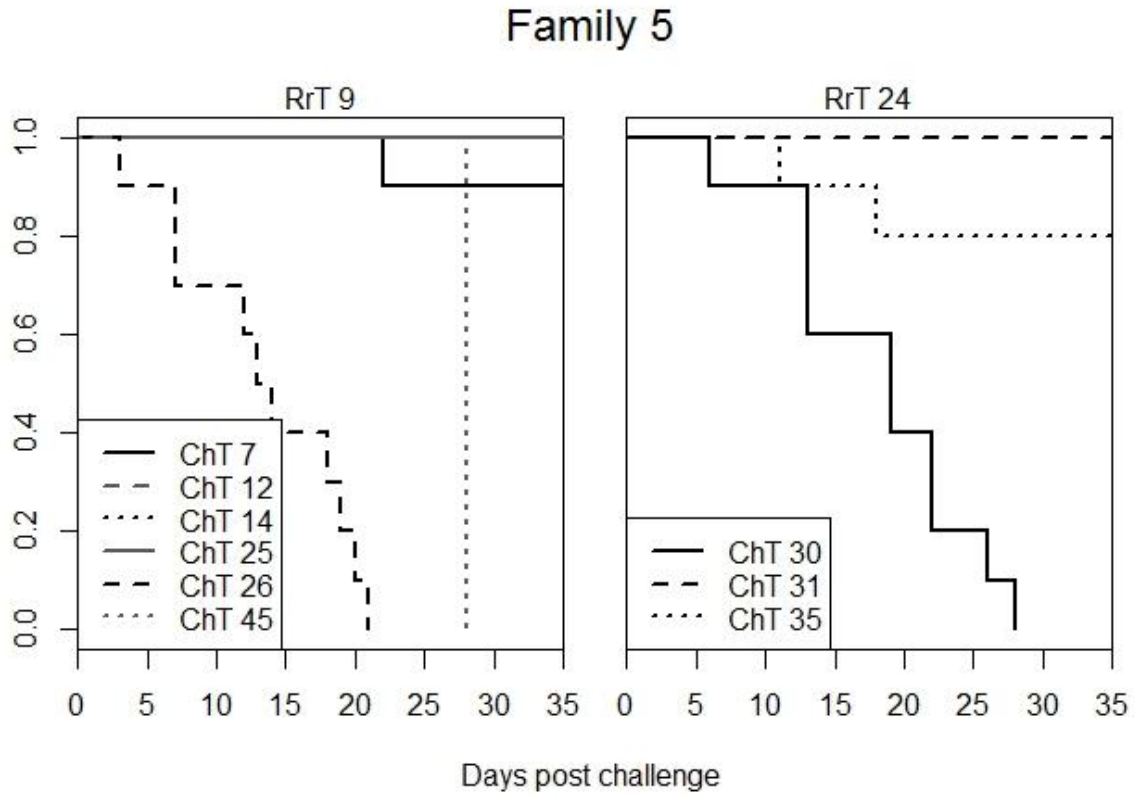


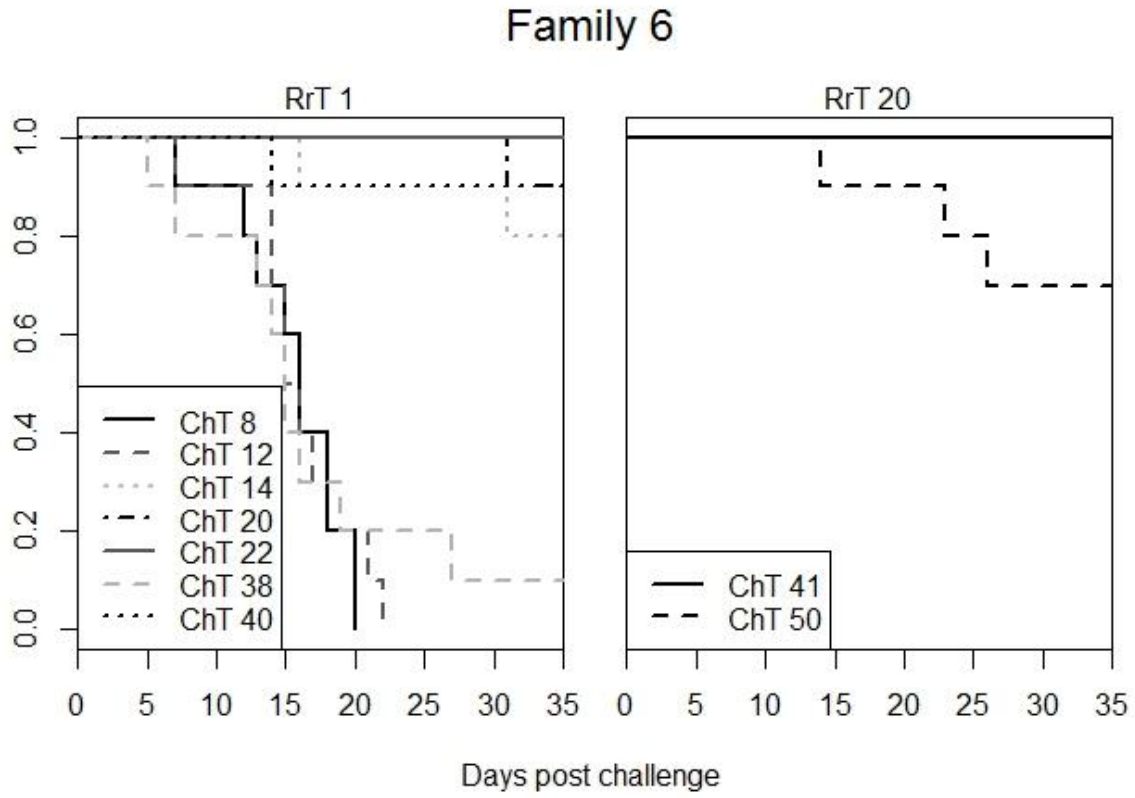
Appendix 2: Kaplan-Meier survival curves of progeny from seven full-sib families reared in replicate rearing tanks and challenged with gill-associated virus in replicated challenge tanks. Survival curves are based on progeny within replicate challenge tanks from the same rearing tank (RrT) and from each family (e.g. Family 1).

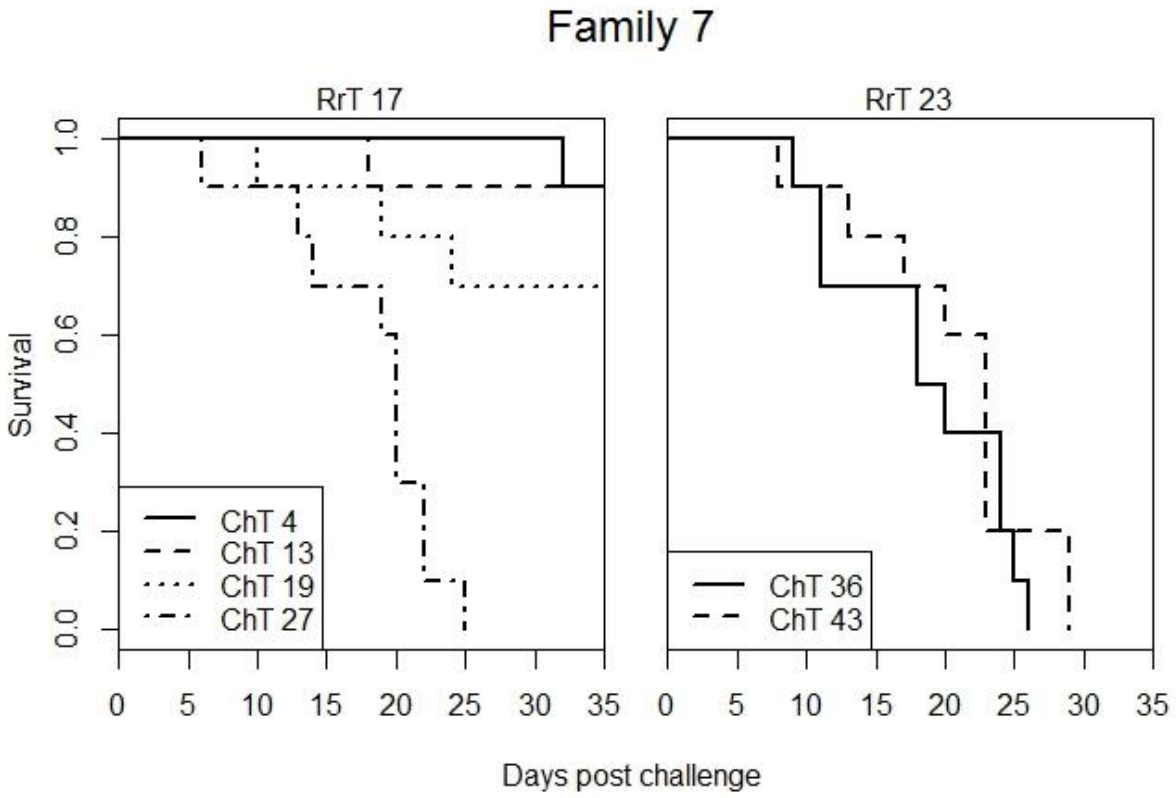


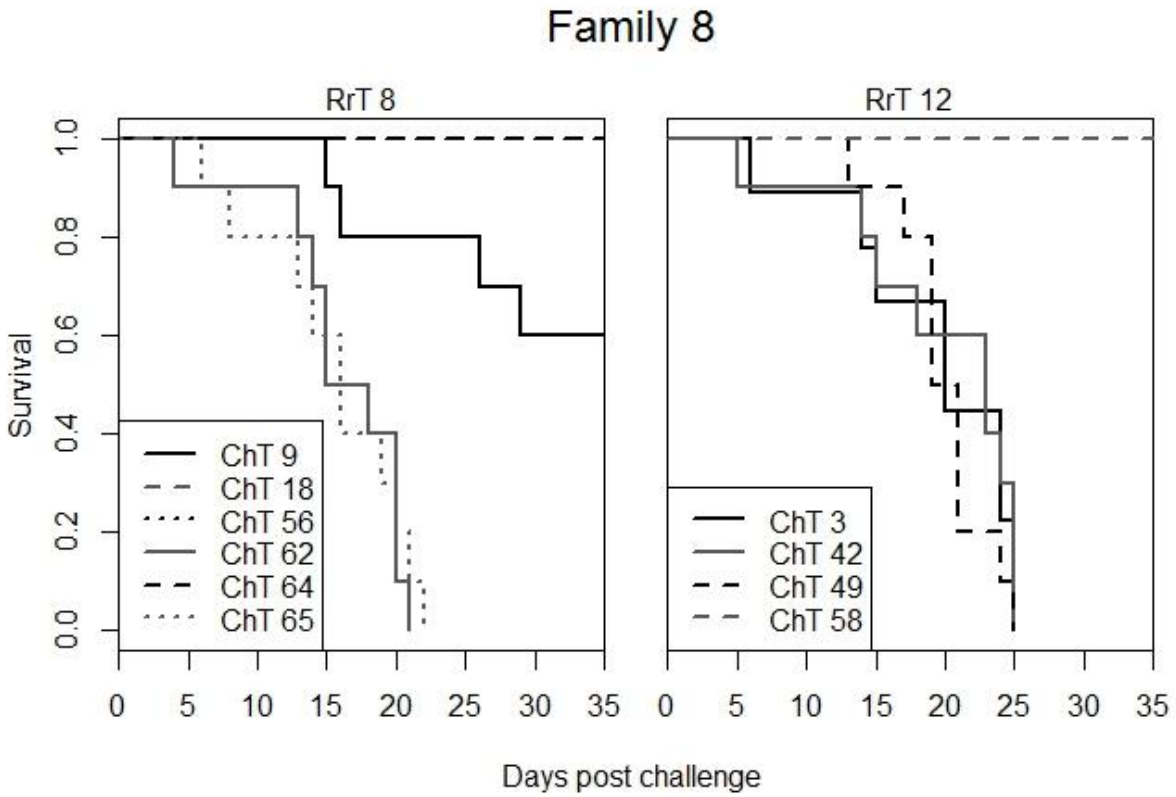



Family 3
RrT 4
RrT 5
RrT 19
Survival
Days post challenge
ChT 5
ChT 10
ChT 11
ChT 23
ChT 25
ChT 36
ChT 42
ChT 45
ChT 52


Family 5
RrT 9
RrT 24
ChT 7
ChT 12
ChT 14
ChT 25
ChT 26
ChT 45
ChT 30
ChT 31
ChT 35
0.0
0.2
0.4
0.6
0.8
1.0
0 5 10 15 20 25 30 35
Days post challenge


Family 6
RrT 1
RrT 20
ChT 8
ChT 12
ChT 14
ChT 20
ChT 22
ChT 38
ChT 40
ChT 41
ChT 50
0.0
0.2
0.4
0.6
0.8
1.0
0 5 10 15 20 25 30 35
Days post challenge


Family 7
RrT 17
RrT 23
Survival
0.0
0.2
0.4
0.6
0.8
1.0
ChT 4
ChT 13
ChT 19
ChT 27
ChT 36
ChT 43
0
5
10
15
20
25
30
35
Days post challenge


Family 8
RrT 8
RrT 12
Survival
0.0
0.2
0.4
0.6
0.8
1.0
ChT 9
ChT 18
ChT 56
ChT 62
ChT 64
ChT 65
ChT 3
ChT 42
ChT 49
ChT 58
0
5
10
15
20
25
30
35
Days post challenge