Understanding and removing the barriers to *Penaeus monodon* domestication

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AUSTRALIAN PRAWN FARMERS ASSOCIATION

Gold Coast Tiger Prawns

CSIRO

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Fisheries Research and Development Corporation

Understanding and removing the barriers to *Penaeus monodon* domestication

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Contents of this report:

This report is a synthesis of the Final Milestone Report which contains all the milestone reports for the project. The Final Milestone Report (444 pp) is available to all project participants and members of the Australian Prawn Farmer's Association (APFA).

2002/209 Understanding and removing the barriers to *Penaeus monodon* domestication

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OBJECTIVES

1. Optimise reproductive output from domesticated *P. monodon* by assessing tank, raceway and pond rearing systems and associated maturation protocols.
2. Assess and minimise chronic and acute health-related barriers to the domestication of *P. monodon*
3. Measure the genetic components underlying the suitability of different strains, families or individuals for domestication.
4. Develop and implement an effective extension, technology transfer and commercialisation program for *P. monodon* domestication.

NON TECHNICAL SUMMARY

OUTCOMES ACHIEVED TO DATE

The results of this project have provided the Australian prawn farming industry with the knowledge and technology to commence the progression of the industry beyond their dependency on wild *P. monodon* broodstock to the production of domesticated broodstock of known health status.

The offspring of wild *P. monodon* broodstock from the East Coast (EC) and from the Gulf of Carpentaria (GoC) were successfully reared through three successive generations in tanks, raceways or specialised ponds. Improvements in the growth, survival and reproductive output were achieved with each generation in each of the rearing environments. The project produced twenty-four third generation families, derived from EC broodstock, as founder stocks for future selective breeding and ten fourth generation families derived from GoC broodstock for on-going stocking of farm ponds and selective breeding.

This project produced new knowledge about the origin and nature of viruses in wild founder stock populations of *P. monodon* and subsequent changes in the level of infections in successive generations of the domesticated stocks. This knowledge has been incorporated into on-going strategies for minimizing and monitoring viral loads in domesticated stocks at research and commercial sites.

The first trials of domesticated stocks in commercial ponds in 2005 demonstrated that growth and survival of the progeny of second generation broodstock was equivalent or superior to that of progeny of wild broodstock. Subsequent to the completion of the project, the second commercial trials in 2006, with third generation stocks, resulted in an average yield of 11.8 tonnes ha^{-1}. The third commercial trials in 2007, with fourth generation GoC progeny, further increased the average yield to 12.8 tonnes ha^{-1}, the highest yields ever recorded for the production of farmed *P. monodon* in Australia.

Over the next few years we anticipate progressive adoption of the results of the project across the whole industry as the progeny of domesticated stocks become increasingly available to more and more farms.

NEED FOR THE RESEARCH

Domestication of *P. monodon* has been the highest research priority for the Australian prawn farming industry for the past several years. In response to this priority the Australian Prawn Farmer's Association (APFA) initiated a major research initiative to understand and overcome the barriers to domesticating *P. monodon*. The initiative integrated research skills from the Australian Institute of Marine Science, CSIRO Marine and Atmospheric Research and Livestock Industries and the Queensland Department of Primary Industries and Fisheries (Bribie Island Research Centre). As core industry partners Gold Coast Marine Aquaculture and Seafarm Pty Ltd provided infrastructure, farm stocks and commercial expertise. The project was co-funded by industry, the research providers and the Fisheries Research and Development Corporation.

SUMMARY OF RESULTS AND CONCLUSIONS

The APFA led multi-partner, multidisciplinary approach adopted in this project has achieved the success that eluded the previous decade of un-coordinated research by individual research institutions and farms. The results of the project have provided the knowledge and technology to permit the industry to begin producing domesticated stocks of *P. monodon* of known health status on a commercial scale.

Broodstock rearing systems

Comparison of different broodstock rearing environments demonstrated the benefits of using controlled environment tanks and raceways to produce mature broodstock within twelve months. This is in synchrony with the annual production cycle of most Australian *P. monodon* farms. Previous studies of the effect of temperature on the growth rate of *P. monodon* indicate an optimum water temperature of around 30°C. This is consistent with the results of this study in which the controlled environment tanks and raceways were maintained at between 28°C and 30°C. The broodstock reared in the unheated ponds at Bribe Island Research Centre (BIARC) had slower growth rates during the winter, thus requiring more time to reach maturity than those grown in temperature controlled environments. However, ponds, particularly those in warmer regions, offer the potential to rear larger numbers of broodstock at lower capital costs. The growth rates of captive reared *P. monodon* reared in controlled environment tanks in this study were a significant improvement over previous trials, with a 200% increase in the average wet weight of 11 month old females compared to previous trials in 1997 and in 2000. This achievement highlights the importance of progressive improvements to the environment, diet, health and husbandry techniques for captive breeding of *P. monodon*.

Broodstock health

This project produced new knowledge about the origin and nature of viruses in wild founder stock populations of *P. monodon* and subsequent changes in the level of infections in successive generations of the domesticated stocks. Viral screening of EC stocks revealed progressive reduction in levels of infections of endemic Gill Associated Virus (GAV) and Mourilyan Virus (MoV) over 3 generations of captive rearing in broodstock ponds at BIARC. Wild GoC stocks were free from GAV but MoV was endemic at low levels. GoC stocks acquired GAV infection during the first generation of domestication, which persisted, along with MoV, in successive generations. These infections did not impair the growth, survival or reproductive output of broodstock or the grow-out performance of their progeny in commercial ponds.

The precise location of GAV and MoV virus particles in *P. monodon* gonads, eggs, larvae and postlarvae was examined using histology and fluorescent *in situ* hybridisation (FISH). Determining the location of these viruses is important in understanding their modes of transmission and the potential effectiveness of egg washing, with or without virucidal compounds. Egg washing can only be effective if the source of the infection is external to the gametes, for example in the spermatophore, seminal fluid or ovarian fluids. No evidence of viral

infection within eggs was evident either prior to or immediately after spawning. The results indicate that horizontal transmission is the main route of GAV infection in early life history stages. However, the results also indicated that MoV could be vertically transmitted via male reproductive tissues and that virus particles are released with seminal fluids during spawning.

The effects of washing newly spawned eggs with different virucidal indicated that Betadine (0.1 ppm) has the best potential to eliminate or significantly reduce levels of GAV without adversely affecting hatch rates. This finding is consistent with the recommendations of the OIE International Aquatic Health Code for washing the eggs of Penaeid prawns (see web page: http://www.oie.int/eng/normes/fmanual/A_00014.htm, Methods for Disinfection of Crustacean Farms). The protocol relies on the use of formalin (100 ppm) for 1 min followed Betadine (0.1 ppm) for 1 min.

Genetic improvement

Improvements in the growth, survival and reproductive output of *P. monodon* broodstock were achieved with each generation in each of the rearing environments. By the second generation reproductive output of domesticated broodstock had reached the level considered to be the minimum required to be commercially viable by industry (mean egg production of >200,000 per spawning and mean nauplii production of >100,000 per spawning). Genetic analysis of domesticated EC stocks reared in tanks indicated high heritabilities for growth and for survival and low to moderate heritabilities for some reproduction traits. These results indicate that future investment in selective breeding of *P. monodon* will result in concurrent improvements in growth, survival and reproductive performance.

Adoption of the results

The project produced twenty four third generation families, derived from EC broodstock, as potential founder stocks for future selective breeding and ten families derived from GoC broodstock for on-going commercial-scale domestication and selective breeding. So far only the core industry participants, Gold Coast Marine Aquaculture and Seafarm Pty Ltd have directly contributed to on farm components of the research and/or directly adopted the results of the project. Seafarm contributed their sixth generation (G6) domesticated stocks to the EC broodstock. Subsequent to the completion of the project, representatives of all the families derived from the EC stocks have been provided to Seafarm for them, and potentially other farms, to commence on-farm trials with these stocks.

The first trials of domesticated stocks in commercial ponds at GCMA in 2005 demonstrated that growth and survival of the progeny of second generation GoC broodstock was equivalent or superior to that of progeny of wild broodstock. Subsequent to the completion of the project, the second commercial trials in 2006, with third generation GoC progeny, resulted in an average yield of 11.8 tonnes ha^{-1}. The third commercial trials in 2007, with fourth generation GoC progeny, further increased the average yield to 12.8 tonnes ha^{-1}.

Over the next few years we anticipate progressive adoption of the results of the project across the whole industry as the progeny of domesticated stocks become increasingly available to more and more farms. The benefits from domestication alone include; eliminating total reliance on wild stocks; all-year round availability of postlarvae and the production of disease free or high health seedstock. All of these will lead to greater profits for the prawn farmers. This in turn will make the Australian prawn farming industry more competitive. Beyond successful, industry-wide, domestication we anticipate subsequent cumulative and permanent genetic gains in commercially important traits and associated economic gains to the industry.

Key Words: Black tiger prawn, *Penaeus monodon*, domestication, broodstock, Gill Associated Virus, Mourilyan Virus, egg washing, heritability.

ACKNOWLEDGEMENTS

This project was initiated by the Australian Prawn Farmer's Association (APFA). The initiative integrated research skills from the Australian Institute of Marine Science, CSIRO Marine and Atmospheric Research and Livestock Industries and the Queensland Department of Primary Industries (Bribie Island Research Centre). As core industry partners Gold Coast Marine Aquaculture and Seafarm Pty Ltd provided infrastructure, farm stocks, technical knowledge and commercial expertise. The project was co-funded by all APFA members, the research institutions and the Fisheries Research and Development Corporation. We wish to acknowledge the collaborative efforts of all the participants which was a key factor in the success of the project. We are indebted to Patrick Hone, Martin Breen and Peter Rothlisberg for their efforts at the inception of the project and for the combined efforts of all other members of the Steering Committee for keeping the project on track. We would also like to acknowledge the assistance of Jan Wakeling in editing and collating the Final Milestone Report and the Final Report

BACKGROUND

In 2002 the Black Tiger prawn *Penaeus monodon* was the dominant prawn species farmed in Australia (65% of farm production value) with an annual production value of $55 M. The industry was totally dependent on wild-broodstock supplies which were erratic both seasonally and annually, sometimes resulting in severe shortages in broodstock and postlarvae. For example, in 1999-2000 a chronic shortage of *P. monodon* broodstock meant that many ponds were either unstocked, stocked late, or were stocked with alternative species. This resulted in an estimated $5 M in lost production for the season. The industry recognized that reliance on wild *P. monodon* was a high-risk practice and identified the need to cost-effectively rear broodstock in captivity (domestication) as their highest research priority. In response to this need a research consortium, led by the Australian Prawn Farmers Association (APFA), was formed in 2002 to develop the knowledge and technology to permit commercially viable domestication of *P. monodon*.

Internationally farmed *P. monodon* comprised more than 65% (US$4 billion) of the annual global production of farmed prawns in 2000 (Rosenberry, 2000). Despite the value of the industry, previous international and national efforts to domesticate this species had met with limited success. The first attempts were by the AQUACOP group at the IFREMER centre in Tahiti (AQUACOP, 1979). Small numbers of *P. monodon* broodstock were successfully reared in captivity but this research did not progress to commercial trials. In Thailand, attempts by the leading producer of farmed *P. monodon* to develop commercial scale domestication did not succeed (Fegan, 1999) despite encouraging research-scale trials (Menasveta et al. 1994, and Fegan, 1999). In the Philippines a domestication program, funded by the private sector, was terminated due to broodstock contamination by viral pathogens. In Indonesia larval production from domesticated *P. monodon* was insufficient to support hatchery requirements. A survey conducted by the Global Aquaculture Alliance in 2000 confirmed the slow progress in the domestication of *P. monodon* throughout S.E. Asia, particularly when compared with the progress that has been made with *P. vannamei*, the dominant species farmed in the western hemisphere (Clifford & Preston, 2001). The survey also revealed strong market demand for Specific Pathogen Free broodstock to supply the US $4 billion grow-out industry in S.E. Asia.

In Australia *P. monodon* domestication programs had previously been initiated by industry and by research agencies. The Australian Institute for Marine Science (AIMS) began a domestication program in the early 1990s (Kenway and Benzie, 1999) and by 2002 domesticated stocks had been reared through three successive generations (G3) in tanks. Research at the Bribie Island Aquaculture Centre (BIARC) had shown that the feasibility of stockpiling wild broodstock is limited (Crocos et al. 1997). Controlled environment trials by CSIRO in 1998 and 1999 demonstrated that tank-reared broodstock can have comparable performance to pond reared broodstock (Crocos et al. 1999). Seafarm ran small-scale *P. monodon* domestication trials for a number of years, and in 2002 had domesticated stocks that had been reared through six successive generations (G6 stocks). However, in common with the experience in S.E. Asia, none of these activities resulted in commercial-scale availability of domesticated broodstock for the industry. What had been shown was that it was possible to raise captive broodstock through to maturity on a small scale and to induce them, on occasion, to produce reproductive output approaching commercial viability. What had not been achieved was consistent success in rearing large numbers of broodstock with viable reproductive performance for commercial production.

The lack of understanding of the causes of the variability in the growth, survival and reproductive performance of captive reared broodstock was identified by the project participants as one of three critical barriers to achieving reliable, commercial-scale supplies of captive-reared broodstock of known health status. The questions posed by the participants in relation to

captive rearing of *P. monodon* broodstock were; what rearing systems are effective for this species, and what viral screening, biosecurity and quarantine protocols are required?

The second barrier identified was the lack of knowledge about the health status of founder stocks and the need to understand the chronic and acute health-related barriers to the domestication *P. monodon*. Previous research had revealed that most of the wild *P. monodon* broodstock supplied to Australian farmers were infected with Gill-Associated Virus (Cowley et al. 2000). There was also evidence that spawner-isolated mortality virus (SMV) is common in Australian *P. monodon* (Owens et al. 1998). These viruses were thought to have been responsible for significant losses to the Australian *P. monodon* industry. They were also thought to have been responsible for very high mortalities in domesticated stocks of *P. monodon* held at AIMS and elsewhere. However, there was no definitive evidence linking the poor reproductive performance of domesticated *P. monodon* in Australia with GAV or other viral infections. Research in Taiwan had demonstrated a correlation between high levels of white spot syndrome virus (WSSV) in *P. monodon* broodstock with a reduced ability to spawn and severely reduced nauplii production (Hsu et al. 1999). In Australian broodstock, GAV had been shown to infect ovaries and spermatophores, sometimes at very high levels, and the virus was thought to be transmitted vertically during mating (Walker et al. 2001; Cowley et al. 2002). A bunya-related virus (Mourilyan virus MoV) had been associated with pathology in tank-reared *P. japonicus* broodstock suffering significant mortality (Sellars et al. 2006). Mourilyan virus was also thought to be common in Australian *P. monodon* (Cowley et al. unpublished) and was considered to have the potential to contribute to disease and mortality in this species. The questions posed in relation to the health of *P. monodon* broodstock were; do virus-free stocks exist in the wild; can viruses be eliminated or reduced in domesticated stocks?; and can viruses be managed to enable efficient broodstock performance in virus infected stocks?

The third barrier identified was the lack of knowledge of the level of variation among different strains of *P. monodon* (i.e. those from different geographic regions), different families or different individuals in their genetic predisposition to captive breeding. Previous research with other species of penaeid prawns farmed in Australia and elsewhere had clearly demonstrated significant variation in reproductive performance among different genetic lines (Preston et al. 2001, Moss et al. 2001). Based on experience with other species it was anticipated that there would be variation in the survival, growth and reproductive performance of the different strains, families and individuals. The questions posed in relation to the genetic components of domestication were; do stocks that have been domesticated over several generations show any genetic adaptation to captive breeding and do some strains, families or individuals have a stronger genetic predisposition to captive breeding than others?

The aim of the project was to provide the answers to the questions posed above about *P. monodon* broodstock rearing system, the health of broodstock and the level of genetic variation in the adaptability of this species to commercially viable domestication.

NEED

The Australian prawn farming industry identified the following needs for research into the barriers to achieving cost-effective, commercial scale domestication of *P. monodon*:

1. The erratic supply of wild broodstock meant that the quantity, quality and timing of supply of postlarvae to farms were all high risk which impaired the productivity and profitability of the industry.

2. The continued use of wild broodstock made it extremely difficult to control introduction of disease. Almost all wild broodstock captured on the East Coast of Australia were known to carry viral infections, most notably with gill-associated virus (GAV), spawner-isolated mortality virus (SMV), and the Mourilyan virus (MoV). There was a critical need to understand the disease status of founder stocks for domestication programs. This included the development and application of rapid and accurate diagnostic tools as well as knowledge of how disease can be managed to permit increased productivity from domesticated stocks.

3. Selective breeding programs to increase farm productivity were impossible in the absence of commercially viable captive reared broodstock.

4. New methods of production that can minimise impacts on the environment, most notably full water recirculation systems, could not work well unless domesticated stock of known health status could be used.

In order to address these needs the project participants identified the following objectives:

OBJECTIVES

1. Optimise reproductive output from domesticated *P. monodon* by assessing tank, raceway and pond rearing systems and associated maturation protocols.

2. Assess and minimise chronic and acute health-related barriers to the domestication of *P. monodon*

3. Measure the genetic components underlying the suitability of different strains, families or individuals for domestication.

4. Develop and implement an effective extension, technology transfer and commercialisation program for *P. monodon* domestication.

1. CHAPTER 1. COMPARISON OF BROODSTOCK REARING ENVIRONMENTS

Optimizing reproductive output from domesticated *Penaeus monodon* by assessing tank, raceway and pond rearing systems and associated maturation protocols.

1.1 Introduction

The objective of this component of the study was to determine the effects of different grow-out and conditioning systems on the growth, survival and reproductive performance of domesticated *Penaeus monodon*. The three different broodstock environments examined in the study were; controlled environment tanks at CSIRO, Cleveland (CMR); controlled environment broodstock maturation tanks, larval rearing tanks and broodstock rearing raceways at Gold Coast Marine Aquaculture (GCMA), controlled environment broodstock maturation and larval/postlarval rearing systems at the Australian Institute of Marine Science (AIMS), controlled environment juvenile rearing tanks and ambient environment broodstock rearing ponds at Bribe Island Aquaculture Centre (BIARC).

The broodstock rearing experiments focused on two separate stocks of *P. monodon* that were selected on the basis of their availability and the results of previous viral screening. One stock was new founder stocks of wild male and female *P. monodon* from the Albatross Bay region of the Gulf of Carpentaria (GoC). This region was selected because viral screening prior to the study had indicated that the stocks were free from Gill Associated Virus (GAV) and had only low levels of Mourilyan Virus (MoV). The other stock originated from the East Coast (EC) in the Cairns/Innisfail region, this stock included third generation males that had been reared at AIMS and new founder stocks of wild males and females. The EC stocks were known to have endemic infections of Gill Associated Virus (GAV) and Mourilyan Virus (MoV).

In order to minimize the risks of transferring viral infections, the EC stocks and GoC stocks were reared at separate locations throughout the study. The GoC stocks were reared in controlled environment tanks at CMR and in controlled environment raceways at GCMA. The EC stocks were reared at AIMS and BIARC. Selected matings were used to produce separate families of postlarvae at AIMS. The postlarvae were then transferred to BIARC where they were reared as separate families until they could be physically tagged. After tagging the prawns were transferred to BIARC ponds for growout to broodstock at ambient pond temperatures.

1.2 Gulf of Carpentaria Stocks

1.2.1 Methods

Capture of founder stocks (G0) from the Gulf of Carpentaria

The Raptis vessel FV "Dampier Pearl" was chartered to trawl for *P. monodon* in a specified area of Albatross Bay (Boyd Bay to Duyfken Point) for a period of 3 days in March 2002 (Fig. 1). The project team were responsible for the collection of data on: trawl location and duration, trawl tracks, steaming tracks, recording catches of all commercial prawn species and catches of endangered species in the by-catch, and to ensure that all operations followed the conditions and guidelines set out in the AFMA scientific permit. Tiger prawn (*P. esculentus & P. semisulcatus*) nets were used for all trawls which were of short duration (15 minutes) in order

minimize stress in captured *P. monodon*. Most trawls were carried out in 2 areas: to the southwest of the Weipa leads, and to the south of Duyfken Point. The scientific permit specified that trawls would be terminated if any evidence of banana prawn (*P. merguiensis*) marks were indicated on the sounder during a trawl. This strategy was intended to minimize any banana prawn catch. No evidence of concentrations of banana prawns (*P. merguiensis*) was observed at any time. The total by-catch of the *P. merguiensis* was very low; with an average catch 0.4 kg per trawl over 52 trawls. Support for the collection of the founder stock was given by the Australian Fisheries Management Authority (AFMA), Raptis & Sons and Gold Coast Marine Aquaculture.

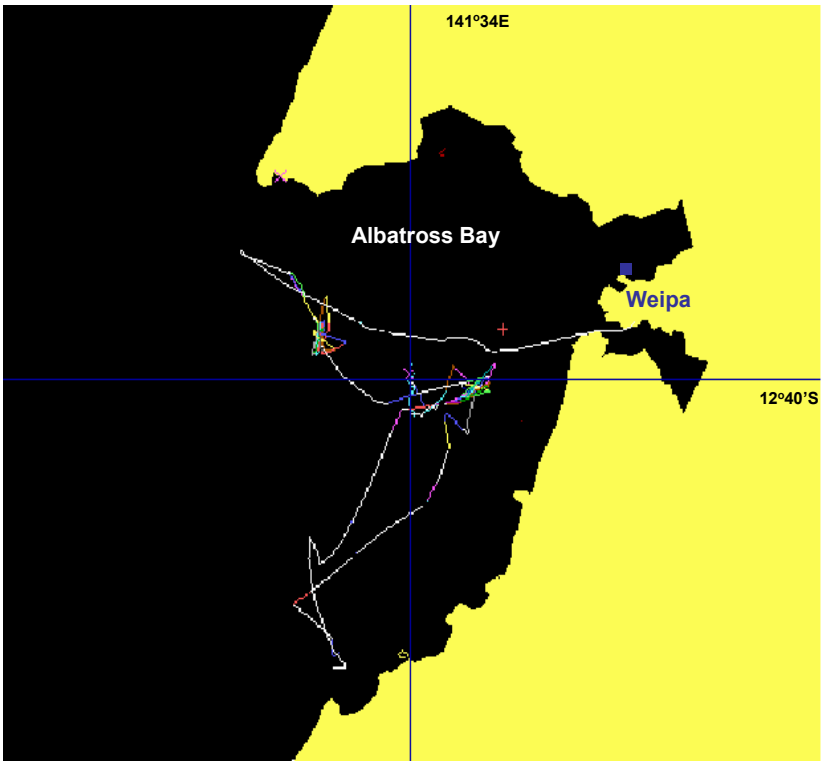

Figure 1. Total course (white lines) and trawl tracks (coloured lines) of chartered trawler used to capture wild male and female *Penaeus monodon* in Albatross Bay, Gulf of Carpentaria (GoC) in March 2002.

Following capture, subsets of the largest *P. monodon* were air freighted to Brisbane and transported by road to CMR and GCMA. A total of 364 *P. monodon* (224 females, and 140 males) were collected from the trawls carried out in the GoC. Of the 364 individuals, 74 females and 46 males were sent to CMR and 150 females and 94 males to GCMA.

Rearing of founder stocks to maturation in tanks and raceways

Previous research indicated that captive reared *P. monodon* need to be at least 12 months old to be suitable to use as broodstock. Therefore the prospective broodstock (estimated to be 5-6 months old at the time of capture) were reared for a further 7 months (April-October, 2002) in the maturation tanks at CMR tanks and raceways at GCMA (Fig. 2).

Production of G1 stocks

The G0 founder stocks at the GCMA maturation facility were ablated in mid-October 2002. In November 2002, two females spawned to produce sufficient numbers of nauplii at the right time to permit commercial-scale domestication trials (Fig. 2). The two GoC-G1 families produced were used to compare the raceway and tank broodstock production systems. The stocks were reared in the hatchery at GCMA until they were approximately 2g (February 2003). Siblings from both families were then transferred to two commercial raceways at GCMA and to 10 ten-

tonne tanks within the quarantine facility at CMR (Fig. 3). The families were reared in these systems until reaching broodstock age (11.5 months) at which time the reproductive performance was assessed at both GCMA and CMR.

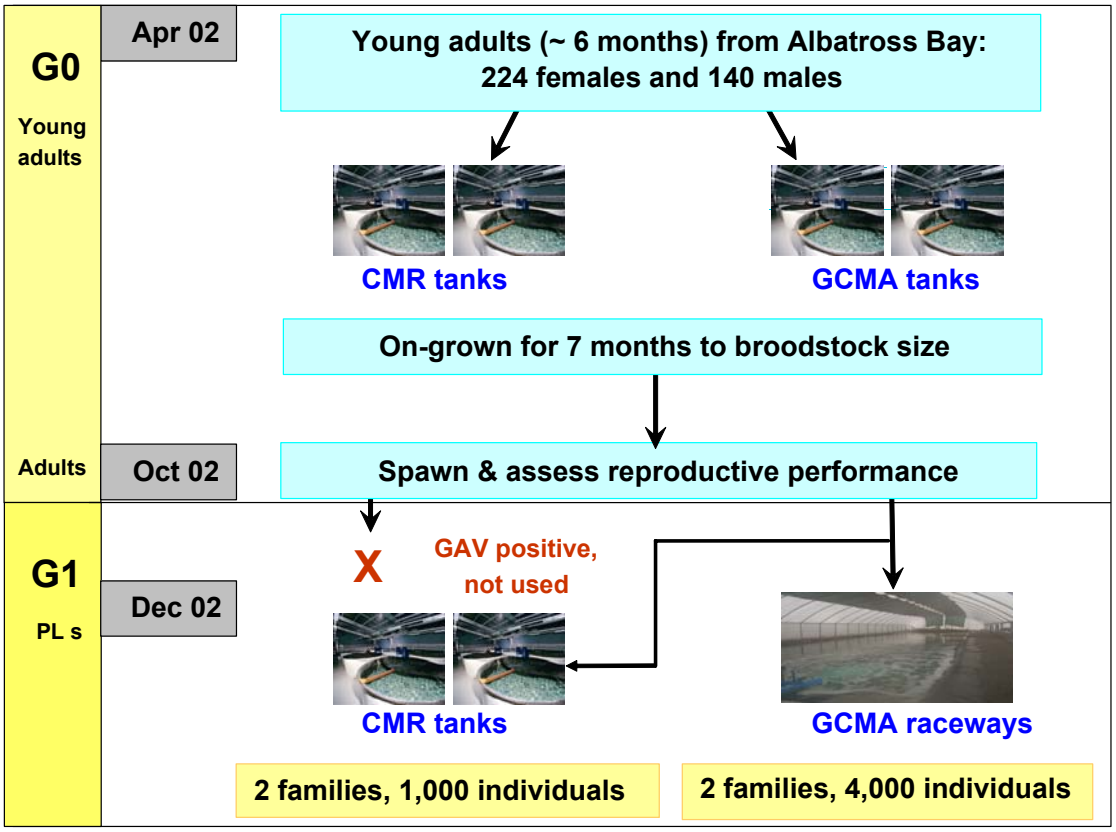

Figure 2. Schematic of the production of G1 *Penaeus monodon* from wild stocks (G0) obtained from Albatross Bay, GoC. The stocks were grown in tanks at CMR and tanks and raceways at GCMA.

Assessing the reproductive performance of broodstock

All females were moult-tagged on stocking into the experimental tanks in order to monitor moulting and ensure females had mated with males from the appropriate family. Females were ablated post-moult and their subsequent reproductive performance monitored over a period of 2 moult cycles at CMR and 1 moult cycle at GCMA. For comparison, two stocks of wild broodstock were included in these trials. One set of wild broodstock were collected from the GoC in March 2002 on-grown in tanks at CMR for eight months, and then spawned in November 2002. The other wild broodstock were EC stocks purchased from a commercial broodstock supplier in November 2002, then spawned immediately at CMR. The reproductive data collected was: percentage of females spawning, number of eggs per spawning, hatch rate and nauplii production.

Rearing of stocks in tanks and raceways

For the experimental stocks (G1, G2, G3 and G4) reared in tanks at CMR, the rearing protocol was as follows. The families were reared to PL15 (15 days post-metamorphosis from mysis to postlarval stage 1) in three replicate 100 L tanks (0.6 m diameter). From PL16 to PL30, the stocks were reared in two replicate 200 L cages suspended within a communal 10,000 L tank (3.6 m diameter). At PL30, prawns from each family were stocked into 10,000 L sand-substrate tanks (Crocos and Coman, 1997) at a density of 10 individuals m^{-2}. When prawns were three months old the densities were reduced to 6 individuals m^{-2} in all tanks. At 5.5 months prawns were measured and the largest 50% of individuals from each family were selected. All tanks had equivalent numbers of individuals from all families. The stocks were reared for a further five to six months until reaching broodstock size.

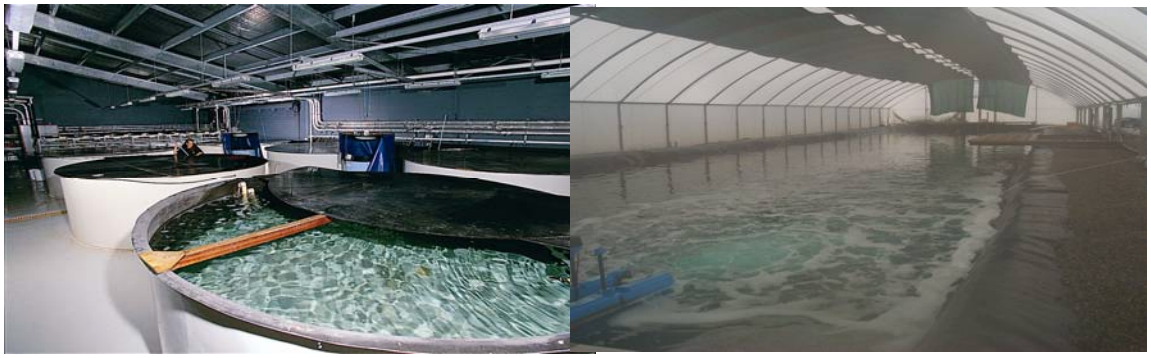

Figure 3. Broodstock rearing systems used for stocks originating from the Gulf of Carpentaria (GoC). Controlled environment 10 tonne tanks at CMR (left). Controlled environment raceways at GCMA (right).

The 10,000 L sand-substrate tanks at CSIRO used to rear the experimental stocks were fitted with a sub-sand water circulation system to maintain the sand substrate in an aerobic condition (Crocos and Coman, 1997). Water quality was maintained under a low-water exchange regime (Coman et al. 2005). Water temperatures and salinities were maintained (mean ± standard deviation) at 29.0 ± 0.2ºC and 35 ± 1‰, and artificial lighting on a 14 h light: 10 h dark photoperiod. From PL30 until 5.5 months of age, shrimp were fed three times daily (0900, 1300 and 1700 h) on a diet consisting of approx. 20% squid (*Photololigo* sp.), 10% bivalves (*Plebidonax* sp.) and 70% commercial pellets (Lucky Star, Taiwan Hung Kuo Industrial Pty Ltd). From 5.5 months to 10.5 months shrimp were fed on a diet of 30% squid, 15% bivalves, 5% polychaetes (*Marphysa* sp.) and 50% pellets.

Production of G2 stocks in tanks at CMR

Seven G2 families were generated at CMR from the G1 tank-reared broodstock (Fig. 4). The G2 tank-reared stocks were reared in larval rearing tanks and cages until they were 1 month old, at which time they were transferred to the 10-tonne maturation tanks for rearing to broodstock age. To examine the effect of different maturation densities on reproductive performance, representatives from all families were reared from 6 months to 11 months at two densities. At 6 months, prawns from all families were randomly allocated to four high density treatment tanks (75 females and 55 males total; 3.3 individuals m^{-2}) and six low density treatment tanks (45 females and 29 males total; 1.3 individuals m^{-2}). At 11 months, prawns were weighed, and rotationally crossed within density treatments, leaving three replicate high density tanks (57 females and 37 males total; 3.2 individuals m^{-2}) and four replicate low density tanks (29 females and 25 males total; 1.4 individuals m^{-2}) for the 12 months reproductive assessment. Because of the focus on generating broodstock on a 12 monthly cycle, combined with the poor survivals in the G1 stocks between 12 months and 15 months of age in the previous year, it was decided that reproductive assessments of the G2 stocks would only be performed at 12 months.

Growth and reproductive performance of the tank-reared stocks were compared between the G2 stocks reared at high density (G2 high), the G2 stocks reared at low density (G2 low), and the G1 stocks, evaluated in the previous year's assessment at the same age, which were reared at a density comparable to the G2 high density treatment. Statistical comparisons of the three generation-treatments were made using values pooled for all families.

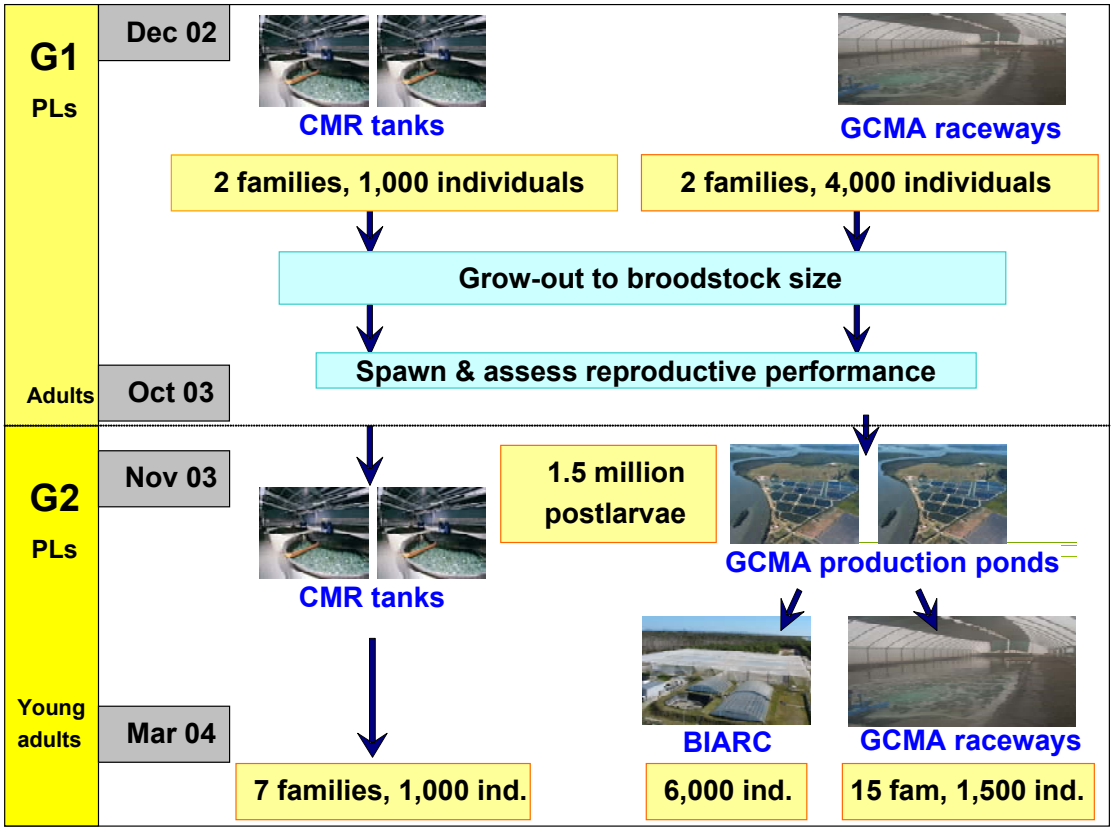

Figure 4. Schematic of the production of G2 *Penaeus monodon* GoC stocks. The stocks were reared in tanks at CMR and in production ponds at GCMA for 6 months then transferred to raceways at GCMA and a broodstock pond at BIARC.

Production of G2 stocks in raceways at GCMA

Fifteen G2 families were generated from the G1 raceway-reared broodstock at GCMA (Fig. 4). Approximately 1.5 million postlarvae from these families were stocked into production ponds at GCMA in November 2003 and reared through to harvest at 6 months of age in March 2004. At harvest, approximately 1500 individuals were each stocked into two raceways at GCMA and 6000 individuals into a broodstock rearing pond at BIARC. The subsequent survival of these stocks was very low in the GCMA raceways and in the BIARC broodstock pond, with too few individuals surviving to maturity to assess their reproductive output.

Production of G3 stocks in tanks at CMR

The growth, survival and reproductive output of the seven G2 families reared in the controlled environment tanks at Cleveland was sufficiently high (~ 70%, see results section) to supply GCMA with post larvae for stocking the GCMA raceways and for the first pond trials in 3 production ponds at GCMA. The progeny supplied to GCMA were siblings of the 9 GoC families that were used to produce the third generation (G3) of GoC stocks in the controlled environment tanks at CMR (Fig. 5). A total of 750,000 nauplii were transported by road to GCMA. These were used to produce 320,000 postlarvae that were stocked into three small (0.25 ha) production ponds and 3,000 juveniles (2 g) for stocking the GCMA raceways (Fig. 5).

Production of G4 stocks in tanks at CMR and raceways at GCMA

Ten G4 families were generated from the G3 tank-reared broodstock at CMR and raceway-reared broodstock at GCMA. Approximately 2 million postlarvae from these families were stocked into 5 commercial production ponds at GCMA in November 2004 (Fig. 6).

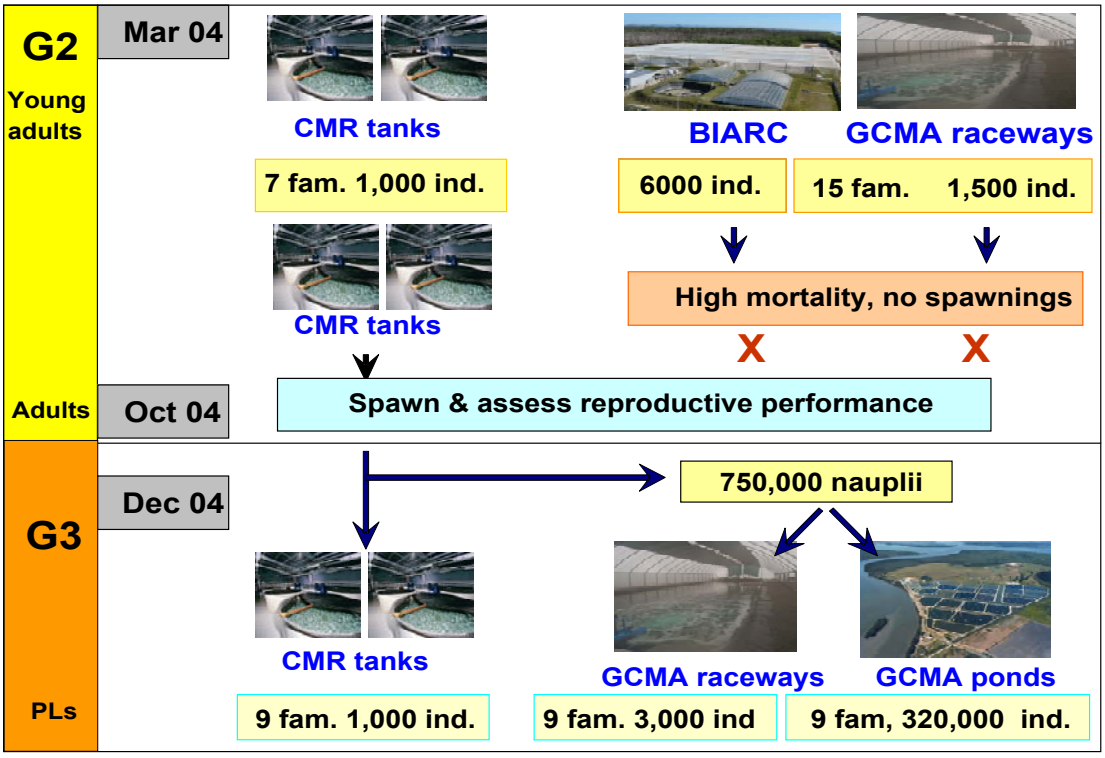

Figure 5. Schematic of the production of G3 *Penaeus monodon* GoC stocks. The G2 stocks that were reared in ponds at BIARC and raceways at GCMA had high mortalities and no viable spawnings. All G3 stocks were derived from the G2 stocks reared in the controlled environment tanks at CMR.

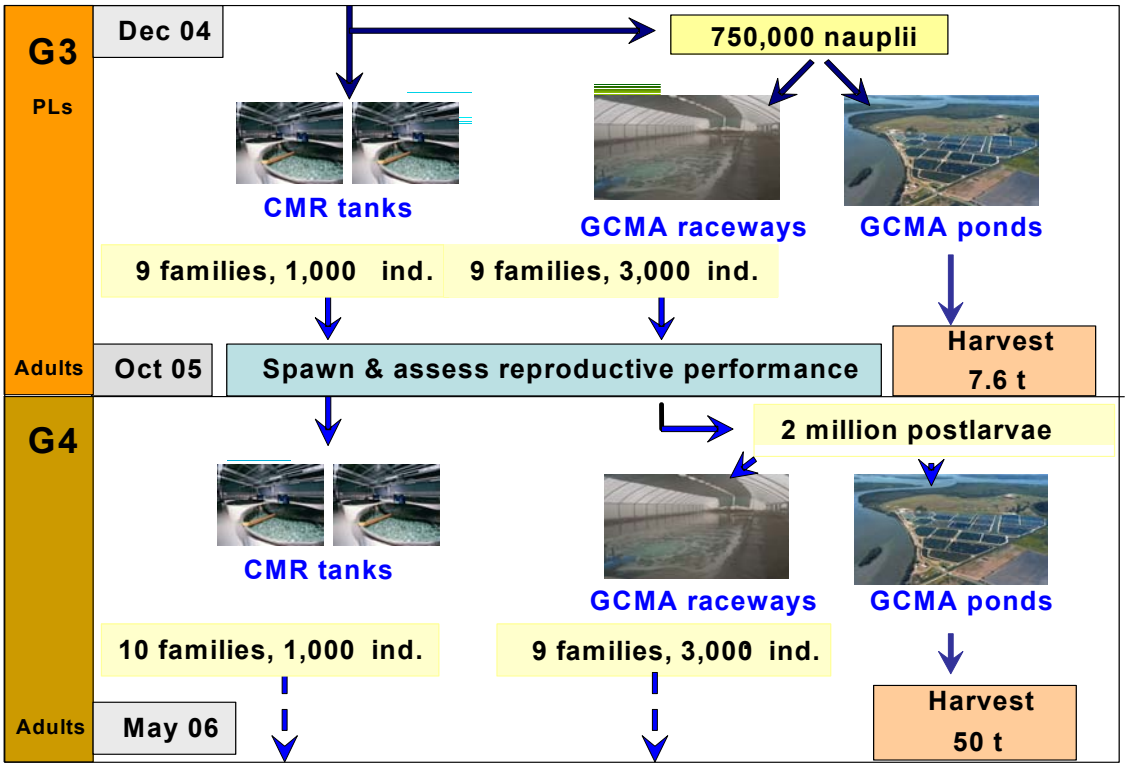

Figure 6. Schematic of the production of G4 *Penaeus monodon* GoC stocks. The G4 stocks were the progeny of CMR G3 tank reared stocks and GCMA G3 raceway reared stocks.

1.2.2 Results

Growth and survival of domesticated GoC broodstock in controlled environment tanks at CMR and controlled environment raceways at GCMA

In the first generation (G1) the growth of the GoC females was similar in tanks and raceways (Fig. 7). By 11 months the average mean weight of females grown in raceways was 120 g, the average mean weight of males was 84 g. Siblings grown in tanks at CMR averaged 115 g (females) and 85.4 g (males). These growth rates were comparable to that of the wild founder stocks (G0) which were reared for 6 months in controlled environment tanks at CMR and GCMA (Fig. 7). The mean survival of the G1 males and females was 80% in both raceways and tanks over the first 11.5 months (the age at which spawning trials commenced). Subsequently mean survival of both males and females declined to an average of 30% by 14 months (including non-ablated females).

In the second generation (G2) it was not possible to accurately monitor the growth of the GCMA production ponds stocks until they were transferred to the raceways (when they were 5.5 months old). Once transferred to raceways the growth rate of females was significantly lower than their siblings that were reared in the controlled environment tanks at CMR (Fig. 7). The growth rate of the tank reared standard density (~3 individuals m^2) was similar to that of the G1 stocks with females reaching a mean weight of 120 g at 10.5 months (Fig. 7). However, significantly higher growth rates were achieved by reducing the rearing density of the G2 stocks (~1 individuals m^2), with females reaching a mean weight of 131 g at 10.5 months (Fig. 7).

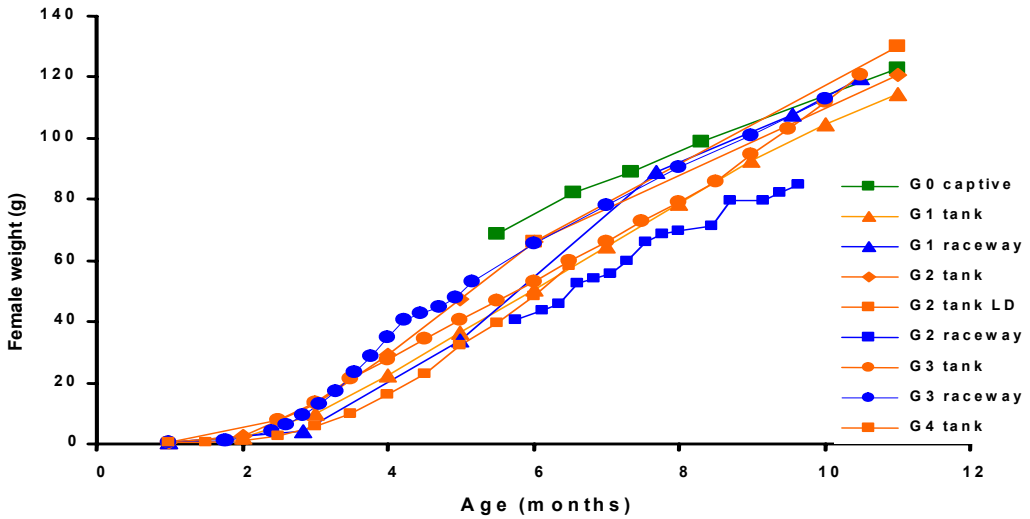

Figure 7. Comparison of the mean growth of successive generations (G0-G4) of female *Penaeus monodon* progeny of Gulf of Carpentaria (GoC) stocks reared in tanks at CMR and raceways at GCMA. LD = Low density.

The survival rates of the G2 stocks that were reared in GCMA production ponds stocks for 5.5 months prior to their transfer to raceways was low (~20%) and none of the surviving females spawned. Mean survival rates of the tank reared females at CMR were 74% with no significant difference in survival between the standard and low density stocks.

In the third generation (G3) the growth of stocks was similar in raceways and tanks with females reaching a mean weight of ~120g in 11 months. The survival rate in tanks and raceways was between 75% and 80%.

Reproductive performance of tank-grown, raceway-grown and wild G1 stocks

The two families of G1 stocks produced at GCMA reached a size of approximately 2 g by February 2003. Siblings from both families were transferred to two commercial raceways at GCMA and to 10 ten-tonne tanks within the quarantine facility at CMR (Fig. 3). These families reached broodstock age at 11.5 months at which time their reproductive performance was assessed.

Percentage of G1 females spawning

In both the tank and raceway grown stocks, family 2 out-performed family 1 (Fig. 8). The highest percentage spawning (71.4%) was from family 2 in the CMR tank grown stock, with a mean of 57% for both families. For the raceway grown stocks, a lower proportion (16.6%) spawned at the GCMA facility compared to the same stock in the CMR facility (39%). For the wild stocks, 37% of the over-wintered stock spawned at least once, but 84.2% of the freshly collected wild broodstock spawned.

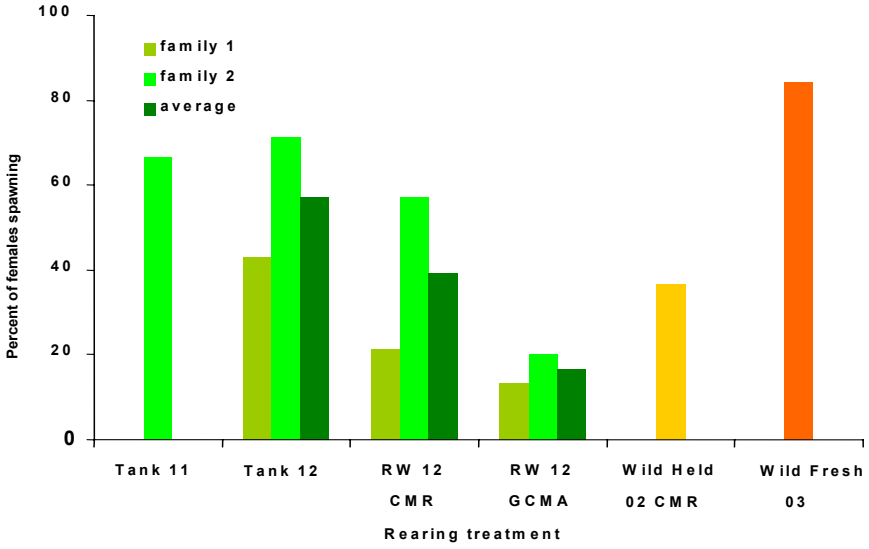

Figure 8. Percentage of all ablated females that spawned at least once. Tank 11: reared in CMR tanks for 11 months, spawned at CMR, Tank 12: reared in CMR tanks for 12 months, spawned at CMR, RW12 CMR: reared in GCMA raceways for 12 months then spawned at CMR, RW12GCMA: reared in GCMA raceways for 12 months then spawned at GCMA, Wild Held (2002) CMR: captured at ~6months reared until 12 months in CMR tanks, spawned at CMR, Wild Fresh 03: captured as mature broodstock (~15 months old), spawned at CMR.

Egg production by G1 broodstock

The mean number of eggs produced per spawning ranged from around 5,000 to 325,000 per spawning (Fig. 9). Despite the wide range of broodstock size (weight), there was no relationship between broodstock size and the number of eggs produced at spawning. The mean number of eggs per spawning for the tank-grown G1 broodstock at CMR was 143,485. For the same families grown in the raceways at GCMA, the mean number of eggs per spawning for the broodstock spawned at CMR was 99,622, but the same stocks spawned at GCMA produced an average of 232,600 eggs per spawning. This difference is likely due to the fact that only the best spawnings were kept and utilized at GCMA, as this was a commercially-based exercise to produce progeny for subsequent pond stocking. The "wild held" overwintered broodstock produced a mean of 221,331 eggs per spawning. The relatively low mean number of eggs per spawning for the "wild fresh" stocks (127,046) was possibly a consequence of the relatively small size of these wild broodstock.

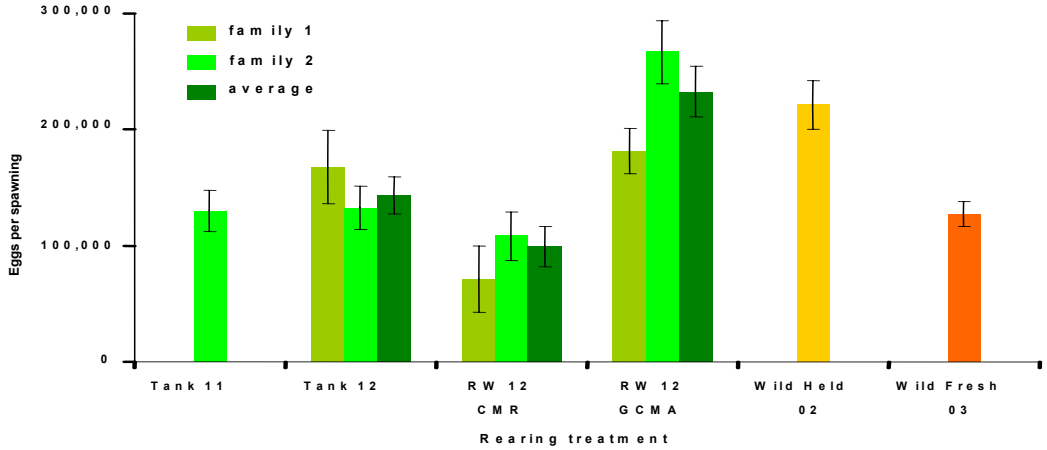

Figure 9. Mean number of eggs produced by tank reared and wild *Penaeus monodon* females at each spawning.

Hatching and hatch rates of G1 broodstock eggs

The percentage of spawnings that hatched was reasonably consistent across the various stock types; between 65.5% and 66.6% for the tank and raceway stocks respectively, 61.6% for the over-wintered wild stocks, and a higher 75.3% for the "wild fresh" stocks.

The hatch rates (i.e. the percentage of eggs that actually hatched from a spawning) for the various stock types were relatively low. Mean hatch rates were 34.2%, 27.3%, 32.5%, 21.6%, and 34.3% for the tank-grown CMR stocks, raceway stocks spawned at CMR, raceway stocks spawned at GCMA, "wild held" stocks at CMR and "fresh wild" at CMR, respectively (Fig. 10). The variability in hatch rate was high, with some around 70 to 80% and a high proportion of low hatches (under 20%). These relatively low mean hatch rates represent one of the main barriers to producing commercial quantities of seedstock from these domesticated stocks. Even so, hatch rates from the wild stocks were also low in this assessment.

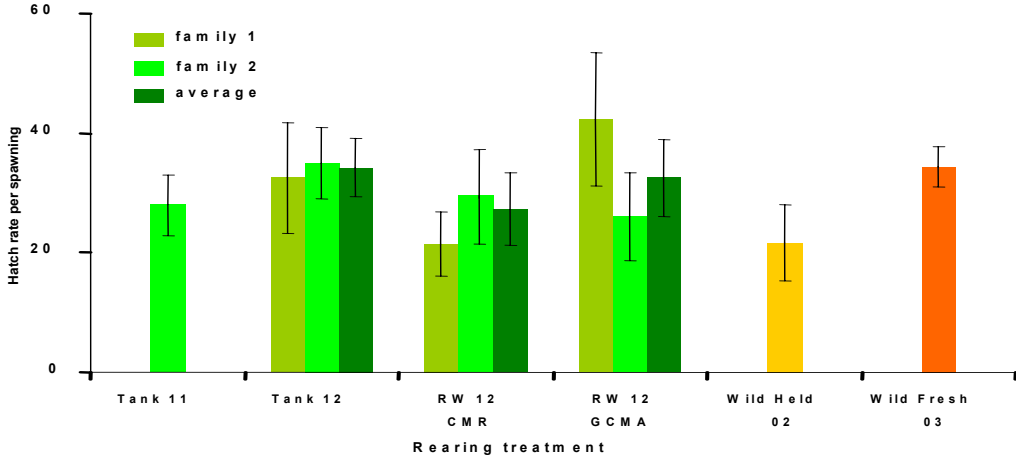

Figure 10. Mean hatch rate per spawning of tank reared and wild *Penaeus monodon* females.

Nauplii production by G1 broodstock

The mean numbers of nauplii produced per spawning were 60,802, 22,002, 126,000 and 51,587 for the tank-grown CMR stocks, raceway stocks spawned at CMR, raceway stocks spawned at GCMA, and "fresh wild" at CMR, respectively. When nauplii production is calculated per ablated female (i.e. including all females which did not spawn), these numbers dropped considerably to 35,283, 15,865, 12,450 and 62,645 for the tank-grown CMR stocks, raceway stocks spawned at CMR, raceway stocks spawned at GCMA, and "fresh wild" at CMR, respectively (Fig. 11).

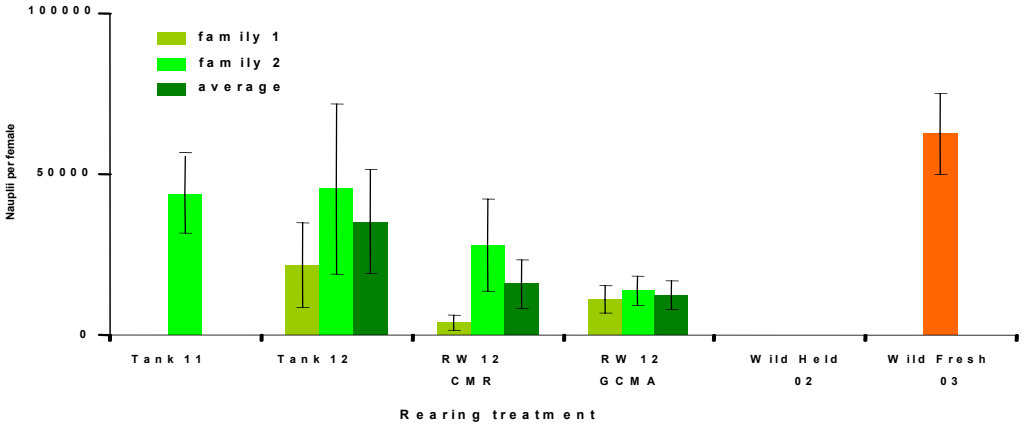

Figure 11. Mean number of nauplii produced for each ablated tank reared and wild *Penaeus monodon* females.

Comparison of reproductive performance in generations: G1, G2, and G3

During the course of the project, 4 generations of *P. monodon* were produced but the G4 stocks had not grown to broodstock size by the end of the project (Fig. 7). The percentage of females that spawned did not differ significantly across the 3 generations under the standard rearing density of 3.3 individuals per m^2 during growout (Fig. 12).

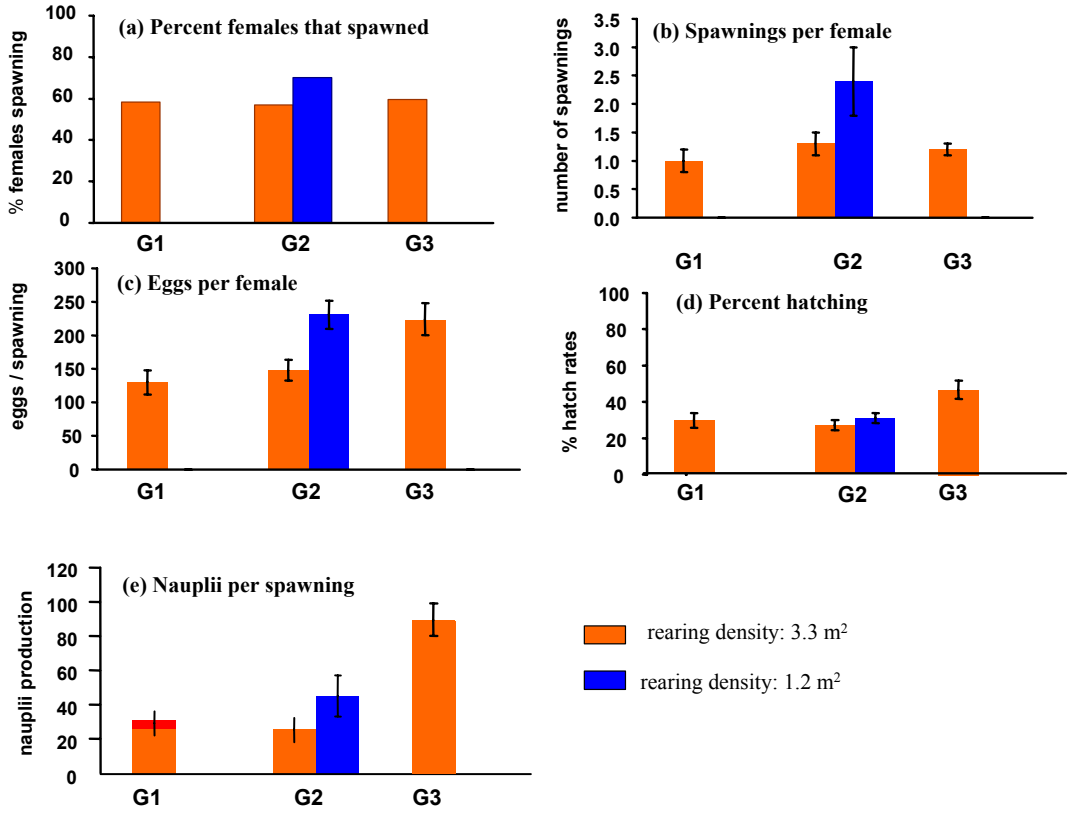

Figure 12. Variation in reproductive performance in three generations (G1, G2, G3) of *Penaeus monodon* broodstock reared at CMR. (a) Percentage of the total population of females spawning, (b) Mean number of spawnings per female, (c) Mean number of eggs produced per spawning, (d) Mean percentage of eggs hatching per spawning, (e) Mean number of nauplii per spawning.

The percentage of females that spawned was 58% for G1, 60% for G2 and 57% for G3 (Fig. 12a). However, when sibling G2 females were reared at a lower stocking density (1.2 per m^2)

there was a significant increase in the proportion of females that spawned, with 70% of females spawning compared with 60% at the higher density (Fig. 12a).

The mean number of spawnings per female was not significantly different across the 3 generations, although G2 and G3 (1.3 and 1.2, respectively) were slightly higher than G1 (1.0) (Fig. 12b). The low density growout for G2 stocks resulted in a much higher (2.4) number of spawns per female.

The mean number of eggs produced per spawning gradually increased from G1 to G3; 130,000 per spawning for G1, 150,000 for G2 and 200,000 for G3 (Fig. 12c). The low density growout treatment for G2 stocks resulted in a much higher (230,000) number of eggs produced per spawning.

The mean percentage of eggs hatching was relatively low in the G1 and G2 generation stocks (27% and 17%, respectively), but was significantly higher for the G3 stocks (40%) (Fig. 12d). In this case, the hatch rate did not improve significantly when G2 broodstock were grown at a lower density; 17% at standard density, 20% at low density.

The mean number of nauplii produced per spawning is the product of egg production and the hatching rate. Nauplii production was more than two fold higher for the G3 stocks (84,000 per spawning) compared to the G1 and G2 stocks (35,000 and 26,000 respectively) (Fig. 12e). The improved performance of the G3 stocks was due to the combination of both higher egg production and higher hatch rates, by comparison with the G1 and G2 stocks. Nauplii production per spawning for the G3 stocks grown at the standard density was still greater (84,000) than the G2 low density treatment (46,000).

When the nauplii per individual spawning result is combined with the higher percentage spawning and the higher number of spawnings per female for the overall broodstock population, the increased nauplii production for the entire broodstock population was significantly higher again for the G3 stocks (Fig. 12e).

1.3 East Coast Stocks

1.3.1 Methods

Production of the first generation (G1) at AIMS and BIARC

In April 2002 wild broodstock were sourced from trawlers fishing in coastal waters off North Queensland's Wet Tropics, in the Cairns/Innisfail region. These prawns were transported to the AIMS maturation facility and separated into males and females. At the same time 17 month old AIMS G3 males were transferred from their out door grow out tanks into a single maturation tank inside the facility. At stocking, all females were given a waterproof moult tag to track subsequent moulting events. Male and female broodstock were fed a conditioning diet of fresh frozen squid (*Photololigo* sp.), New Zealand mussels (*Pema canaliculus*), pipis (*Plebidonax* sp.) and an in-house maturation pâté containing astaxanthin.

Female broodstock were artificially inseminated, two days post-moult. Two spermatophores were extracted from each male, cut in half, and used to inseminate up to four females depending on the number of females available for mating each day (i.e. those that had moulted two days previously). While this was possible for most wild males, the strategy could not be implemented for the majority of the AIMS G3 males because of the small size and the low number of sperm bundles in the spermatophores of these males.

Immediately after artificial insemination each female was unilaterally ablated using heated wire snips and then returned to their respective tanks. Ablated females were thereafter monitored each afternoon, for signs of ovarian development. Females with fully mature ovaries were transferred to individual spawning tanks. The following morning, eggs were collected and sub-samples taken for estimating egg number for each batch. For the purposes of this experiment only the first batch of eggs, produced within the first inter-moult period post-ablation, was retained from each female.

All the artificial inseminations for this experiment were undertaken from April 24 to May 27, 2002. A total of 28 half-sib families (i.e. 14 pairs) were produced from the wild x wild treatment while only 3 half-sib families were generated for the AIMS G3 x wild treatment (Table 1). These families were stocked for larval rearing from 4 May to 5 June 2002 (Fig. 13). Although parents of these families were a mixture of wild stocks and domesticated stocks (the AIMS G3 males) the terminology was simplified by referring to successive generations as G0 (wild), G1 (first generation produced at AIMS/BIARC using a mixture of EC stocks), G2 (second generation produced at AIMS/BIARC using a mixture of EC stocks and domesticated Seafarm stocks) and G3 (third generation produced at AIMS/BIARC using a mixture of EC stocks).

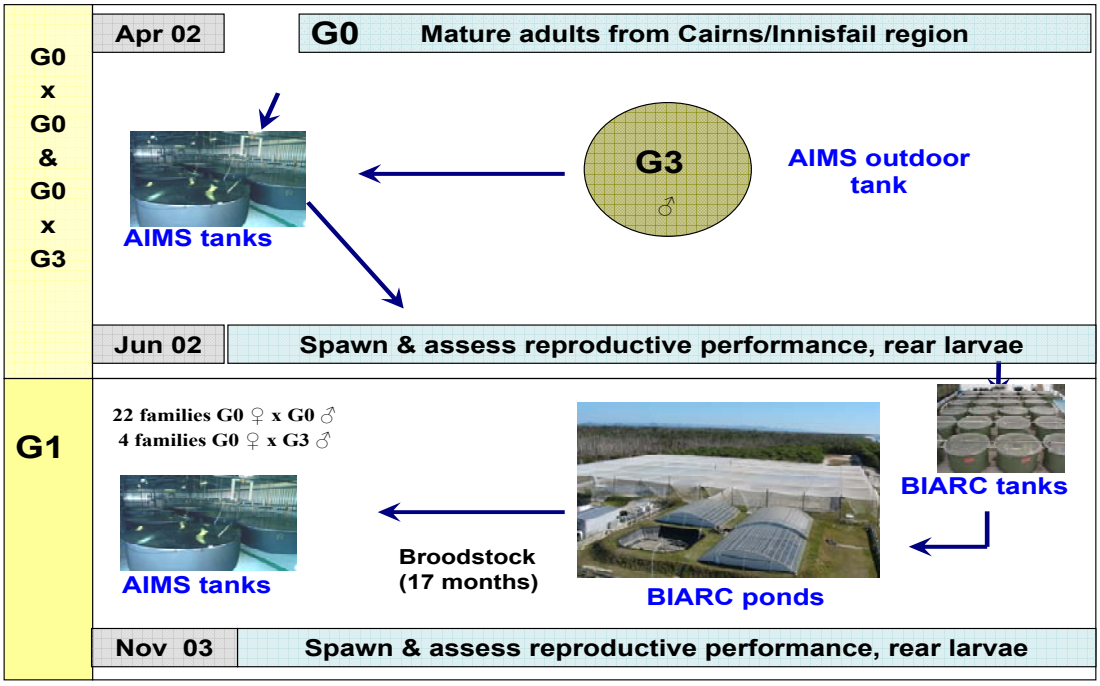

Figure 13. Schematic of the production of G1 of *Penaeus monodon* EC stocks. The G1 stocks were the progeny wild (G0) EC males and females and wild females crossed with G3 males reared in tanks at AIMS. The stocks were reared to postlarvae at AIMS, transported by air to BIARC where they were reared to juveniles in cages in tanks then to mature adults in ponds.

A total of 94 batches of larvae (47 batches each with two replicates) were reared in 50 L larval rearing tanks following the standard AIMS larval rearing protocol (Benzie et al. 1997), from 4 May to 5 June 2002. These included the priority half-sib families for the wild x wild treatment (22 half-sib families) and those for the AIMS G3 x wild treatment (4 half-sib families). In addition seven reserve half-sib families were reared from the wild x wild treatment in case any of the priority batches failed to reach the required postlarval stage (PL15). Replicate batches were also reared from three full-sib families from the AIMS G3 x wild treatment. This was

necessary because of the low number of half-sib families generated in this treatment. On the 4th June, 2002 the first shipment of *P. monodon* postlarvae were air-freighted from AIMS to the BIARC. These shipments continued until the 2nd July 2002.

Table 1. Summary of the numbers of wild and domesticated G3 males used for spermatophore (SPTM) extraction, the number of wild females inseminated and the number of families produced at AIMS.

	Males used For SPTM extraction	Wild females artificial inseminated	Females that died post ablation without spawning	Females that spawned at least once	Egg batches hatched	Families reared to PL 15	No. pairs of half-sib families
Wild males	29	85	24	60	41	41	14 (total of 28 half sib families)
G3 males	31	67	22	27	6	6	1(total of 3 half-sib families)

Rearing of the G1 families at BIARC

On arrival at BIARC each batch of postlarvae was placed into a 10 L bucket. Family number, replicate, water quality, and survival records were taken for each batch. Postlarvae were acclimated for a period of 60 minutes with the addition of heated seawater in a temperature-controlled room at a rate not exceeding 1°C per 15 minutes. Each family (≈150 individuals) was placed into floating cages labelled with their family code and feed rate (Fig. 14). Of the 26 families stocked, 17 of these were stocked into duplicate cages and each cage placed in a separate 10,000 L tank in order to spread the risks of losing families. Due to low postlarval survival, 8 families were housed as single cage replicates only.

A total of 76 cages were allocated to and suspended in 4 x 10,000 L tanks, with each cage receiving its own food supply, air and exchange water (Fig.14). Each cage received approximately 526 litres of exchange water per day or the equivalent of 100% exchange of the holding tank. The exchange water was sourced from a commercially stocked banana prawn grow-out pond at BIARC and heated to 28°C. Pond water was added to stimulate natural productivity in the 10,000 L holding tanks.

The postlarvae in each cage were fed 4 times per day (0800, 1100, 1400 and 1700 hours) according to total prawn biomass with 3 artificial feeds (Frippak Ultra PL+ range of diets initially and then Higashimaru Ebi Star) and 1 live feed of newly hatched Artemia (for the first 4 weeks). Water quality was measured twice daily including temperature (27°C to 28°C), salinity (35 ppt to 37 ppt), DO (> 6ppm), TAN (<2ppm) and nitrite (<0.05ppm). Holding tanks were vacuumed on a bi-weekly basis or as required.

Figure 14. Mesh cages used for rearing *Penaeus monodon* postlarvae at BIARC (left) and mesh cages used for rearing *P. monodon* postlarvae suspended in a 10,000 L fibreglass tank (right).

After 112 days a total of 3,288 prawns (average weight of individuals = 4.2 grams) from 26 families were tagged with an average of 78 representatives from each family. At this stage the pond temperatures precluded stocking into grow out ponds (1600m^2) so the families were grown in a heated nursery pond for a further 45 days until the end of October 2002, when the grow-out pond temperature reached 23°C. The families were harvested and all surviving prawns (average wet weight 15.15 g) were relocated into a 1600m^2 grow out pond (Fig.15). Stocking density was reduced from 16m^2 to 2m^2 in order to maximize growth rates. Average size and harvest biomass estimates indicated that survival from nursery stocking until harvest had been extremely high, approximately 95%.

On the 15th May 2003, due to falling water temperatures, the grow out pond was harvested, and the remaining prawns weighed and counted and all surviving prawns relocated into one of two heated nursery ponds. All prawns continued to be grown and maintained in these nursery ponds until required for reproductive assessment or selection purposes.

In September 2003 the prawns were harvested and weighed, sorted into families, and stocked into 24 x 2 tonne holding tanks. These prawns were subsequently eye tagged with colour coded and numbered budgerigar rings; the former to identify family, the latter to mark individuals within families, in preparation for air freighting to AIMS. The retagging enabled rapid sorting of these stock at AIMS to minimise stress and facilitate assignment of families to specific tanks in order to implement the preferred mating protocols.

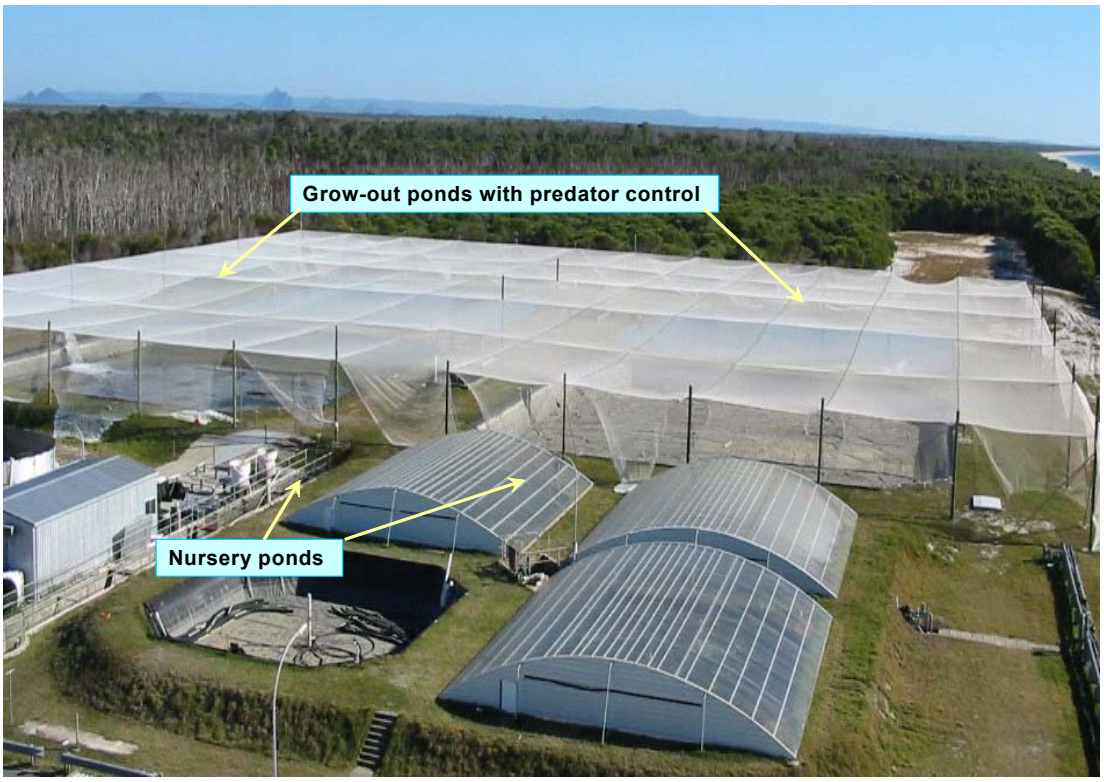

Figure 15. Nursery and Growout ponds at BIARC, Queensland.

G1 Broodstock shipments

Up to 12 (6 female and 6 male) selected broodstock per family were air freighted to AIMS for spawning and reproductive assessments between 30 September and 6 October 2003. At this time, these broodstock were approximately 17 months old. In addition to the broodstock from BIARC, Seafarm provided a batch of 53 untagged broodstock containing 18 females and 35 males, from a domesticated sixth generation (G6) stock, as a late inclusion into this experiment

(Fig.17). These prawns were road freighted to AIMS on 13 October 2003 and treated as a single family group for the purpose of this experiment. They were incorporated into the mating design to produce a number of distinct crosses (Fig.16).

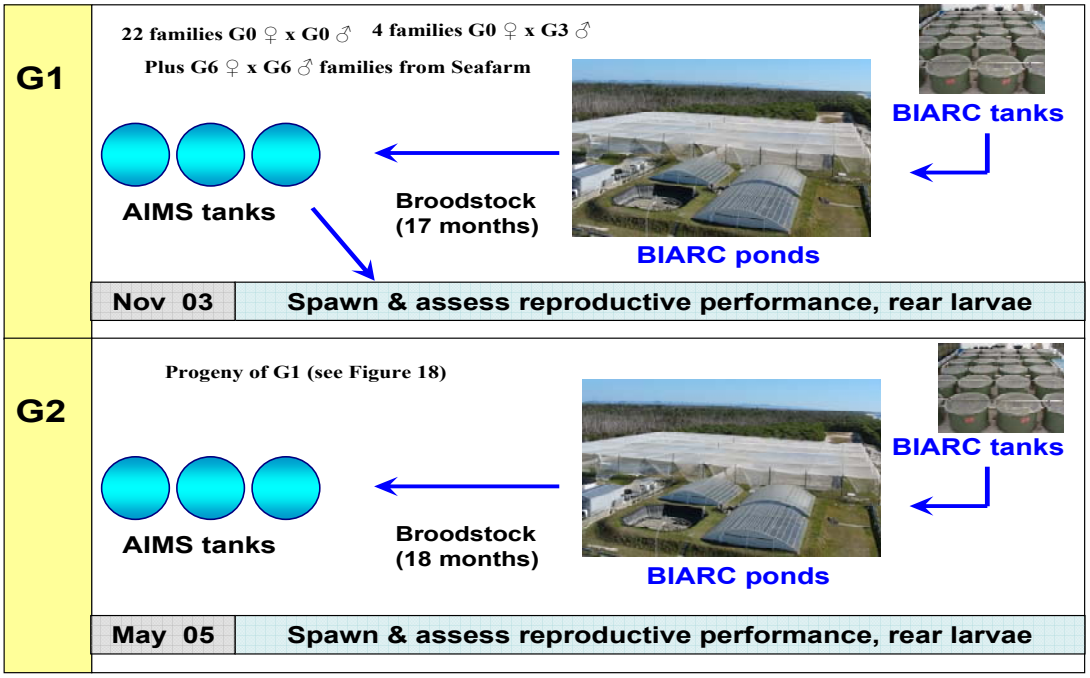

Figure 16. Schematic of production of the G2 of *Penaeus monodon* EC stocks. The G2 stocks were the progeny of G1 stocks spawned and reared to postlarvae at AIMS, transported to BIARC where they were reared to juveniles in cages and tanks then reared to mature adults in broodstock ponds.

Broodstock stocking and husbandry of G1 stocks at AIMS

All incoming prawns were separated into male and female groups and stocked into 4m diameter tanks in the AIMS maturation facility. A total of 4 female only tanks were stocked, each with an average of 38 broodstock. Incoming males were split between the remaining 3 tanks. At stocking, females were weighed, moult tagged with waterproof spots, and eye tagged with colour coded rings. The former allowed moulting events to be followed, the latter enabled individuals to be identified within tanks without handling. In a similar fashion males were eye tagged with colour coded and numbered rings to identify them to the family and individual level.

Throughout the experiment broodstock were fed the AIMS standard maturation diet of frozen squid, NZ greenlip mussels, pippies and an in-house pate based on molluscs and enriched with a vitamin supplement and astaxanthin. Polychaete worms were also fed on a daily basis at a rate of 1 worm for 3 broodstock. For the duration of the experiment protocols for monitoring and feeding were the same as those detailed in Benzie et al. 1999.

Production of G2 stocks at AIMS and BIARC

The mating protocol used to produce the second generation (G2) stocks included a mixture of parental stocks that had been domesticated for different periods of time (e.g. the AIMS G3 males used to produce the G1 stocks and the Seafarm G6 males and females) (Fig. 17). The overall aim was to maximise production of unique full sib families where a particular male line was crossed with a unique female line. This was achieved by selecting a line of males (i.e. from a single family) and then using spermatophores from that line of males to only inseminate females from a single family line. Because of the non-synchronous moulting of females within a particular family, multiple spermatophore extractions from a single male were sometimes required to achieve this, particularly in those male lines where only a few individuals remained. Typically each female received 1 spermatophore during artificial insemination.

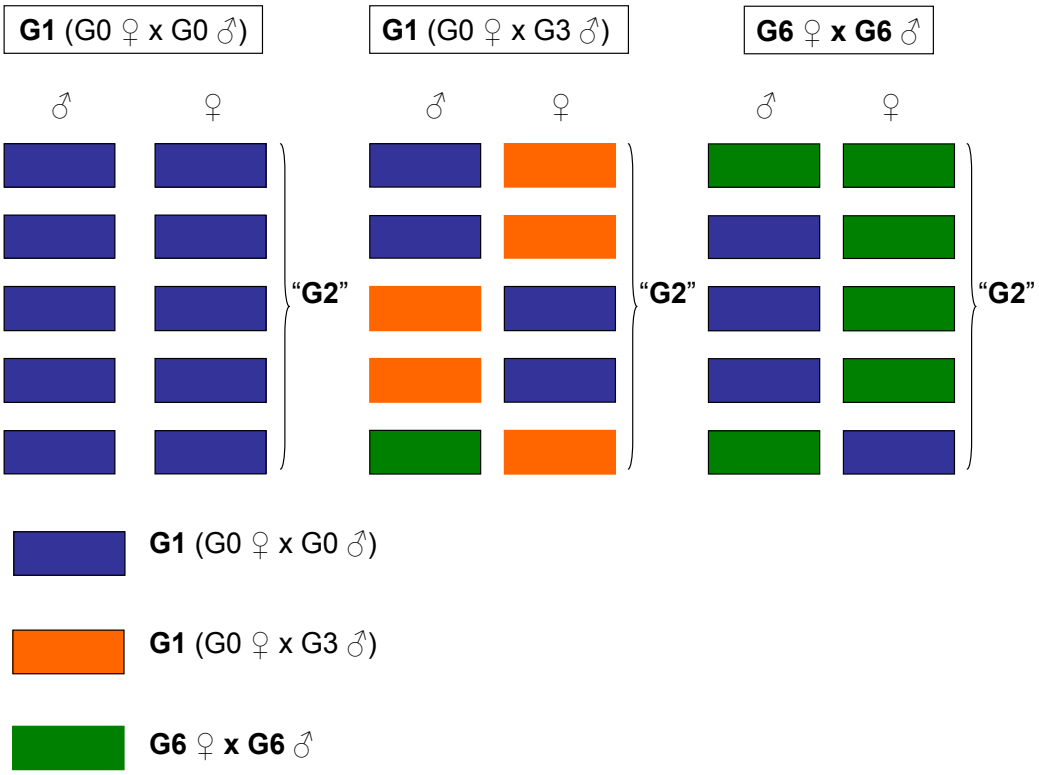

Figure 17. Schematic of the mating protocol used to produce the G2 *Penaeus monodon* EC families. The G2 stocks were the progeny of G1 stocks spawned and reared to postlarvae at AIMS, transported to BIARC where they were reared to juveniles in cages in tanks, reared to mature adults in broodstock ponds then transported to AIMS for production of the G2 families

The protocols for spermatophore extraction, artificial insemination, ablation, monitoring of gonad development, spawning, egg collection and counting, nauplii collection and counting and the assessment of nauplii condition were the same as those used for the production of the G1 families. Females that produced high priority families that were stocked for larval rearing were culled within 3 days of spawning and tissue samples taken for DNA pedigree data and for viral load analysis (pleopods and gill tissue respectively). As a result most females were culled during the first inter-moult period after ablation. Males that produced high priority families were also culled and tissue samples taken. Parental pleopod samples were stored at AIMS in case there is a future need for progeny verification.

Initially a total of 64 separate families were produced from the cohort of G1 broodstock. During the course of the experiment this was reduced to 32 families using a process of culling lower priority families as high priority families became available. As the priority was to generate as many independent full sib families as possible, families that were discarded included families from a cross where another family derived from a cross between the same parental lines was already stocked. From each of these families, two replicate batches of 4,000 nauplii each were stocked and reared through to PL15 at AIMS with assistance from BIARC staff. This occurred over a 4 week period from 19 October to 20 November 2003.

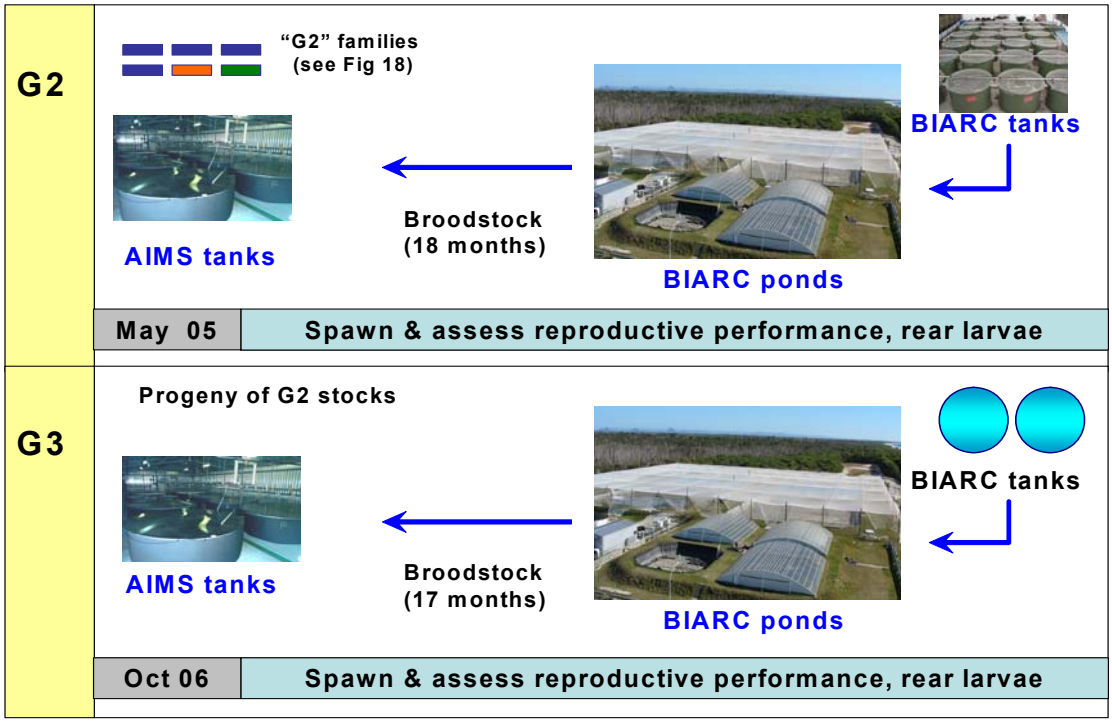

Figure 18. Schematic of production of the G3 *Penaeus monodon* EC stocks. The G3 stocks were the progeny of G2 stocks spawned and reared to postlarvae at AIMS, transported to BIARC where they were reared to juveniles in cages in tanks then reared to mature adults in broodstock ponds.

Harvest of G2 families at AIMS and shipment to BIARC

At the end of the hatchery phase PL15's were harvested and a total count obtained to establish survival. From each family, 150 PL15's from the 2 replicates were then sampled and placed in two 1.5 L bags containing 300 ml seawater, one for each replicate. In replicates where survival was low, PL's from the other replicate for that family were used to make up the numbers to ensure that two bags of 150 PL15's per family were obtained. After stocking, bags were filled with industrial grade oxygen, placed in a styrofoam box (up to 12 bags per box) and air-freighted to BIARC the same day. The first consignment of families was sent on 17 November 2003, and the last on 5 December 2003. A total of 22 distinct families were freighted to BIARC in 5 consignments. The 22 families comprised the following crosses; 12 (G1 x G1), 4 (G1 x G4), 4 (G1 x G6), 1 (G4 x G6) and 1 (G6 x G6). On arrival at BIARC the postlarvae were stocked into a floating cage nursery system (Fig. 14). The postlarvae were reared to juveniles in the nursery system at BIARC until 24th February 2004, when they were harvested, tagged with elastomer implants and stocked into covered broodstock ponds (Fig. 15).

Production of G3 stocks at AIMS and BIARC

In March and April 2005 mature G2 broodstock were harvested from the BIARC growout pond sorted by family and stocked into 24 x 2 tonne holding tanks. These prawns were subsequently eye tagged with colour coded and numbered budgerigar rings; the former to identify family, the latter to mark individuals within families, in preparation for air freighting to AIMS.

The production, growth and survival of the G3 cohort of families will be reported in the follow-on project (Practical, feasible and low-cost genetic selection of *Penaeus monodon* for increased profitability), together with the reproductive performance of these stocks.

1.3.2 Results

Growth and survival of G1 broodstock in controlled environment tanks, heated nursery ponds and ambient temperature ponds at BIARC.

26 families of first generation (G1) EC stocks were sent from AIMS to BIARC as postlarvae during June and July 2002 (Fig. 16). The families were all successfully reared in cages suspended in 10,000 L fibreglass tanks at BIARC. After 52 days post-hatching the average weight of the *P. monodon* juveniles was 0.3 grams with an average survival of 71%. After 86 days the average weight was 1.8 grams and survival between 52 and 86 days was 83%. After 112 days the average weight had increased to 4.2 grams, with survival from 86 to 112 days of 95 %. A total of 3,288 prawns (average weight of individuals = 4.2 grams) from 26 families were tagged with an average of 78 representatives from each family. At this stage the pond temperatures precluded stocking into grow out ponds (1600m^2) and the families were grown in a heated nursery pond for a further 45 days until the end of October 2002 when the grow-out pond temperature reached 23°C. The families were harvested and all surviving prawns (average wet weight 15.15 g) were relocated into a 1600m^2 grow out pond (Fig. 15). Stocking density was reduced from 16m^2 to 2m^2 in order to maximize growth rates. Average size and harvest biomass estimates indicated that survival from nursery stocking until harvest had been extremely high, approximately 95%.

On the 15th May 2003, due to falling water temperatures, the grow out pond was harvested and all surviving prawns (average weight 55.5 g ♂ and 91.9 g ♀) were again relocated into one of two heated nursery ponds. Average size and harvest biomass estimates indicated that survival in the grow-out pond had been reasonable, approximately 63%. All prawns continued to be grown and maintained in these nursery ponds until required for reproductive assessment or selection purposes. Although the stocks were over wintered in the heated nursery ponds the relatively low water temperatures (compared to the controlled environment tanks at CSIRO and raceways at GCMA) resulted in significantly lower growth rates of the broodstock reared in ponds at BIARC.

In September 2003 the prawns were harvested, sorted into families, and up to 12 (6 female and 6 male) selected broodstock per family were air freighted to AIMS for spawning and reproductive assessments according to agreed mating protocols. The average weight of males was 63 g and the average weight of females was 103 g. (Fig.19).

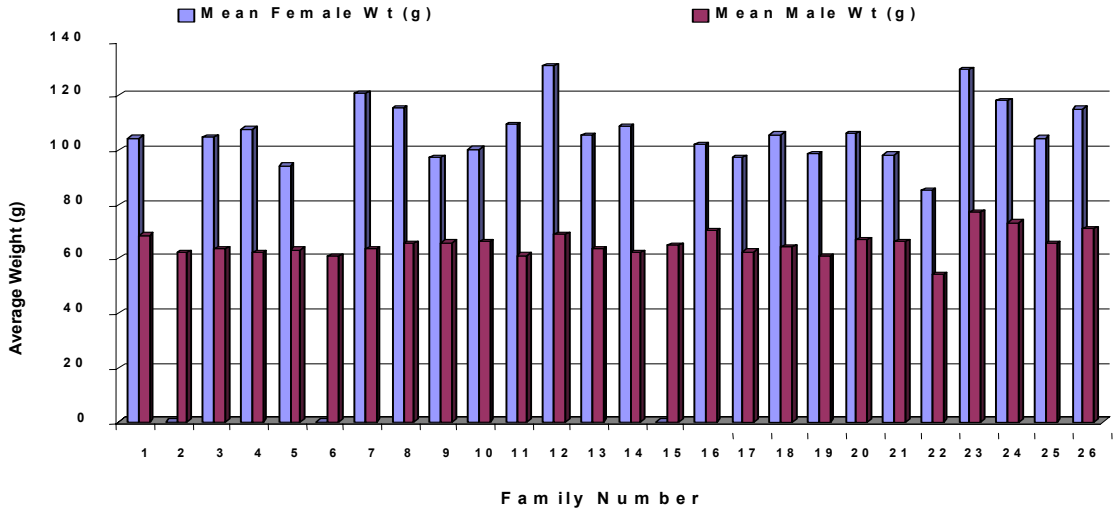

Figure 19. Mean harvest weight of 17 month old males and females from 26 families of G1 EC families of *Penaeus monodon* reared to juveniles in cages and nursery ponds then reared to mature adults in broodstock ponds at BIARC.

Survival of G1 broodstock during transport and condition on arrival

A total of 348 G1 broodstock (157 females and 191 males) were air freighted to AIMS in 5 consignments between 30 September and 6 October 2003. During transit from BIARC to AIMS, 6% of the broodstock sent from BIARC died. Upon arrival, survivors were lethargic and exhibited a range of coloration from normal (i.e. for pond reared broodstock as distinct from the bright colouration-white, olive and black, of wild caught stock) to crimson red over the entire body, the latter generally being regarded as an indicator of stress. By the end of the first week less than 75% of males and females survived. Major mortalities continued and by the end of the second week less than 50% of females remained alive. As prawns began to moult however, they appeared to recover condition and became more active. Coloration also changed, with these prawns becoming much closer to the coloration of wild caught stock. Of particular note were the bright red setae on the pleoplods, uropods and antennules, typical of freshly caught wild broodstock. This may have resulted from the inclusion of polychaetes in the diet.

The apparent recovery of females was short lived however, as 2 days after moulting they were captured, artificially inseminated and ablated. This caused many to regress back to a stressed coloration although a significant proportion did not exhibit this change immediately. In view of the poor condition of the surviving prawns and because BIARC had previously reported high mortalities during reproductive assessments, especially when biopsies (e.g. gill samples for disease testing) were taken, it was decided to focus on the production of the G2 families as the key priority. This meant that the reproductive assessment of the G1 broodstock at AIMS was limited to their first moult cycle following ablation. From the starting batch of 157 females, 81 survived in the AIMS maturation tanks to be ablated 2 days after moulting.

Post-ablation mortalities in G1 Broodstock

The progressive deterioration of ablated females is highlighted in Figure 20 which shows the distribution of mortalities during this period. A total of 40 natural deaths were recorded from the initial batch of 81 females that were ablated. Most occurred within the first 10 days after ablation. The mean period from ablation to death was 10.2 days. This data set is incomplete because the females that produced priority families were culled in order to obtain DNA samples for pedigree verification. If these prawns had not been culled, natural mortalities would have been higher as many of the females in this category appeared stressed (reddish coloration and lethargic) and would probably have died in the system before moulting.

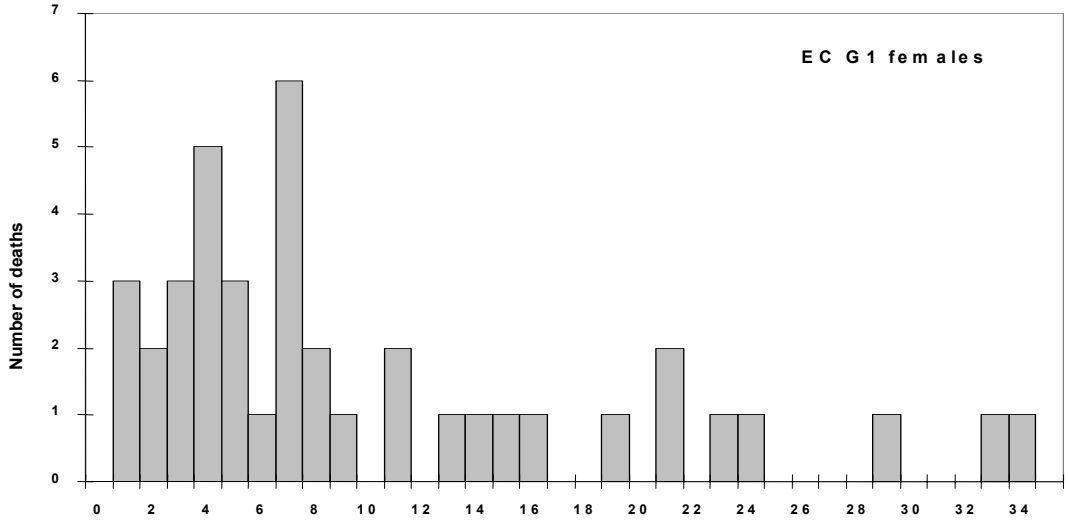

Figure 1. The number of deaths each day following ablation of female EC G1 broodstock.

Days to first maturation for G1 Broodstock

Days to first maturation is recorded as the number of days from ablation to the first time a female's ovaries reached Stage III development. From the initial batch of 81 ablated females, most reached Stage III within 7 days, with a mean of 5.3 days.

Days to first spawning for G1 Broodstock

Just under half of the females (43%) that reached Stage III ovarian development either experienced ovarian regression or died before spawning. A total of 36 females from the ablated batch of 81 spawned at least once. The mean number of days from ablation to spawning for this latter cohort was 8.8 days, with most spawning occurring during the first 15 days after ablation (i.e. within the first moult cycle which is generally around 16-18 days for domesticated broodstock held in the AIMS system).

Total spawnings for G1 Broodstock

A total of 71 spawnings (53 where eggs were harvested and 18 where no samples were taken) were recorded. As with the data for post-ablation mortalities this is an underestimate of the number of spawnings that would have been possible from this batch of broodstock as many females that generated priority families were culled soon after their first spawning to ensure that tissue samples were obtained. It is reasonable to assume that if these females had not been culled some would have produced additional batches of eggs before their next moult.

Egg production by G1 Broodstock

During this trial a total of 53 batches of eggs were harvested from this cohort of females. An additional 18 batches were also produced by the cohort but samples from these batches were not collected due to staff shortages during some peak periods of activity. The harvested batches were divided into "complete" and "partial" spawnings based on the degree of egg evacuation during spawning. Egg batches where the female had released all visible eggs during a spawning event were scored as "complete" spawnings. "Partial" spawnings were recorded when the female did not completely evacuate her ovaries and significant numbers of eggs remained in the ovaries the morning after spawning. Mean recorded egg production for complete spawnings was 188,381 ± 16,814 (n=43) and for partial spawnings was 148,640 ± 32,911 (n= 10). If these data are pooled mean recorded egg production was 180,883 ± 15,009.

Hatch rates of G1 Broodstock eggs

All matings during this experiment used artificial insemination with 1 spermatophore. From the 53 egg batches that were harvested, 11 batches did not hatch (i.e. zero hatch rate) from within the "complete" spawning category whereas 2 batches from within the "partial" spawning category did not hatch. If both categories are pooled, 25% of all egg batches sampled during this experiment showed no hatching. For complete spawnings, mean hatch rate (including zero hatches) was 30.6 ± 4.7% (n= 43) and for partial spawning mean hatch rate (including zero hatches) was 22.5 ± 7.5% (n=10). If all spawnings are pooled, mean hatch rate was 29.2 ± 4.1 (n=53).

Nauplii production by G1 Broodstock

From the 53 eggs batches that were harvested during this trial, a total of 2,683,020 nauplii were produced. Mean nauplii production from 43 complete (C) spawnings was 55,334± 10,021 and from 10 partial (P) spawnings was 30,366 ± 12,679. If all 53 sampled spawnings are pooled, mean nauplii production per female was 50,623 ± 11,965 (n=53).

Summary of results

This experiment highlighted the susceptibility of *P. monodon* broodstock to handling stress, as evidenced by the high proportion (48%) of female broodstock that died following transport from BIARC to AIMS and prior to ablation. However, it should also be recognised that, due to

various delays, the animals were 17 months old by the time they were shipped to AIMS, which probably meant that their tolerance to stress was extremely low.

The spermatophores produced by the male broodstock were superior to those from AIMS tank-reared domesticated EC males (G3 males) in the past. This correlated with superior performance compared to AIMS G3 males. However, better spermatophores did not necessarily translate into high fertility, as approximately 25% of spawnings had zero hatch rate, despite use of AI ensuring females were 'mated'. This indicates the need for more work in this area to determine the cause of male-associated fertility problems.

Overall numbers of egg and nauplii production per spawner ablated (although underestimates) are comparable to previous results achieved with domesticated broodstock. However, positive developments were:

- improved spermatophore morphology, which did correlate with higher fertility than for tank reared animals even if there remain additional issues associated with male fertility
- relatively rapid response to ablation, with a mean time to first spawn of 8.8 days
- reasonable overall percentage of animals that spawned following ablation (44%), for domesticated broodstock
- relatively high hatch rate, with a mean hatch rate of 30% across all spawnings assessed, including the 25% of spawnings which had a zero hatch rate.

Growth and survival of G2 broodstock in controlled environment tanks, heated nursery ponds and ambient temperature ponds at BIARC

22 families of second generation (G2) EC stocks arrived from AIMS in batches from November 17, 2003 until December 5, 2003 (Fig.16). At the end of this first nursery cage period (December 22, 2003), the average weight of juveniles was 0.28 g with an average survival of 89%. At the end of the second nursery cage period (24, February 2004), their average weight was 4.63 g and average survival was 78%. Juvenile prawns were then harvested from the nursery system in February 2004 and a total of 2,510 prawns from 22 families were tagged using a visible implant elastomer (VIE) tag with an average of 78 representatives from each family stocked into two covered broodstock ponds.

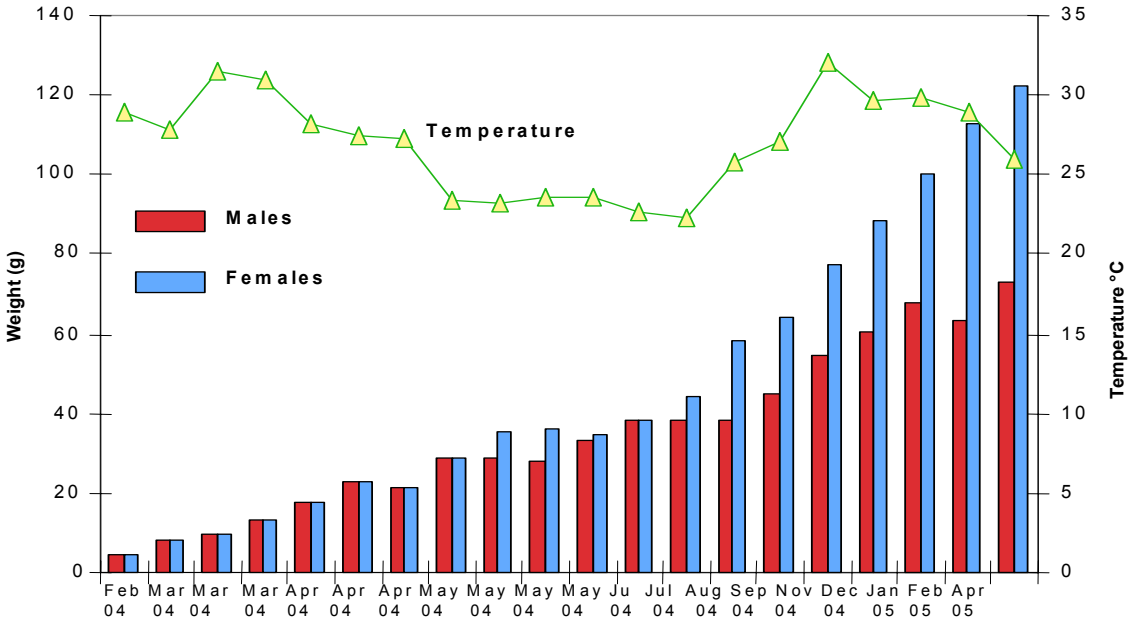

Figure 21. Growth of G2 EC *Penaeus monodon* reared for 14 months in broodstock ponds at BIARC.

After 14 months in the covered broodstock ponds, the first of these ponds (N1) was drain harvested in April 2005 and 291 (125 females and 166 males) broodstock removed and stockpiled into 26 x 2000 L plastic tanks. On the May 4, 2005, after analysis of pond survival at BIARC and transport survival of the first shipment to AIMS the second broodstock pond (N2), was drain harvested and again the 193 (89 females and 104 males) broodstock were stockpiled into 26 x 2000 L plastic tanks for shipment to AIMS.

The females had reached an average wet weight of 118 g and males 63 g (Fig. 21). Overall survival of the pond stocks was low (approximately 20%). The broodstock were sent to AIMS over three days (April 19 – 21, 2005). There was significant variation in growth and survival amongst families in both males and females (Fig. 22 and 23) but no attempt was made to exclude slow growing or lower surviving families at this stage. A total of 214 female and 270 male broodstock were produced at BIARC and air freighted to AIMS.

Survival of G2 broodstock during transport and condition on arrival

Of the 214 females and 270 males air freighted to AIMS, 23 females and 30 males died in transit. Most of these mortalities occurred in the first batch, with survival improving in later shipments after packing protocols were modified by increasing water volume in the transport bags (i.e. housed inside the polystyrene air freight boxes) and by using filtered seawater instead of pond water. This reduced transport mortality to approximately 6%. Density of females was also reduced but with little significant effect.

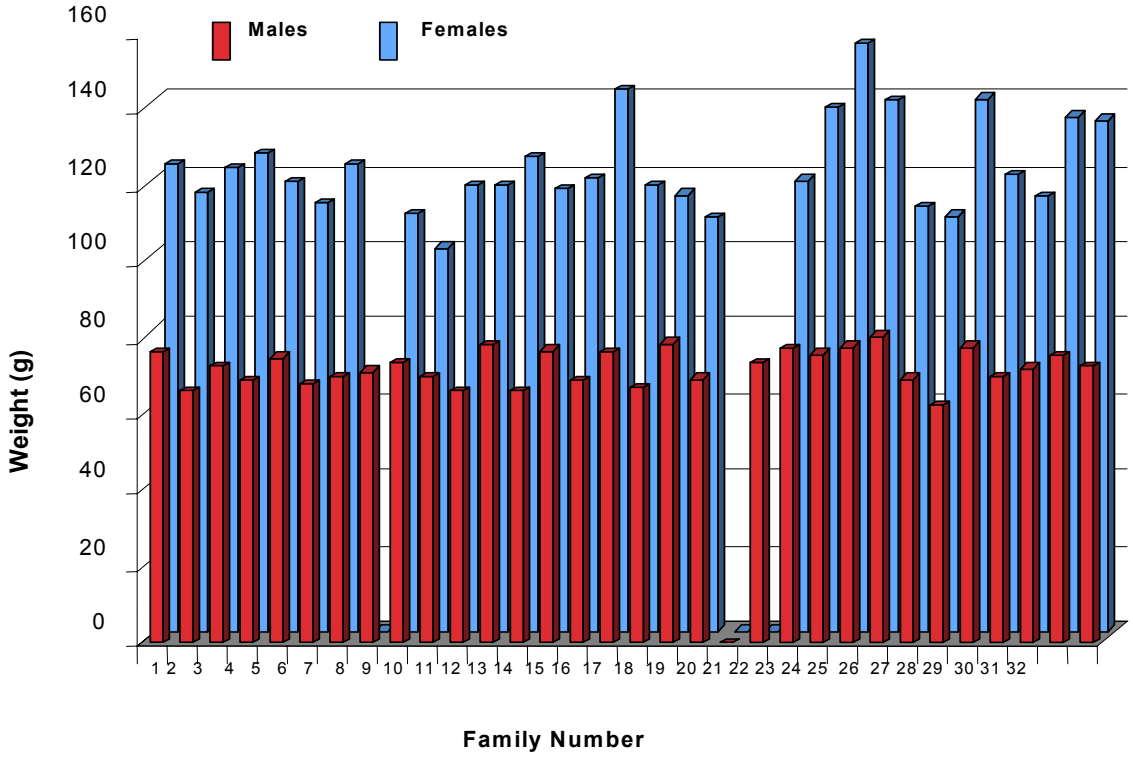

Figure 22. Variation in harvest weight among males and females from 32 families of 14 month old G2 EC *Penaeus monodon* reared in broodstock ponds at BIARC.

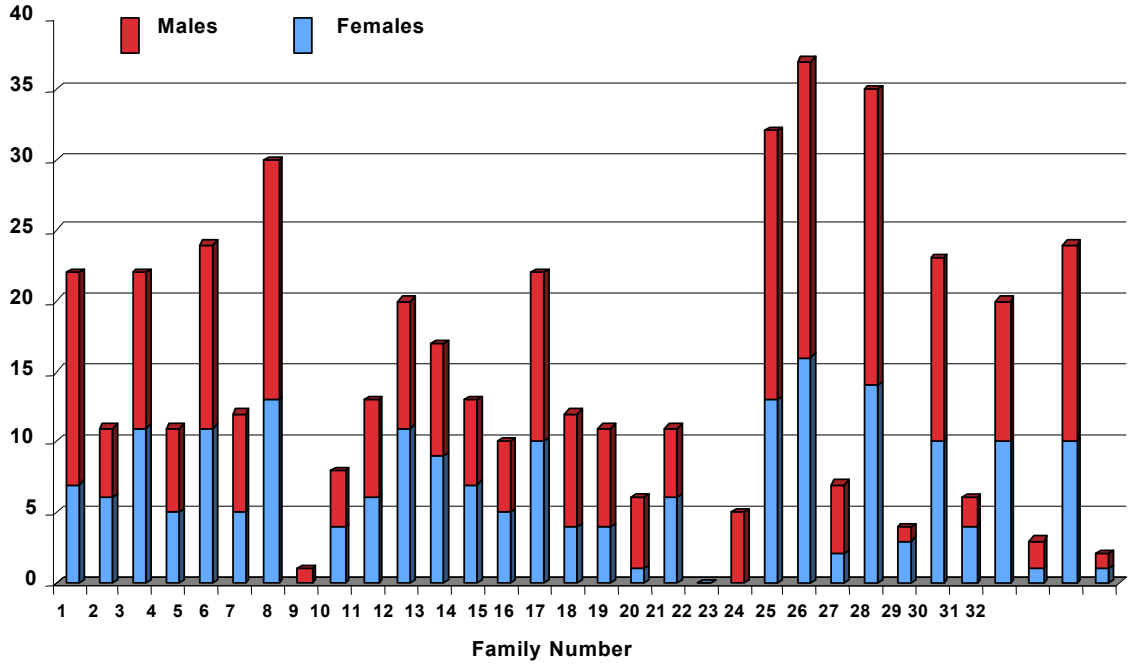

Figure 23. Variation in survival among males and females from 32 families of 14 month old G2 EC *Penaeus monodon* reared in broodstock ponds at BIARC.

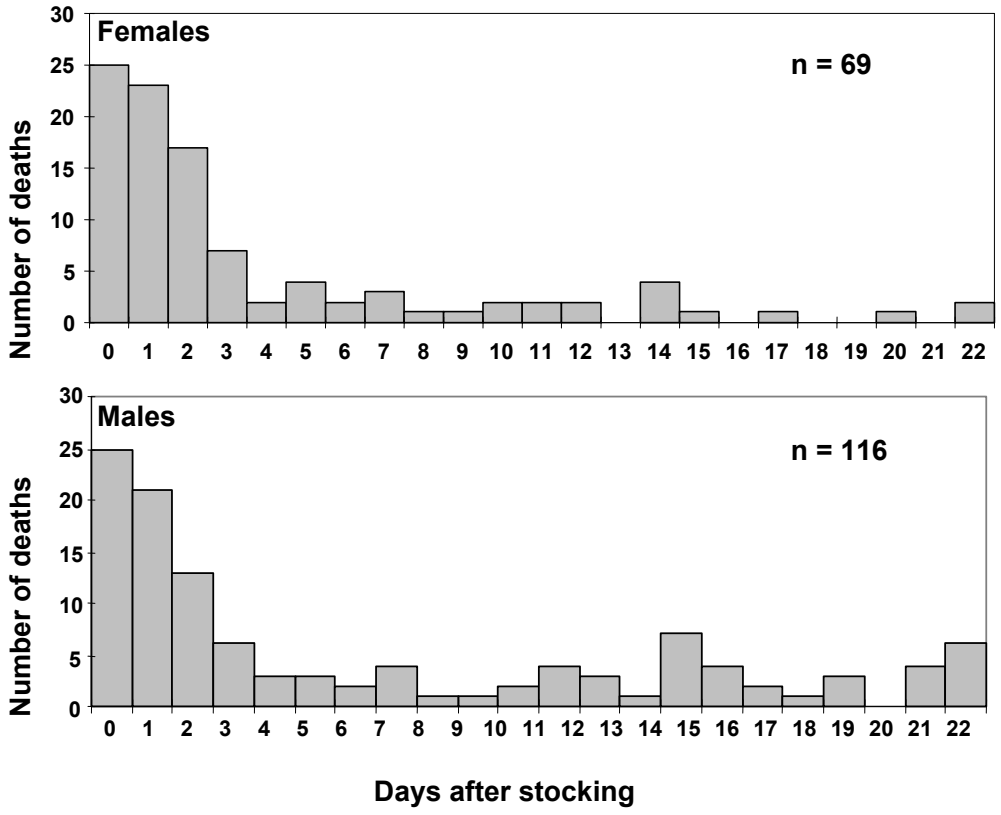

Figure 24. Post-stocking mortalities of G2 EC female and male *Penaeus monodon* held in tanks at AIMS tanks after being transported by air and road from BIARC.

Post-stocking (pre-ablation) mortalities of G2 broodstock

The majority of female broodstock for this experiment arrived in a stressed condition, as evidenced by their reddish colouration. After stocking many appeared to recover and regained their normal coloration. This was particularly noticeable immediately after moulting, when the majority developed a brighter coloration pattern, typical of newly harvested pond reared broodstock. Despite the apparent recovery, post-stocking mortalities were high in this cohort of females, with 69 dying before they could be ablated (Fig. 24). Most of these mortalities occurred within the first 3 days of stocking, probably as a result of harvest and/or transport stress. Shipment 1 accounted for 60% of all mortalities up to 7 days post stocking. In addition to in-tank mortalities, 8 females were rejected and subsequently culled when their VIE tags could not be identified to family level (i.e. they could not be used for breeding). Post stocking mortalities of G2 females and males had similar patterns with most occurring in the first 3 days after stocking (Fig. 24). Some of the mortalities after this period occurred when males that were electro-ejaculated failed to recover.

G2 Females ablated

Of the 214 females sent to AIMS, 113 were ablated for this experiment. Female weight ranged from 79.0g to 163.4g, with a mean of 125.9 ± 1.6g.

Post-ablation mortalities and mean inter-moult period of G2 broodstock

During the first inter-moult period after ablation 48 of the 113 ablated females died (Fig. 25). The mean inter-moult period of surviving females was 19.7 ± 0.5 days. In the second inter-moult period, 65 females were artificially inseminated for a second time. Of these 24 died and 35 were culled during the inter-moult period, the latter to ensure that maternal DNA was collected for pedigree analysis. The remaining 6 females were culled soon after moulting a third time.

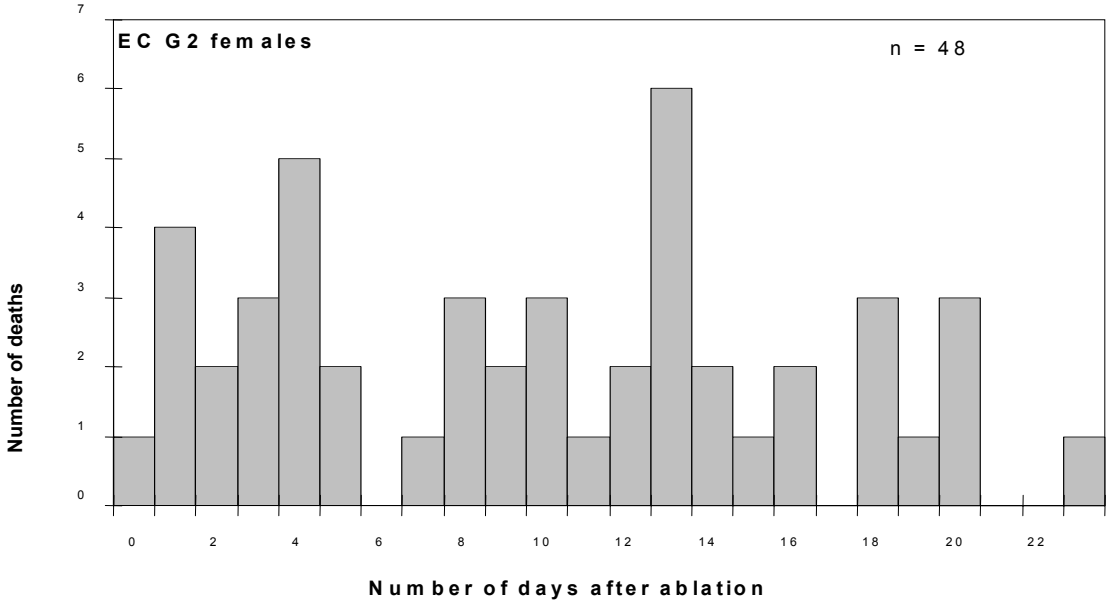

Figure 25. The number of deaths each day following ablation of EC G2 females. n = total number of deaths.

Spermatophore morphology

More than 100 pairs of spermatophores were extracted for artificial inseminations during this experiment. Although there was some variability, particularly amongst those extracted from males that were ejaculated a second time, most spermatophores from G2 males appeared to have similar gross morphology, based on colour, presence/absence of tail and presence of sperm bundles to spermatophores extracted from wild males during their peak breeding season in spring. In terms of size, spermatophores extracted from G2 males were slightly smaller (≤ 75%)

than spermatophores from wild caught males of similar weight. There was no obvious difference in spermatophore morphology between families.

Days to first maturation (i.e. ovarian stage GIII) in G2 broodstock

From the initial batch of 113 ablated females, 82 reached GIII. The mean interval from ablation to first maturation in this cohort was 5.9 ± 0.5 days.

Ablated G2 broodstock that spawned

Of the 82 females that reached GIII development, 75% went on to spawn. In the first inter-moult period 54 females spawned (i.e. 48%). An additional 12 females that had not spawned during the first inter-moult, spawned during the second inter-moult period. In total, 66 females, which represented 58% of the initial batch of 113 females that were ablated, spawned during this experiment (Fig. 26). This was an improvement on the previous generation of EC stocks that were transported from BIARC when handling and transport resulted in high stress and mortalities.

Days to first spawning for G2 broodstock

Mean number of days from ablation to first spawning was 7.3 ± 0.4 days for the group of 66 ablated females that spawned during this experiment.

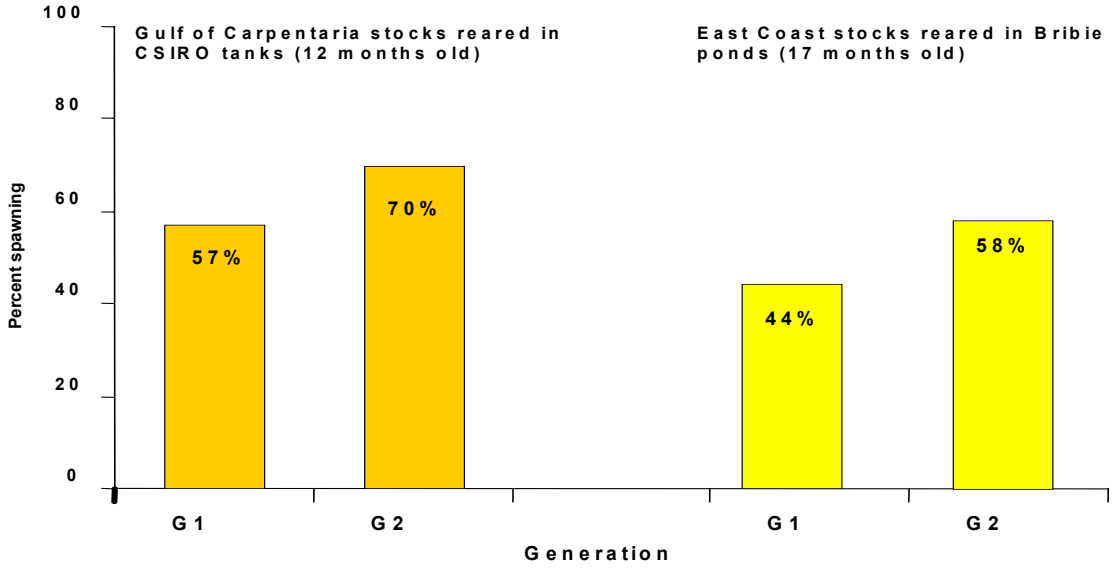

Figure 26. Comparison of the percentage of ablated female *Penaeus monodon* broodstock that spawned for the G1 and G2. GoC stocks reared in tanks at CSIRO when 12 months old and G1 and G2 EC stocks reared in ponds at BIARC then transported by road and air and spawned at AIMS when 17 months old.

Egg and nauplii production by G2 broodstock

Egg samples were collected from 107 of the 129 spawnings recorded during this experiment. A total of 33,365,200 eggs were produced in these 107 batches. Individual batches ranged from 1,200 to 702,000, with mean production of 311,824 ± 16,089 eggs per spawning. From the 107 batches of eggs that were sampled during this experiment 12,832,383 nauplii were produced. Overall mean nauplii production per spawning, calculated from those batches that were sampled (includes batches that did not hatch), was 119,929 ± 9,698 (Fig. 27).

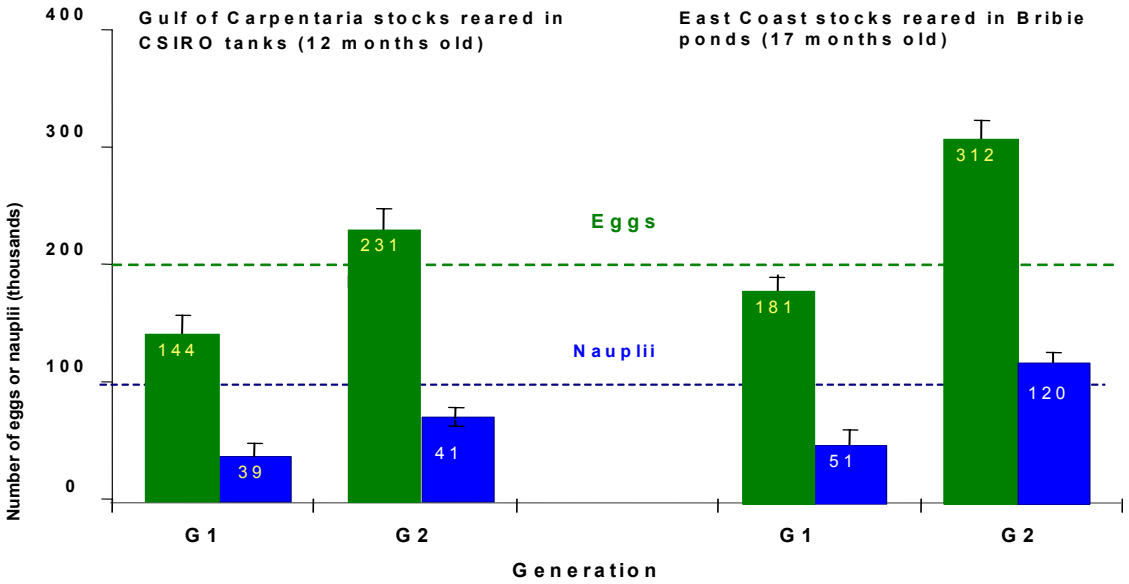

Figure 27. Comparison of mean number of eggs and nauplii from individual spawnings of G1 and G2 GoC stocks reared in tanks at CMR and spawned at 12 months and G1 and G2 EC stocks reared in broodstock ponds at BIARC, transported by air and road to AIMS and spawned at 17 months. The dashed lines show the target of achieving mean production of > 200,000 eggs and 100,000 nauplii (required industry standard).

Condition of Nauplii produced by G2 broodstock

Based on an assessment of activity, phototaxis, presence/absence of deformities, the degree of fouling and setae condition there was no noticeable difference in the quality of nauplii, within and between batches. All batches from this EC-G2 cohort were of similar condition and comparable to the high quality batches of wild EC broodstock produced during their peak breeding period in spring (late September-October 2003).

1.4 Discussion

The results of this study demonstrated successive improvements in the growth survival and reproductive output with each generation of both GoC and EC stocks of domesticated *P. monodon*. By the third generation the reproductive output of 12 month old GoC broodstock, reared in controlled environment tanks or raceways, had reached the level considered to be commercially viable by industry. By the second generation the reproductive output of 17 month old EC broodstock, reared in the broodstock ponds at BIARC, was the highest that has been reported for domesticated *P. monodon* derived from founder stocks from Australian waters, with a mean nauplii output of 120,000 per spawning. The growth rates of captive reared *P. monodon* achieved in study were a significant improvement over previous trials, with a 200% increase in the average wet weight of 11 month old females compared to trials in 1997 (Coman et al. 2006). This achievement highlights the importance of improvements to the environment, diet, health and husbandry techniques for captive breeding of *P. monodon* in advance of, and in parallel with, the development and implementation of selective breeding. The improvements in husbandry developed in this project are immediately applicable to new first generation founder stocks that will be required to increase the genetic diversity in future *P. monodon* selective breeding programs.

The comparatively high reproductive output from the EC-G2 cohort was offsest by significant post-harvest, in-transit and post-stocking mortalities. High transport mortalities represent a significant barrier to commercial scale domestication, especially when broodstock are air-freighted to distant hatcheries. The results of laboratory experiment on stress (de La Vega,

2004) indicate that the observed mortalities were due, in part, to the stress associated with drain harvesting and the high level of post-harvest handling these broodstock were subjected to. The latter was an unavoidable result of implementing specific breeding protocols that required sorting and stockpiling broodstock into family groups after harvest. Further stress was incurred when these broodstock were then net harvested, packed into transporter bags, air-freighted, acclimated, unpacked, weighed, eye-tagged and moult-tagged before being stocked into maturation tanks.

Further evidence that the mortalities of the EC broodstock were largely due to harvesting and post-harvest handing stress is that most deaths occurred in the first 3 days after transportation and stocking into maturation tanks. Broodstock appeared to recover condition soon after this period, especially when they commence feeding and become more active in the maturation tanks. One sign that some broodstock were able to recover was a change in their colouration; from a reddish carapace and abdomen back to a bright white, black and olive coloration, characteristic of newly harvested pond-reared broodstock.

Although broodstock condition improved markedly soon after they were stocked into maturation tanks their recovery was short lived as they were subjected to further stresses due to artificial insemination, re- tagging and ablation, 2 days after moulting. However, post-ablation mortalities did not show the same modal peak (i.e. first 3 days) nor were they of the same magnitude as post-stocking mortalities. In view of the difference it is possible that if post-harvest and post-stocking survival could be improved through better harvesting and handling techniques this may improve post-ablation survival and hence the reproductive output of domesticated broodstock (i.e. starting with a healthier and less stressed cohort of broodstock). There will be an opportunity to test this hypothesis in future studies with the next generation of these stocks.

The absence of GAV in gill samples at levels detectable by conventional 1- and 2-step Polymerase Chain Reaction (PCR) (see Chapter 2) confirmed previous findings that the GAV infection loads in the EC-G2 prawns were low. It should also be noted that female broodstock were sampled after they had been eyestalk ablated and spawned, and that the stress associated with this might have been expected to elevate virus loads, if infections were present.

Male prawns from this cohort also showed significant post-stocking mortalities. However, for those that survived and were used for breeding, their spermatophores were morphologically similar, albeit slightly smaller, than those from wild caught males of similar weight. The apparent “high” quality of these spermatophores was confirmed with 94% of egg batches hatching and an overall hatch rate of 38% recorded for this reproductive assessment. It is probable that these males mated many times and produced multiple spermatophores after reaching maturity in the BIARC ponds, before being harvested for this experiment. It is not known whether younger males will produce spermatophores of comparable quality and the consortium has no data on seasonal effects on spermatophore quality from pond reared males.

An interesting result from this experiment was the uniformity in hatch rate between egg batches where broodstock were inseminated with 1 spermatophore of “normal” (average or above) size and those inseminated with 2 “smaller” (below average) sized spermatophores. This may indicate that hatch rate is linked to spermatophore size (1 “normal” equivalent to 2 “smaller” spermatophores based on gross assessment) although this has yet to be tested. Irrespective of the difference AI provides a useful technique to achieve reliable hatch rates, particularly if there are issues with achieving natural matings in tanks. However implementation of AI in commercial hatcheries is more labour intensive (due to moult tagging, stripping spermatophores and AI) although the benefit may warrant the expense.

Despite the post-harvest and handling stressors on the EC-G2 cohort and the fact they arrived in a stressed condition, they achieved commercially acceptable nauplii production. If these initial

stressors could be reduced and post-harvest survival improved it may be possible to further improve reproductive output.

Having reached the level of reproductive output considered by industry to be commercially viable, future efforts to improve the growth and reproductive output of domesticated *P. monodon* should integrate incremental improvements in husbandry and nutrition with the commencement of selective breeding of the domesticated stocks. The results of this study have clearly demonstrated that one of the key targets of future research efforts should be achieving further improvements to the hatch rates of captive reared *P. monodon*.

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2. CHAPTER 2. ASSESSING HEALTH

Assessing and minimizing chronic and acute health-related barriers to the domestication of *Penaeus monodon.*

2.1 Introduction

The objective of this component of the study was to assess pathogen-related constraints to *Penaeus monodon* domestication and develop strategies to minimize their impact. At the start of the project it was well established that production of *P. monodon*, like other penaeid species, can be affected by a range of bacterial, fungal, and viral pathogens. Of the bacterial pathogens, several *Vibrio* species can cause significant mortalities in hatcheries and ponds. Gill-fouling protozoan (e.g. *Zoothamnium sp*. and *Epistylis sp*.) and fungal (e.g. *Fusarium sp*.) infections also occur commonly in farmed prawns. However, these are largely opportunistic epicommensal infections that can be controlled by good hatchery practices, simple chemical bathing treatments and maintaining a high quality pond culture environment. Globally, good managerial and husbandry regimes have overcome most production risks due to bacterial, fungal and protozoan pathogens.

In contrast, viral pathogens are far more problematic, being the largest global contributor to losses of farm stocks and profits. Viruses known to infect *P. monodon* include Gill-associated virus (GAV), Spawner-isolated mortality virus (SMV), Mourilyan virus (MoV), Monodon baculovirus (MBV), Infectious haematopoietic necrosis virus (IHHNV), Yellow head virus (YHV), White spot syndrome virus (WSSV), Taura syndrome virus (TSV) and Hepatopancreatic parvovirus (HPV). Of these nine viruses, GAV, SMV, MoV and MBV are known to be endemic (occur commonly) in the Australian East Coast (EC) population of *P. monodon.* A conclusive list of the viruses infecting other populations of Australian *P. monodon* such as the Gulf of Carpentaria (GoC) was not available when this project commenced. The remaining five viruses (IHHNV, YHV, HPV, WSSV, TSV) were thought to be exotic at the start of this project, but considered significant threats to the Australian industry. Consequently, routine screening for these five viruses in new stocks from remote locations is a critical component for any domestication program.

When this project commenced there was no known method of eliminating viruses from contaminated stock. The only method of avoiding the presence of specific viral pathogens in culture systems was to try to obtain broodstock that were free of specific viruses (specific pathogen free; SPF) and to maintain them in their SPF state. Against this background, the objectives of this component of the project were to;

- Develop rapid, highly sensitive, quantitative viral screening techniques for monitoring the level of viral infections (both endemic and exotic viruses) in the project stocks
- Verify that GAV, SMV, MoV and MBV are the only endemic viruses occurring in the EC founder stocks and establish a conclusive list of endemic viruses present in the GoC founder stocks
- Monitor the prevalence of endemic viral pathogens in progeny of successive domesticated generations of EC and GoC lines
- Assess the relationship between viral infections and reproductive output (Chapter 5),
- Determine the precise location of viral infections in prawn tissues, including gametes (Chapter 6)
- explore the potential for eliminating viruses from infected stocks via chemical treatment in hatcheries (Chapter 7)

In the year prior to the project commencing, viral screening had found that almost all (approaching 100% prevalence) wild EC broodstock sampled were infected with GAV, as were their farmed progeny. In this report viral prevalence refers to how many individuals in a sample are infected and viral level refers to the amount of virus detected in individuals.

It was also known that SMV occurred in wild EC broodstock, but below 25% prevalence. Less was known about the more recently discovered MoV, although preliminary screening had detected this virus at very high prevalence levels similar to GAV in wild EC broodstock.

In contrast to the EC stocks, preliminary viral screening indicated that wild GoC stocks were either GAV-free (i.e. SPF for GAV) or infected at such low levels that detection was beyond the capability of the test. Preliminary screening detected MoV in some GoC prawns but at extremely low levels that challenged the capability of the test. These preliminary indications that there were significant differences in the viral infection status of wild EC and GoC stocks was the main reason that the project participants decided to keep these stocks separate throughout the course of the project.

Viral infections are not eliminated through moulting or by administration of chemical treatments routinely employed to remove bacterial and fungal pathogens. However, several important prawn viruses, including GAV and WSSV, infect gonad tissue and are thought to be transmitted vertically during spawning from parent to offspring. If this transmission occurs through the presence of virus particles in extracellular seminal or ovarian fluid, egg washing could potentially result in virus-free larvae. Examining the possibility of removing GAV from *P. monodon* embryos, in parallel with the pathogen screening strategies outlined above, was a key objective of this study.

In summary, a lack of knowledge about pathogens of *P. monodon*, particularly viral pathogens, was considered by industry to be a key barrier to successful domestication of this species, and a significant threat to the long-term maintenance of domesticated lines. Locating SPF stocks (e.g. GAV-free stocks from the GoC) and maintaining them in biosecure conditions was one of the key strategies adopted. This approach also demanded rigorous pathogen screening of all project stocks (see Chapter 1) in order to assess the impact of potential pathogens, particularly viruses, on reproduction and growth performance (Chapter 3).

In parallel with rigorous viral screening, the potential to develop a means of eliminating viruses from infected stock, with a secondary goal of obtaining sufficient information on the relationship between levels of viral infection in broodstock and reproductive output to enable improved management and successful utilization of infected broodstock was explored.

2.2 The development of high-throughput screening tests for endemic and exotic viruses

The goals of this component were to:

- Develop high-throughput screening tests to detect endemic and exotic viruses
- Quantify a conclusive list of viruses present in the founder stocks whilst also quantifying their prevalence and level of infection, and
- Monitor infection prevalence and viral level in successive generations of domesticated lines.

Two PCR methods were employed for detecting viruses in prawn stocks; conventional 1-step and 2-step PCR, and quantitative real-time PCR (qPCR). For RNA viruses such as GAV and

MoV (i.e. their genome is made of ribonucleic acids, RNA as opposed to deoxyribonucleic acids DNA). Prior to conducting PCR test for GAV and MoV, viral RNA must be copied into DNA (termed cDNA) to determine its presence in the sample. This copying process is called reverse transcription and abbreviated RT. For DNA viruses such as SMV, WSSV and IHHNV, this copying step is not required.

PCR is a very accurate way to detect the presence of a virus in a sample. During a PCR, short artificial pieces of DNA called primers (that contain identical pieces of genetic sequence to the virus been screened for) are used to identify virus genetic sequence in a sample. These artificial pieces of DNA bind to the identical fragment of the virus genetic sequence and, with the help of enzymes in the PCR mix, millions of copies of the viral genetic sequence are made, so the virus can be detected on a gel under UV light or by fluorescence under a fluorescence microscope. For conventional PCR, 1-step primers identify a piece of the virus genetic sequence that is larger in length than the sequence that the 2-step primers identify. As a result, samples are analysed initially by 1-step PCR and, if negative for the presence of the virus, the 1-step PCR product is put into the 2-step reaction to look further for the presence of the virus, making it the more sensitive of the two detection reactions.

In this report samples that were positive by 1-step PCR are reported as having high levels of infection and samples that were positive at 2-step PCR are reported as having low levels of infection.

Conventional 1 and 2 step PCR methods use a DNA stain and determine the presence or absence of a virus by the presence or absence of the stain. Quantitative real-time PCR (qPCR) uses a complex fluorescent detection system that calculates the amount of DNA being made in a PCR reaction over time and can therefore quantify the level of viral infection, and is 100 to 1000-fold more sensitive than conventional PCR.

qPCR has many advantages over conventional PCR due to this difference in detection method. qPCR is much faster (i.e. 2 hours compared to 4 hours for physically making the millions of DNA copies), more sensitive (i.e. can detect very low level infections), is quantitative (i.e. gives actual numbers of viral DNA fragments present compared to a yes or no result) and reduces the chance of cross-contamination between samples (as it is all performed in one tube that is shut the whole time compared to 1-step and 2-step PCR which is performed in different tubes). Turn-around time from point of sampling to a reportable result for qPCR can be 8 hours compared to 2 days for conventional PCR.

The output value from qPCR is called a cycle threshold value (Ct). This value represents the number of PCR amplification cycles required for fluorescence to reach a specified threshold level, with highly positive samples having lower Ct values. For the purposes of this report we will report on Ct's of less than 25 as having a high-level of infection and Ct's of more than 25 as having a low level of infection.

At the start of this project, qPCR tests for some prawn viruses (see list below) were not available. In addition, many of the conventional PCR tests reported in the literature were not able to detect new viral strain variants recently identified (e.g. the genetic sequence of MBV that occurs in Australia is slightly different to the genetic sequence of MBV that occurs in Taiwan and therefore, as the PCR primers are highly specific, if they are designed in regions of the GAV genetic sequence that vary between geographical locations, the PCR test will only detect one of the two strains). For these reasons we aimed to establish new conventional and qPCR tests that accommodated, where possible, all strain variants known at this time.

Endemic viruses
- Gill-associated virus (GAV)
- Spawner-isolated mortality virus (SMV)
- Mourilyan virus (MoV)
- Monodon baculovirus (MBV)
- Infectious hypodermal or haematopoietic necrosis virus (IHHNV)*

Exotic viruses
- Yellow head virus (YHV)
- White spot syndrome virus (WSSV)
- Taura syndrome virus (TSV)
- Hepatopancreatic parvovirus (HPV)

*During this study IHHNV was detected in stocks from the Gulf of Carpentaria (GoC) and East Coast (EC).

Gill-Associated Virus (GAV).
The qPCR assay for GAV was developed using genome sequence information already determined by CSIRO Livestock Industries (CLI) (prior public domain information). The qPCR targeted the same genome region as the conventional PCR assays for GAV.

Mourilyan virus (MoV) and Spawner Mortality Virus (SMV)
The qPCR assay for MoV was developed using genome sequence information already determined by CLI that was not in the public domain at the time of development. The qPCR assay for SMV was developed using genome sequence information supplied by Dr Leigh Owens (James Cook University; JCU) under agreed conditions that the assay only be used for research purposes. The MoV qPCR and the SMV qPCR assays target the same genome regions targeted by the conventional PCR tests for MoV at CLI and SMV at JCU.

IHHNV and other exotic viruses (YHV, WSSV, TSV and HPV)
Samples of infected prawn tissue were obtained from national and international collaborators that collectively contained all of the endemic and/or exotic viruses listed above and all known variants or strains. Some samples were extracted in foreign countries and the DNA or RNA was sent to Australia to eliminate any chance of introducing exotic viruses. DNA or RNA from all other samples was extracted at CLI. The collected sample set was used as positive-controls in the development and conduction of PCR tests.

PCR tests were designed in such a way that all different genetic variants of each virus could be detected. This was achieved by making primers in locations where sequence similarity (or conservation) occurs between variants. For IHHNV and TSV, primers were targeted to DNA sequences conserved amongst all known variants, thus providing a wide range of strain variation coverage. For YHV and HPV, the sequence variation among genotypic variants was too great to allow the design of primers for generic detection of all strains. Accordingly, primer combinations specific to the virulent Thailand YHV isolate and the Thailand HPV isolate identified to infect *P. monodon* were used, as these strains were considered to be the most important for exclusion from founder prawns and domesticated breeding lines. For WSSV, primers were designed that would allow detection of all known variants, and their sensitivity and ability to do so were extensively tested at the Australian Animal Health Laboratory (AAHL) in Geelong.

Conventional PCR as well as qPCR tests were established for all nine viruses and their specificity and sensitivity assessed using the positive control material. All tests were highly specific and sensitive except MBV. However, control DNA from MBV-infected *P. monodon* was obtained from Taiwan and sequence analysis of DNA identified that it was genetically

different from the Australian MBV isolate. The MBV genetic sequence needs to be characterized from locally infected *P. monodon* and used to design primers that will detect Australian MBV.

2.3 Gulf of Carpentaria Stocks

2.3.1 Methods

Details of the production of successive generations of domesticated lines originating from the GoC founder stocks are described in Chapter 1. A summary of the stocks sampled for pathogen monitoring at CSIRO Marine Research (CMR), Cleveland Laboratory, and Gold Coast Marine Aquaculture (GCMA), Woongoolba is shown in the flow chart in Figure 1.

Founder (G0; generation zero) stocks
April 2002: Founder (G0) *P. monodon* stocks that were collected from the GoC were held under quarantine conditions in tanks at CMR and GCMA. Gill filaments were collected non-sacrificially (i.e. prawns remain alive) from 30 prawns held at GCMA. Nucleic acid (RNA and DNA) was isolated from gill filaments pooled from six prawns sampled from five tanks. Conventional 1- and 2-step PCR was used to test the isolated nucleic acid for the presence of the four viruses (WSSV, YHV, GAV and MoV) which were considered highest priority at the time (Tables 1 and 2).

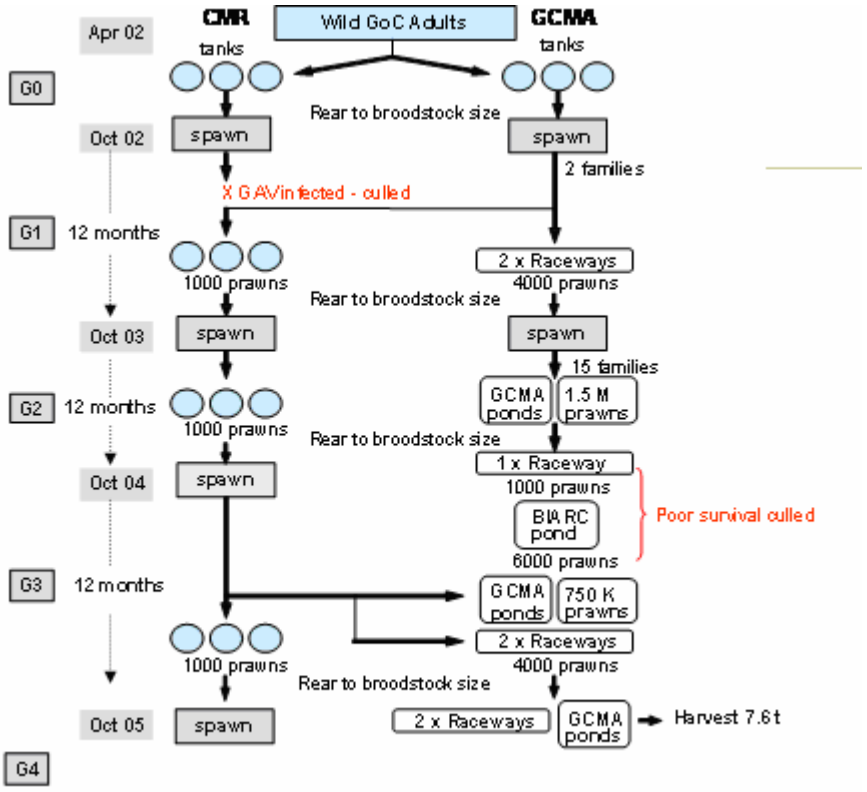


Figure 2. Flow chart of the GoC lines reared at CMR and GCMA. G0 = founder stocks, G1 = generation one etc.

April 2002: To examine the viral status of GoC-G0 stocks more rigorously, gill fillaments, lymphoid organ, haemocytes and hepatopancreas tissue were collected for PCR screening from ten prawns held at GCMA. Half of each prawn head was processed for histology, examined and

tested for the presence of MoV and GAV using a technique called *in situ* hybridisation (ISH) which employs fluorescent DNA probes to detect the presence of virus within specific cells. Nucleic acid was isolated from gill filaments, lymphoid organ, haemocytes and hepatopancreas tissues and used in conventional 1- and 2-step PCR tests for WSSV, GAV, YHV and MoV.

Since the GoC stocks were found to be GAV free and with very low levels of MoV, some GoC-G0 prawns held at GCMA were relocated to tanks at CMR for rearing to reproductive maturity through the winter months. This was done to spread the risk of GoC stocks becoming infected with viruses that were not endemic in their natural population (i.e. it is less likely that stocks held at two different facilities will both become infected).

October 2002: Representatives of the GoC-G0 stocks held at CMR were screened for GAV and MoV by conventional 1- and 2-step PCR just prior to spawning. Gill filaments, lymphoid organ, haemocytes, hepatopancreas and gonad tissues were sampled from twelve prawns and half heads of all prawns were processed for histology (Table 1).

November 2002: Representatives of the GoC-G0 stocks held at GCMA were screened for GAV and MoV by conventional 1- and 2-step PCR just prior to spawning. Gill filaments, lymphoid organ, haemocytes, hepatopancreas and gonad tissues from eleven prawns were sampled (Table 2).

First generation (G1) stocks

November 2002: GoC-G1 stocks reared in tanks at GCMA were tested at stage PL 15 for GAV and MoV using conventional 1- and 2-step PCR (Table 2).

March to October 2003. GoC-G1 stocks reared in tanks at CMR and raceways at GCMA were sampled in March/April, June and September/October 2003 and tested for GAV and MoV using conventional 1- and 2-step PCR (Tables 1 and 2).

Second generation (G2) stocks

February to November 2004. GoC-G2 stocks reared in tanks at CMR were tested for GAV and MoV in February, March and May using conventional 1- and 2-step PCR , and in November 2004 using qPCR (Table 1). At GCMA, GoC-G2 postlarvae were initially stocked into two production ponds where they were reared for five months prior to transfer to raceways. When the stocks were approximately four months old (March 2004) samples were collected from the production ponds and tested for GAV and MoV using conventional 1- and 2-step PCR (Table 2). The stocks were tested again for GAV and MoV using conventional 1- and 2-step PCR in May and July 2004 after they had been transferred to the raceways at GCMA (Table 2).

Third generation (G3) stocks

December 2004 and March 2005. Due to the poor survival of the GoC-G2 stocks at GCMA following their transfer from commercial ponds to raceways, all GoC-G3 stocks were generated from GoC-G2 stocks grown in tanks at CMR. The GoC-G3 stocks reared in tanks at CMR were sampled in February 2005 and tested for GAV and MoV using qPCR (Table 1). The stocks reared in raceways at GCMA were tested in December 2004 for GAV and MoV using conventional 1- and 2-step PCR, then tested again in March 2005 for GAV and MoV using qPCR (Table 2).

2.3.2 Results

WSSV, YHV, TSV and HPV were not detected in any of the founder stocks tested.

Founder (G0) stocks

CMR G0 stocks: In April 2002, shortly after founder stocks arrived at CMR, GAV was not detected in any of the animals screened using conventional 1- and 2-step PCR (Table 1), but MoV was detected at very low prevalence and low levels of infection. In October 2002, seven months after rearing at CMR and shortly before spawning, GAV was detected at high prevalence and high-level of infection by conventional 1- and 2-step PCR, whilst MoV was not detected (Table 1). *In situ* hybridization (ISH) analysis confirmed the presence of GAV but not MoV in these prawns. The results indicate that the GoC-G0 founder stocks acquired GAV infections during the seven months that they were reared to broodstock age in the CMR tanks.

Table 1. Results of conventional 1- and 2-step PCR and qPCR for GAV and MoV conducted on representatives of the GoC-G0 founder stocks, G1, G2 and G3 stocks reared at CMR.

Conventional 1- and 2-step PCR data								
					GAV		MoV	
Gen	Date	Av. wt.	Tissue	no	1-step	2-step	1-step	2-step
G0	Apr 02	75 g	LO	19	0	0	0	1
G0	Oct 02	120 g	LO	12	12	ND	0	0
G1	Apr 03	28 g	LO	10	0	0	0	0
G1	Jun 03	59 g	LO	10	0	7	0	8
G1	Oct 03	80 g	LO	10	0	0	0	10
G2	Feb 04	14 g	LO	24	24	ND	0	2
G2	Mar 04	40 g	LO	12	11	ND	0	0
G2	May 04	66 g	LO	16	16	ND	0	8
qPCR data					GAV		MoV	
					Ct<25	Ct>25	Ct<25	Ct>25
G2	Nov 04	116 g	LO	19	19	0	0	13
G3	Feb 05	6 g	gill	46	9	37	0	0

LO = lymphoid organ, ND = not done, Gen = generation, Av. wt. = average weight, no. = number of pawns sampled, g = grams, Ct = cycle threshold value (Ct<25 refers to a high-level infection whilst Ct>25 refers to low-level infection)

GCMA G0 stocks: In April 2002, shortly after founder stocks arrived at GCMA, GAV was not detected in any of the animals tested using conventional 1- and 2-step PCR (Table 2), but MoV was detected at moderate prevalence, at low levels of infection in gill tissues and at low prevalence and low levels of infection in lymphoid organs (Table 2). In November 2002, eight months after rearing at GCMA and shortly before spawning, neither GAV nor MoV were detectable by conventional 1- and 2-step PCR. These data indicated that, in contrast to the stocks reared in tanks at CMR, the stocks reared in parallel in raceways at GCMA had not acquired GAV infections.

First generation (G1) stocks

CMR G1 stocks: In April 2003, neither GAV nor MoV were detected by conventional 1- and 2-step PCR in juvenile GoC-G1 (~28 g) prawns reared at CMR (Table 1). Two months later (June 2003) when tested as sub-adults (~59 g), GAV and MoV were detected by conventional 1- and 2-step PCR at high prevalence at low levels of infection. In October 2003, when the prawns were tested as adults (~80 g), GAV was not detected in any prawns whilst MoV was detected at high prevalence but at low levels. The infection of GoC stocks at CMR with GAV compromised the SPF status of these stocks and, at the time, was also considered to pose a risk to the health and survival of the stocks. Accordingly these stocks were culled. In an effort to avoid cross-

stock viral contamination at CMR, a new maturation facility with a higher level of biosecurity was constructed on site at CMR. The new maturation facility was restocked with sibling GoC postlarvae from GCMA in February 2003, which screening had indicated were SPF for GAV (Table 2).

Table 2. Results of conventional 1- and 2- step PCR and qPCR for GAV and MoV conducted on representatives of the GoC-G0 founder stocks, G1, G2 and G3 stocks reared at GCMA.

Conventional 1- and 2-step PCR data								
					GAV		MoV	
Gen	Date	Av. wt.	Tissue	no.	1-step	2-step	1-step	2-step
G0	Apr 02	75g	gill	30	0	0	0	18
G0	Apr 02	75g	LO	10	0	0	0	2
G0	Nov 02	120g	LO	11	0	0	0	0
G1	Nov 02	Pl-15	whole	-	0	0	0	0
G1	Mar 03	25g	LO	10	0	0	0	5
G1	Jun 03	53g	LO	29	0	2	0	16
G1	Jun 03	53g	gill	29	0	2	0	3
G1	Sep 03	110g	LO	10	0	8	0	0
G2*	Mar 04	19g	LO	20	17	19	0	10
G2	May 04	38g	LO	20	20	ND	12	15
G2	Jul 04	58g	gill	11	11	ND	11	ND
G3	Dec 04	Pl	whole	20	17	18	0	1
qPCR data					GAV		MoV	
					Ct<25	Ct>25	Ct<25	Ct>25
G3	Mar 05	43g	LO	20	18	2	0	13

*Production pond (prior to transfer to raceways), LO = lymphoid organ, PL = postlarvae, ND = not done, Gen = generation, Av. wt. = average weight, no. = number of pawns sampled, g = grams, Ct = cycle threshold value (Ct<25 refers to a high-level infection whilst Ct>25 refers to low-level infection)

GCMA G1 stocks: In November 2002, GAV and MoV were not detected in G1 postlarvae at GCMA (Table 2). Subsequent sampling of these stocks in March, June and September showed that levels of both GAV and MoV remained low or undetectable. However, there was an increase in the prevalence of low GAV infections in adult stocks (Table 2).

Second generation (G2) stocks

CMR G2 stocks: In February 2004, GAV was detected at high prevalence and high levels of infection by conventional 1- and 2-step PCR in juvenile (~14 g) GoC-G2 prawns sampled from CMR tanks. The high prevalence and high level of infection by GAV persisted in sub-adults (~40 g) sampled in March and adult prawns (~66 g) sampled May 2004. In November 2004, prior to spawning, GAV was detected at high prevalence and high levels (19/19 prawns). The results for MoV showed a progression from undetectable or low infection in February and March to moderate prevalence at low levels by May 2004 in adult prawns.

GCMA G2 stocks: In March 2004, four months after stocking GoC-G2 prawns into two production ponds, GAV was detected at high prevalence and high levels. This high prevalence and high-level of GAV infection persisted in samples collected in May and July 2004, after the prawns had been transferred to the raceways at GCMA (Table 2). In March 2004 MoV was detected at moderate prevalence and low levels. Subsequently, MoV progressed to high prevalence and high levels of infection in May and July 2004 (Table 2).

Third generation (G3) stocks
CMR G3 stocks: In February 2005 GAV was detected at high levels in a few prawns using qPCR (note Ct values equivalent to detection by 1-step PCR) and most prawns (37/46) with low levels of infection. MoV was not detected in these stocks (Table 1)**.**

GCMA G3 stocks: GoC-G3 postlarvae used to stock the GCMA raceways originated from CMR stocks. In December 2004, GAV was detected at high prevalence and high infection levels. MoV was detected at low prevalence and low levels of infection (Table 2). In March 2005, testing of sub-adults (~43 g) using the qRT-PCR showed that GAV had persisted at high prevalence and high levels. Testing for MoV by qRT-PCR showed moderate prevalence at low levels of infection.

2.4 East Coast stocks

2.4.1 Methods

Details of the production of successive generations of domesticated lines originating from EC founder stocks are described in Chapter 1. A summary of the stocks sampled for pathogen monitoring at AIMS and BIARC is shown in Figure 2.

Founder stocks (G0)
June to September 2002: Founder stocks of *P. monodon* used to generate domesticated EC lines were wild EC broodstock (G0) and AIMS-reared third generation (G3) domesticated males that were descendent from wild EC broodstock (Fig. 2). Representatives of AIMS G3 males and females were tested for GAV and MoV using conventional PCR. The wild EC-G0 founder broodstock were not tested for their viral status. However, wild EC broodstock captured at the same time from the same area and sent to GCMA were screened for GAV and MoV (Table 3).

First generation (G1)
March and May 2003: Five month old, BIARC reared EC-G1 progeny generated at AIMS (from wild EC-G0 crosses and wild EC-G0 x domesticated AIMS G3 crosses) and domesticated sixth generation (G6) males and females supplied by Seafarm (SF G6) were screened for GAV and MoV using conventional 1- and 2-step PCR (Table 3).

Second generation (G2)
May to November 2004: Broodstock used to generate EC-G2 lines originated from G1 lines derived from wild parents (EC-G0 females and males) as well as wild EC-G0 females crossed with AIMS G3 males. A few EC-G2 lines were also derived from crosses undertaken between EC-G1 broodstock and domesticated sixth generation (G6) females and males supplied by Seafarm. For details of these stocks see Chapter 1 (Fig. 19). These stocks were screened for GAV and MoV using conventional 1- and 2-step PCR (Table 3).

Third generation (G3)
March to April 2006: Representatives of the EC-G3 lines were screened for GAV and MoV using conventional 1- and 2-step PCR in March and April 2006 as part of the PMO2 follow-on project. These results are included here for completeness.

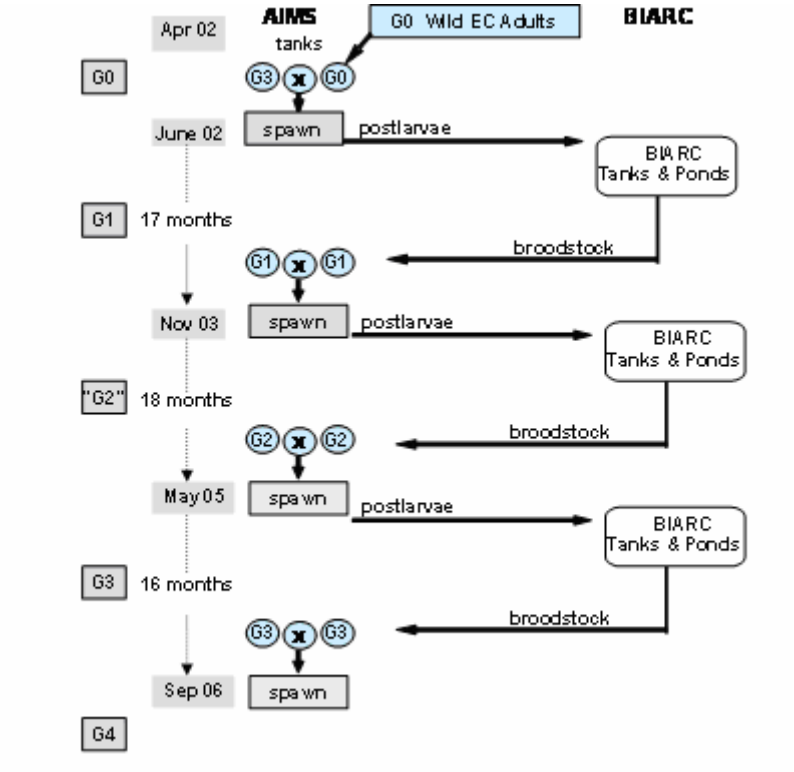


Figure 2. Flow chart of the EC lines reared at AIMS and BIARC. G0 = founder stocks, G1 refers to generation one and so on.

2.4.2 Results

Founder stocks (G0)

AIMS (EC-G0 x EC-G0 and EC-G0 x AIMS-G3): In June 2002 GAV was detected at moderate prevalence and low levels of infection in AIMS-G3 females using conventional 1- and 2-step PCR and in the AIMS-G3 males, at moderate prevalence and high levels of infection (Table 3). MoV was detected at high prevalence and high levels of infection using conventional 1- and 2-step PCR in AIMS-G3 females and males. The AIMS-G3 females were not used to generate EC-G1 lines but results have been included for completeness.

Samples were not collected from the wild EC-G0 broodstock used by AIMS to generate EC-G1 lines in September 2002. However, wild EC broodstock from the same area (North Queensland) obtained from the GCMA hatchery were tested for GAV and MoV. These broodstock had high prevalence and high levels of infection for both viruses (Table 3).

First Generation (G1)

AIMS/BIARC (progeny of EC-G0 x EC-G0 and EC-G0 x AIMS-G3): In March 2003 GAV was detected at moderate prevalence and low levels of infection in EC-G1 progeny generated at AIMS and reared at BIARC (Table 3) using conventional 1- and 2-step PCR. MoV was detected at low prevalence and low levels of infection.

In May 2003 the domesticated EC-G6 prawns obtained from Seafarm had low prevalence but high levels of infection of GAV using conventional 1- and 2-step PCR (Table 3). MoV was detected at low prevalence and low levels of infection.

Table 3. Results of conventional 1- and 2- step PCR and qPCR for GAV and MoV conducted on representatives of the EC founder stocks, AIMS-G3, G1, G2 and G3 stocks of *Penaeus monodon* produced at AIMS and reared in ponds at BIARC.

Conventional 1- and 2-step PCR data								
					GAV		MoV	
Gen	Date	Av. wt.	Tissue	no	1-step	2-step	1-step	2-step
AIMS-G3 ♀	Jun 02	85 g	LO	12	0	4	12	12
AIMS-G3 ♂	Jun 02	78 g	LO	5	3	5	5	5
G0*	Sep 02	50 g	LO	14	13	14	13	14
G1	Mar 03	15 g	LO	16	4	8	1	2
SF-G6#	May 03	25 g	LO	11	3	11	0	2
G2	May 04	30 g	LO	20	0	4	0	9
G2	Nov 04	80g	LO	20	0	5	0	2
G3 (P)	Apr 06	28 g	Gill	21	0	17	21	ND
G3 (N)	Apr 06	31 g	Gill	12	0	8	12	ND

qPCR data					GAV		MoV	
					Ct<25	Ct>25	Ct<25	Ct>25
G2	Nov 04	80 g	LO	76	1	48	0	49
G3 (P)	Mar 06	33 g	LO	22	0	1	22	ND
G3 (P)	Apr 06	28 g	Gill	21	0	17	21	ND
G3 (N)	Apr 06	31 g	Gill	12	0	8	12	ND

* Wild EC-G0 broodstock sampled from GCMA hatchery
# SF-G6 = Seafarm-G6 domesticated broodstock ND = not done. (P) = grow-out ponds at BIARC, (N) = nursery ponds at BIARC

Second Generation (G2)
AIMS/BIARC (progeny of EC-G1 x EC-G1 and EC-G1 x Seafarm-G6): In May 2004 the EC-G2 progeny generated at AIMS and reared at BIARC had low prevalence and low levels of GAV infection (Table 3) and moderate prevalence and low levels of MoV infection using conventional 1- and 2-step PCR. In November 2004 (6 months later, when the stocks were 12 months old) tests for GAV again showed low prevalence and low levels of infection. The prevalence and levels of MoV infection were also low using conventional 1- and 2-step PCR and qPCR (Table 3).

Third Generation (G3)
AIMS/BIARC (progeny of EC-G2 x EC-G2): In March 2006, GAV was detected at low prevalence and low levels of infection using qPCR (Table 3) in the EC-G3 progeny generated at AIMS and reared in grow out ponds at BIARC, but MoV was detected at high prevalence and high levels of infection. One month later, in April 2006, the prevalence of GAV had increased to high, but the level of infection remained low using both conventional 1- and 2-step PCR and qPCR. In April 2006, sibling stocks that had been reared in the nursery ponds at BIARC had moderate prevalence of GAV at low levels of infection using both conventional 1- and 2-step PCR and qPCR, but MoV was detected at high prevalence and high levels of infection.

2.5 Discussion

For ease of communication in this discussion we have combined the results for prevalence and level of infection and categorized the viral status of stocks as *high* (high prevalence and high level of infection), *moderate* (low prevalence and high level of infection, or high prevalence and low level of infection) and *low* (low prevalence and low level of infection). We have used these terms to summarize and discuss the variation observed in both GAV and MoV infection status in progressive generations of domesticated *P. monodon* produced in the project (Figs 3 and 4).

Gill-associated virus (GAV)

The results of this study confirmed previous observations that *P. monodon* stocks in the Gulf of Carpentaria are either free from GAV or have levels that are too low to be detected by conventional 1- and 2-step PCR and qPCR. However, following translocation of GoC prawns to CMR and to GCMA, GAV infections became established in stocks at both sites. GAV infection was first detected in the wild broodstock that had been held in the CMR tanks for seven months from April to October 2002, just before the females were spawned. Although the precise source and mechanism of infection was not determined, both CMR and GCMA also held stocks of *P. monodon* from East Coast (EC) populations and it is likely that, despite the quarantine and biosecurity measures in place at both sites, the EC stocks were the source of infection. Because the GoC stocks were never in direct contact with EC stocks, the transmission must have occurred via free virus particles introduced to the rearing environment.

GAV persisted at high levels in the first generation (G1) of domesticated GoC stocks at CMR but was also only detected sporadically and at low levels in the GCMA G1 stocks (Fig. 3). The source of GAV infection in the CMR stocks is likely to have originated via vertical transmission from the parent stocks. In support of this observation, studies of wild EC *P. monodon* broodstock have revealed the presence of virus in spermatophores and mature ovarian tissue, as well as the association of virus with fertilized eggs, and to a lesser extent, nauplii spawned from wild-impregnated females (Cowley et al. 2002). These observations, together with evidence of matching genome sequences of GAV isolates detected in parent broodstock and their progeny, provide evidence that GAV in parent stocks is a key source of infection in offspring (Cowley et al. 2002). Experimental mating of wild EC stocks and analysis of various larval and postlarval stages also indicated that GAV is likely to be attached to the egg surface, with most viral particles being shed during hatching, resulting in no or very low levels of virus being detectable in nauplii and protozoea (Cowley et al. 2002). However, further studies to identify the location of virus particles found in association with eggs, which might also benefit from the experimental mating of uninfected and GAV-infected broodstock, are needed to determine the mode by which GAV is transmitted vertically. If further studies confirm that GAV is associated with the egg surface, the practice of egg washing and disinfection (see Chapter 4) might significantly reduce or even eliminate the transmission of GAV to offspring.

It has been established that handling stress can lead to rapid increases of GAV infection levels in chronically-infected *P. monodon* collected from the wild (de la Vega et al. 2004). However, although the GoC stocks at both CMR and GCMA acquired GAV infections, the domesticated stocks still had good growth, survival and reproductive performance (Chapter 1).

Prior to the project, conventional 1- and 2-step PCR screening had indicated that the prevalence of GAV infection in wild broodstock from the Cairns to Townsville region in north-east Queensland approaches 100% (Cowley et al. 2000, Walker et al. 2001). The results of this study were consistent with these observations, with founder EC stocks being infected at high prevalence with moderate to high levels of GAV infection (Fig. 3).

Despite the high prevalence of GAV infection in founder wild EC stocks, there was a progressive decline in prevalence and infection levels in successive generations of these stocks (Fig. 3). This was a positive and unexpected outcome for the project. The identification of many prawns in which GAV was not detectable, and its presence at extremely low levels when detected, indicates significant future potential to develop and maintain stocks that are certifiable as specific pathogen free (SPF) for GAV.

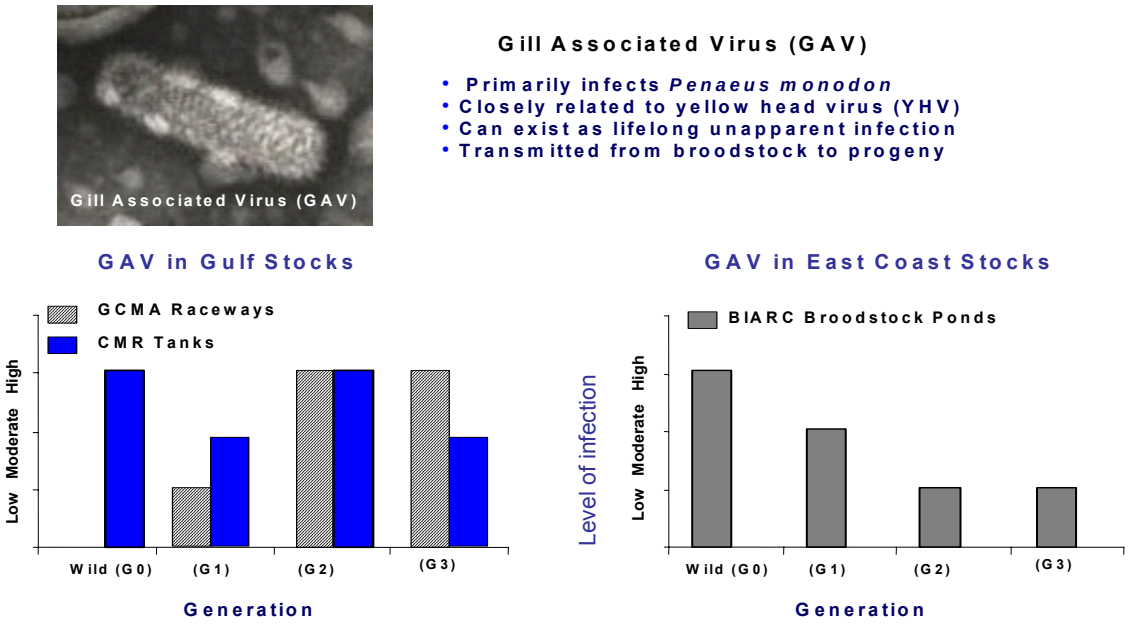


Figure 3. Characteristics of Gill Associated Virus (GAV) and summary of prevalence and levels of infection in progressive generations of *Penaeus monodon* stocks produced from founder stocks from the East Coast (EC) of Australia and Gulf of Carpentaria (GoC) and reared in ponds at BIARC, raceways at GCMA and tanks at CMR.

The mechanisms leading to the decline in GAV in successive generations of EC stocks were not determined. It is likely that these included the progressive loss of individuals that were susceptible to GAV and that stress of harvesting and transport of these stocks exacerbated such losses (de la Vega et al. 2004). This in turn would have led to fewer broodstock harbouring higher level infections and thus lower levels of vertical transmission of the virus. Genetic variation affecting the capacity of some prawns to tolerate infection and thus survive through each successive generation is also likely to have contributed to the reduction in GAV. The potential for genetic selection for tolerance and/or resistance to GAV infections needs to be explored further. In other species of farmed prawns, particularly *Litopenaeus vannamei*, the development of both SPF and specific pathogen resistance (SPR) stocks is widely acknowledged as a key contributor to the recent increases in production and profitability of the Asian industry (Funge-Smith and Briggs, 2003). The development of *P. monodon* stocks that are both SPF and SPR for GAV would be of considerable benefit to the Australian industry.

Mourilyan Virus (MoV)

The results of this study confirmed previous observations that MoV exists in *P. monodon* stocks in the Gulf of Carpentaria (GoC) but at very low prevalence and levels of infection. In the GoC-G1 stocks reared at CMR and GCMA, MoV was detected only at low prevalence and low levels. In the next generation, the GoC-G2 prawns grown at GCMA developed moderate level MoV infections at high prevalence, in contrast to the CMR stocks in which MoV was detected at low prevalence and levels similar to that existent in the previous generation. The results indicate that the increase in MoV in the GoC-G2 stocks at GCMA is likely to have resulted, at least in part, from their transfer to farm grow-out ponds. Chronic stress induced in grow-out pond stocks, due to fluctuations in physical and chemical conditions, may influence the capacity of prawns to tolerate MoV as has been demonstrated with other viral infections. Some species in the biota that commonly colonise grow-out ponds may also be carriers of MoV and thus provide additional sources of infection. Although detailed information on the host range of MoV has yet to be determined, MoV is known to occur in *P. japonicus* (Cowley et al. 2002) and *P. merguiensis,* and possibly pond biota (Cowley, this study). Captive breeding experiments with *P. japonicus* have demonstrated that broodstock reared in controlled environment systems are

less at risk from MoV, due to lower infection rates or a greater capacity to tolerate infections, than putative broodstock sourced from farm ponds (Sellars et al. 2006).

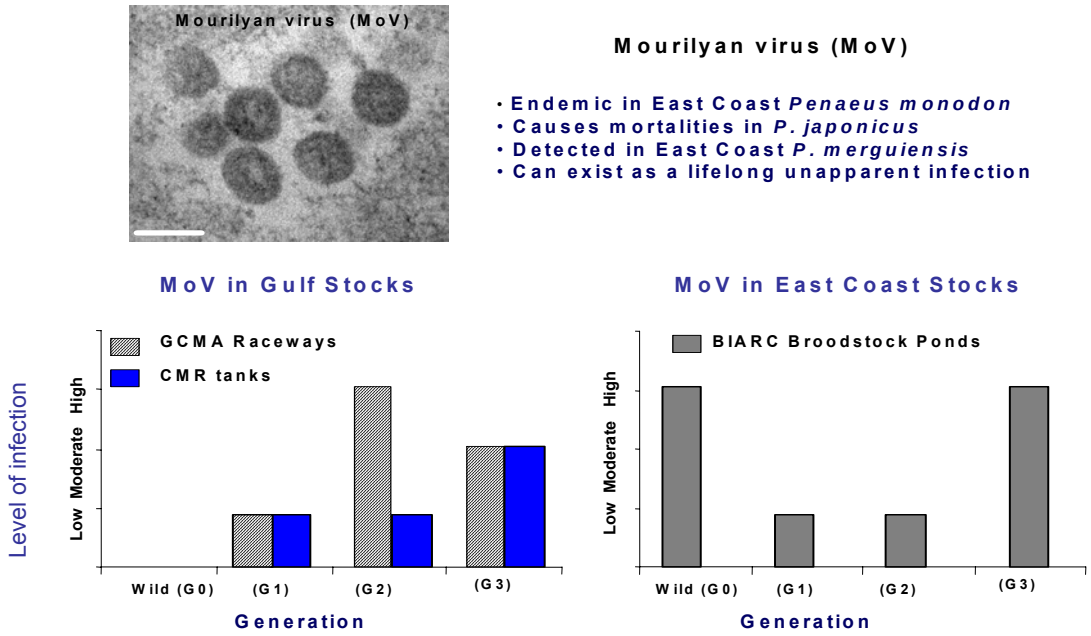


Figure 4. Characteristics of Mourilyan virus (MoV) and summary of levels of infection in progressive generations of *Penaeus monodon* stocks produced from founder stocks from the East Coast (EC) and Gulf of Carpentaria (GoC) and reared in ponds at BIARC, raceways at GCMA or tanks at CMR.

The results of this study confirmed previous observations that MoV is endemic in wild EC stocks, with moderate to high level infection occurring at high prevalence. There was a pronounced decline in the prevalence and level of MoV infection in the first generation EC prawns reared in the broodstock ponds at BIARC, and this low infection state persisted through to the second generation (Fig. 4). However, in the next generation, EC-G3 stocks reared in the both nursery and grow-out ponds at BIARC developed a high prevalence and high-level MoV infections. As noted above for the GoC-G2 stocks reared in grow-out ponds at GCMA, the increased MoV infection state could have resulted from a reduced capacity to tolerate infections due to stressful fluctuations in pond conditions and/or horizontal transmission of MoV from carrier species co-existing in the ponds.

The fact that elevated MoV infection prevalence and levels occurred in some GoC and EC stocks produced as part of this project emphasises the importance of maintaining replicate stocks in biosecure environments, the need for further studies to determine the sources of MoV infection, and the need to develop breeding stocks that are both SPF and SPR for MoV and GAV.

3. CHAPTER 3. STRESS TESTING AND VIRUS DETECTION

In situ stress testing of *Penaeus monodon* broodstock from the GoC to determine Gill Associated Virus (GAV) and Mourilyan Virus (MoV) status

3.1 Introduction

The objective of this component of the study was to conduct *in-situ* stress tests in *Penaeus monodon* broodstock captured in the Gulf of Carpentaria in order to assist in determining whether Gill Associated Virus (GAV) and/or Mourilyan Virus (MoV) are endemic in stocks in this region. The rationale was that if these viruses are endemic but at levels that are too low to detect, then subjecting the stocks to severe stress could result in elevation of viral loads to detectable levels. (de la Vega et al. 2004).

The original founder stocks of *P. monodon* broodstock captured at Weipa in the Gulf of Carpentaria (GoC) in March 2002 screened negative by 1- and 2-step conventional PCR for GAV (see Chapter 2). However, following relocation of broodstock to quarantine facilities at two independent sites in Southeast Queensland, GAV was detected in stocks being maintained in quarantine tanks at CMAR, Cleveland, approximately 6 months after transfer. As the stocks held at the GCMA showed no signs of GAV infection, it was speculated that the infections had been acquired from stocks of *P. monodon* from the east coast (EC) of Queensland, which existed at the CMAR site, through a breakdown in biosecurity.

A critical question that needed to be answered was whether or not the infections that arose in the GoC-G0 stocks might have been due to some of the wild Weipa broodstock carrying GAV infections which escaped detection.

It is possible that GAV might have pre-existed in the Weipa broodstock. To address this question *in situ* stress testing of wild *P. monodon* at the CMAR facility at Weipa was carried out. Conducting the experiment at Weipa, coupled with strict precautions with personnel and equipment eliminated East Coast (EC) stocks of *P. monodon* as a source of infection. As discussed in Chapter 2, both GAV and MoV are endemic and highly prevalent in EC *P. monodon* stocks.

3.2 Methods

Capture and Rearing
223 juvenile prawns were collected at 5 sample sites along the Embley River, Weipa (Lat: 12° 67` Long: 141° 92`) and held at the CMAR compound at Evans Landing (Fig. 1). GPS coordinates of sample sites were obtained (Table 1) using an Electronic Chart Display Information System (MAXSEA).

Prawns were maintained in two round 2.5 m diameter (1500 L) plastic-lined pools at a stocking density of 100 prawns per pool (~20 prawns m^{-2}). Temperature, ammonia and nitrate were monitored daily using an aquarium test kit (Sera). When concentrations of ammonia reached 3 ppm, 50% of the tank water was exchanged with fresh seawater.

Prawns were held for a total of 14 days. During this period, haemolymph samples (100 µl) were collected on days 5, 10 and 14. This repeated stress has previously been shown to induce higher-level GAV infections in chronically-infected *P. monodon* (de la Vega et al.. 2004).

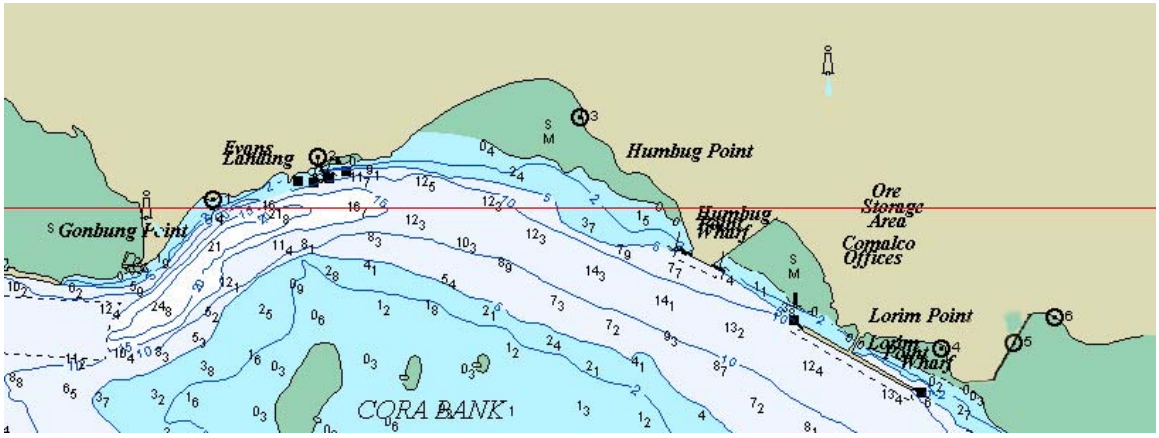


Figure 1. MAXSEA map indicating Weipa sample site (numbered) and facility locations identified in Table 1.

Table 1. Approximate positions of sampling sites at Weipa, Queensland

#	Sample Site	Latitude	Longitude
1	Gonbung Point	12" 39.985'	141" 50.595'
2	CSIRO Evans Landing	12" 39.884'	141" 50.847'
3	Boat Area	12" 39.787'	141" 51.486'
4	Picket Point Beach	12" 40.326'	141" 52.365'
5	Hornibrook Lagoon	12" 40.323'	141" 52.533'
6	Hornibrook Beach	12" 40.263'	141" 52.631'

Captured prawns were weighed, measured (length from tip of antennal scale to tip of uropod) and tagged (Hallprint Pty Ltd, streamer-tag) laterally between the 2nd and 3rd abdominal segments.

Haemocytes collected at intervals over the 2 week period as well as gill and lymphoid organ tissues collected at the end of the stress experiment were tested using quantitative real-time (q-RT) PCR to detect the presence of GAV and MoV in the stressed prawns down to a 2% prevalence threshold.

Sampling

Haemolymph (100 µl) was removed from the ventral sinus at the basal segment of a periopod using a 20 gauge needle (Terumo) attached to a 1 ml syringe (Terumo) pre-filled with 500 µl anticoagulant (450 mM NaCl, 10 mM KCl, 10 mM HEPES, 10 mM EDTA) prepared using DEPC-treated MilliQ water.

Haemocytes were pelleted by centrifugation at ~12,000 rpm for 5 minutes. The supernatant was discarded and haemocytes were preserved for RNA isolation using RNAlater solution (Ambion). Samples in RNAlater were maintained at 4°C for 24 hours then stored at -20°C.

On day 14, following the third bleeding, prawns were immediately sacrificed by severing the ventral nerve cord at the 1st abdominal segment and puncturing the heart. Under aseptic conditions, a small biopsy of gill and entire lymphoid organs were removed and placed in RNAlater solution and stored as for haemocytes. Muscle tissues from the base of the pleopod were also removed and placed in 95% ethanol for subsequent DNA extraction.

Prawns that became moribund or had recently died were sampled immediately they were observed. Samples of haemolymph, lymphoid organs, pleopod and gill were collected as previously described. The remainder of the corpse was left in the tank.

Initially, only lymphoid organ tissues were processed for PCR to confirm the presence or absence of viral infection. All tissue RNA extractions were completed in sets of 24 samples, which also included positive and negative controls comprising GAV inoculum and pearl oyster mantle, respectively.

3.3 Results

A total of 215 juvenile *P. monodon* were captured and transferred to plastic lined ponds. There were 120 males from 3 g to 32 g wet weight (mean weight 11 g) and 95 females from 3g to 42 g wet weight (mean weight 11.5 g).

Water quality in the plastic lined pools was monitored over the 2 week period of the experiment (Table 2). Ammonia levels maintained at acceptable concentrations through water exchange.

Table 2. Physical and chemical conditions in plastic lined pools used to hold *Penaeus monodon* over the course of the stress experiment.

Parameter	Pool 1 minimum	Pool 1 maximum	Pool 2 minimum	Pool 2 maximum
Temperature	26 °C	31 °C	26 °C	31 °C
Ammonia (NH3)	0.5 ppm	4 ppm	0.5 ppm	3 ppm
Nitrite (NO2)	<0.1 ppm	0.9 ppm	<0.1 ppm	0.9 ppm

Survival over the course of the experiment was recorded from the day of capture rather at the start (i.e. Day 0) of the experiment. Highest mortalities occurred immediately after prawns were captured. Accumulated mortalities (~ 10%) were low during the course of the 2 week stress experiment, (Fig. 2).

For GAV, the only PCR-positive results originated from the positive control samples. Although 2 RNA samples generated Ct values <40 in 1 of the 2 duplicate wells that were very close to the detection threshold, these samples were GAV-negative when re-tested. MoV was detected at very low to low levels in 5/34 samples. However, as MoV was similarly detected at very low levels close to the detection threshold in 2 of the 9 pearl oyster negative controls, the validity of the positives obtained with the *P. monodon* samples is questionable. qRT-PCR analysis of the pooled RNA samples provided evidence that GAV was absent (95% confidence of a GAV prevalence less than 2 %) from the 160 juvenile *P. monodon* sampled from 5 habitats near Weipa and stressed whilst being maintained in captivity for 2 weeks.

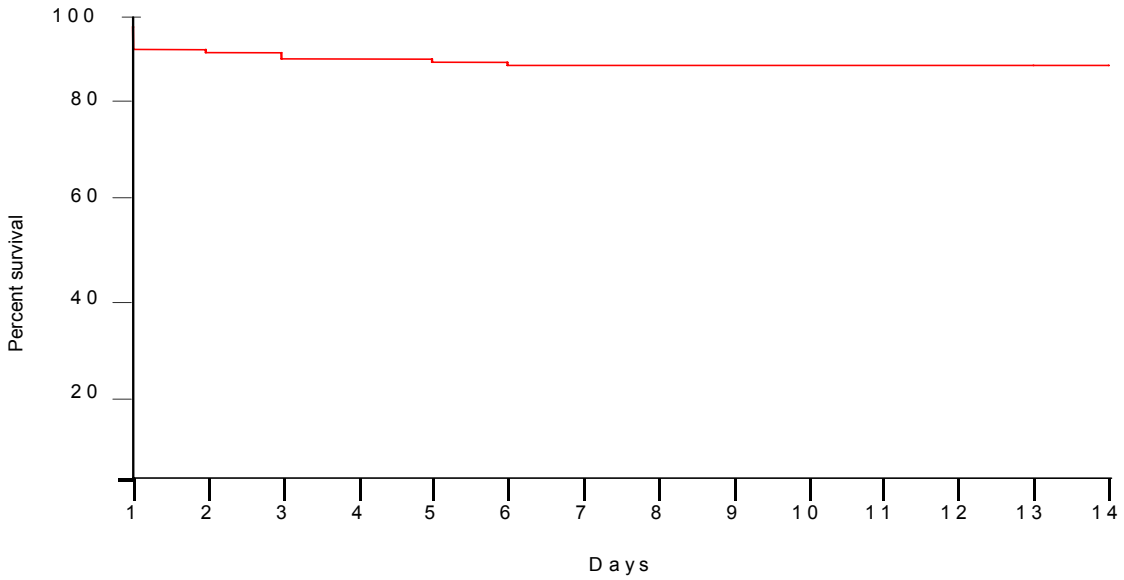


Figure 2. Survival of juvenile *P. monodon* maintained in plastic lined pools at Weipa over the course of the two week *in situ* stress experiment (n = 216).

Table 3. Results of qPCR testing for GAV and MoV conducted on representatives of *Penaeus monodon* GoC-G0 stocks collected from Weipa and stressed for 2 weeks in ponds at the CMR site at Weipa.

qPCR data

					GAV		MoV	
					Ct<25	Ct>25	Ct<25	Ct>25
Gen	Date	Av. wt.	Tissue	# batches				
G0	May 05	20g	LO	34 (=150 prawns)	0	0	0	5

3.4 Discussion

The results from the *in situ* stress experiment support previous observations that the *P. monodon* population in the Gulf of Carpentaria (GoC) is GAV-free (Chapter 2). The juvenile prawns (average weight 11.5 grams) used in the experiments at Weipa were much smaller that the adults *P. monodon* (average weight 62 grams) used by de la Vega et al, (2004) in stress experiments in which elevated GAV loads were induced. It has yet to be determined whether juvenile and adult *P. monodon* differ in their ability to tolerate physical or chemical stresses and, if so, whether this influences viral loads.

The results also supports previous observations that MoV is endemic in GoC stocks, but at low levels of infection. The presence of low levels of MoV was also detected in oyster tissue that was used as a negative control. As it is not known whether MoV can infect oysters, or as filter feeders whether oysters can simply acquire MoV from seawater, the significance of these results is unclear.

The fact that only very low levels of MoV were detected in RNA that had been pooled from several individuals indicates a high likelihood that some individuals were uninfected. Although screening of individuals would be required to confirm this, the results of the current study indicate that the GoC population of *P. monodon* is a source of stocks that, through screening and elimination, will be certifiable as specific-pathogen-free (SPF) for both GAV and MoV, with significant benefits for both research and industry.

4. CHAPTER 4. GENETIC VARIATION IN GAV AND MOV

Genetic variation between GAV and MoV in the GoC stocks and EC stocks determined.

4.1 Introduction

The objective of this component was to determine whether there are detectible genetic differences in Mourilyan virus (MoV) and gill-associated virus (GAV) isolated of stocks from *Penaeus monodon* from different geographic regions. The expectation was that if genetic variation between viral strains from different regions could be identified, this information would be very useful in determining the source(s) of infection. Of particular interest to this study was whether the viral infections in the GoC founder prawns were pre-existing or were acquired subsequent to their relocation to S.E. Queensland.

4.2 Methods

The samples of MoV and GAV PCR products were amplified from:

- GoC-G0 prawns sampled soon after their relocation from GoC
- GoC-G0 stocks after 6 months in culture at CMR and GCMA
- GoC-first generation (G1) and second generation (G2) stocks at times viruses were detected
- EC prawns present at CMR and GCMA at the same time as GoC prawns

RNA isolation
RNA was isolated from prawn tissue using TRIzol™ Reagent (Invitrogen) as described by the manufacturer, re-suspended in water and stored at -80°C. The RNA concentration was estimated by determining the absorbance at A_{260nm}.

Total nucleic acid isolation
Total RNA and DNA were isolated using a CTAB buffer followed by sequential extractions with equal volumes of phenol:chloroform:iso-amylalcohol (25:24:1) and chloroform:iso-amylalcohol (24:1) and precipitation under 0.9 volume isopropanol. The RNA/DNA pellet was washed with 70% ethanol, air dried, re-suspended in water and stored at -80°C.

cDNA synthesis
cDNA was synthesised in a 10 µl reaction volume containing 1.0 µg total RNA and 50 ng random hexamer primers using either Superscript II or III reverse transcriptase (Invitrogen) according exactly to the manufacturers protocols and stored at -20°C.

Conventional 1- and 2-step (nested) PCR for GAV
The nested PCR to amplify GAV RNA employed primer pairs GAV5/6 and GAV1/2 for the primary (618 bp amplicon) and nested PCR (317 bp amplicon) tests, respectively, and was conducted essentially as described previously (Cowley et al. 2000) with some modifications. An aliquot of the cDNA representing 100 ng total RNA was amplified in the first-step PCR (GAV5/6) and, when necessary, 2 µl of the primary PCR was amplified in the nested PCR (GAV1/2).

Conventional 1- and 2-step (nested) PCR for MoV
The nested PCR to amplify MoV RNA employed primer pairs MoV24F/25R and MoV148F/149R for the primary (610 bp amplicon) and nested PCR (322 bp amplicon) tests, respectively, and was conducted as described previously (Cowley et al. 2005).

Usually 5-8 µl of the PCR was resolved in a 2.0% agarose gel containing 0.5 µg /ml ethidium bromide and the size and amount of DNA product were visualized under UV light.

DNA sequencing and sequence analysis
DNA amplified in the PCR tests was purified using QIAguick™ columns (Qiagen). Both DNA strands of PCR amplicons were sequenced using various primers for GAV (GAV5, GAV5, GAV1 or GAV2) and Big Dye reagent (Applied Biosciences Inc., ABI) according the manufacturers instructions. Sequences were generated at the Australian Genome Research Facility, University of Queensland. Sequence chromatograms were edited using SeqEd (ABI) and sequence alignments were constructed using the MacVector 7.0 software (Oxford Molecular Ltd.). Only the sequences inside the primer regions of amplicons (GAV5/6 = 577 nt, GAV1/2 = 274 nt, MoV24F/25R = 562 nt, MoV148F/149R = 274 nt) were used in the sequence comparisons.

4.3 Results

Origin of P. monodon from which GAV sequence data was generated
The origin and location of EC and GoC *P. monodon* from which GAV PCR products were amplified and sequenced are listed in Table 1. These included wild EC *P. monodon* used at the commercial GCMA hatchery in September 2002 (Group 1), founder GoC-G0 prawns from CMR in which moderate-level GAV infection had became established by October 2002 (Group 2), GoC-G1 prawns being reared in tanks at CMR and raceways at GCMA in which low levels of GAV were detected at the half way point of the rearing cycle in June 2003 (Group 3) and CMR GoC-G2 prawns found to be infected with GAV at moderate levels and high prevalence when the stock was sampled for the first time in February 2004 (Group 4).

Table 1. The location and origin of the *Penaeus monodon* used to generate GAV sequences.

Group	Date	Location	Origin	Sample	Number	Codes	GAV primers
1	Sept. 2002	GCMA	EC	broodstock	5	(1872 -1880)	5/6
2	Oct. 2002	CMR	GoC-G0	broodstock	4	(1918 -1924)	5/6
	Oct. 2002	CMR	GoC-G0	broodstock	3	(2040 -2042)	
	Oct. 2002	CMR	EC	broodstock	4	(1926 -1932)	
3	June 2003	GCMA	GoC-G1	sub-adult	2	(2048, 2050)	1/2
	June 2003	CMR	GoC-G1	sub-adult	2	(2066, 2068)	
			EC	(test control)	1	(2064)	
4	Feb. 2004	CMR	GoC-G2	juvenile	3	(2294, 2295)	5/6
			EC	(test control)	1	(2303)	

Group 1
GAV sequences were determined for GAV PCR products amplified from 5 wild-caught EC *P. monodon* broodstock sourced from North Queensland and collected from the commercial hatchery at GCMA in September 2002. The broodstock were sampled post-spawning and PCR

screening had indicated that moderate-level GAV-infection was universally present in all 10 prawns tested. In the 577 nucleotide region between the GAV5/6 PCR primers, GAV sequences from 2 of the prawns were identical, 2 others possessed a single nucleotide substitution at the same position whilst the other GAV isolate possessed 2 substitutions. Thus, the level of sequence identity (>99.7) was extremely high among the GAV isolates present in the EC broodstock. In addition, the sequences were <1.5% variant from the consensus sequence of GAV isolates identified in wild and farmed EC *P. monodon* stocks over previous years dating as far back as 1996. The GAV genotype present in the GCMA EC broodstock was thus within the normal range (ie. <2.5% sequence variation) expected due to quasi-species variation of this GAV strain in individual prawns that had been determined from previous analyses of prawns from the EC *P. monodon* population.

GAV sequence comparisons

The sequences were thus representative of the GAV genotype found at high prevalence in the wild EC population of *P. monodon*, provided a representative measure of the expected quasi-species sequence variation in individual prawns and provided a baseline sequence for comparisons with GAV sequences detected in the CMR GoC-G0 prawns and in the GoC-G1 prawns reared at both CMR and GCMA.

Group 2

GAV sequences were determined for GAV5/6 PCR products amplified from 4 wild-caught EC *P. monodon* broodstock (sjc1926, 1928, 1930, and 1932) sampled from CMR on the same day (29-10-2002) as 12 representatives of the GoC-G0 stocks were sampled. Although CMR did not have information from the commercial broodstock supplier on the exact location the prawns had been caught. However the supplier was based in Innisfail in North Queensland and the prawns originated from the local area.

Of the GAV isolates sequenced from the 4 CMR EC prawns, 1 isolate possessed a sequence with only a few nucleotide variations (5-6/577 = 1.0%) from the GCMA EC prawns (Group 1). GAV sequences obtained from the other 3 prawns were identical. However, the GAV sequences in these 3 prawns were markedly divergent (24/577 = 4.2% nucleotide variation) from the GAV sequence obtained with the other CMR EC prawn. A similar level of divergence (20-21/577 = 3.6% nucleotide variation) was evident with the 4 EC prawns sampled from GCMA (Group 1) and the 4 GoC-G1 prawns sampled from CMR as the same time (see below).

GAV sequences were also determined for GAV5/6 PCR products amplified from 4 out of 12 GoC-G0 *P. monodon* broodstock (sjc1918, 1920, 1922, and 1924) sampled from CMR on the same day (29-10-2002) as were 4 EC broodstock. The GAV sequences generated from 3 of these prawns were identical and only a single nucleotide change was detected in the other isolate. In comparison to GAV sequences detected in the GCMA EC broodstock (Group 1), only 1 to 2 nucleotide changes were evident in any of the 4 CMR GoC-G1 prawns.

Group 3

GAV was detected in the GoC-G1 prawns reared at both GCMA and CMR when the stocks were sampled in June 2003. Virus load was very low, however, and only 2-step GAV1/2 PCR products were detected. PCR products amplified from 2 GoC-G1 prawns from either CMR (sjc 2066, 2068) or GCMA (sjc2048, 2050) were sequenced together with a PCR product (sjc2064) generated from a control EC prawn. The GAV sequences obtained from the 2 GCMA GoC-G1 prawns were identical to the EC control. GAV present in the 2 CMR GoC-G1 prawns showed some differences (7/274 = 2.6% nucleotide variation) at a level that was outside the acceptable range normally seen among quasi-species variants. Because of this and the fact that some EC *P. monodon* sampled from CMR in September 2002 also showed considerable variation (3.6% - 4.2% nucleotide differences) from the known EC GAV genotype, the sequences of these

amplicons were compared to representatives of the EC type (sjc1926) and variant GAV genotype (sjc1928). In this alignment, the 2 variant GAV sequences (sjc2066, 2068) obtained in the CMR GoC-G1 prawns were identical to the variant sequence found in one of the CMR GoC-G0 prawns (sjc1928) in which GAV infection was detected at high prevalence. This is highlighted in the phylogenetic tree shown in Figure 1.

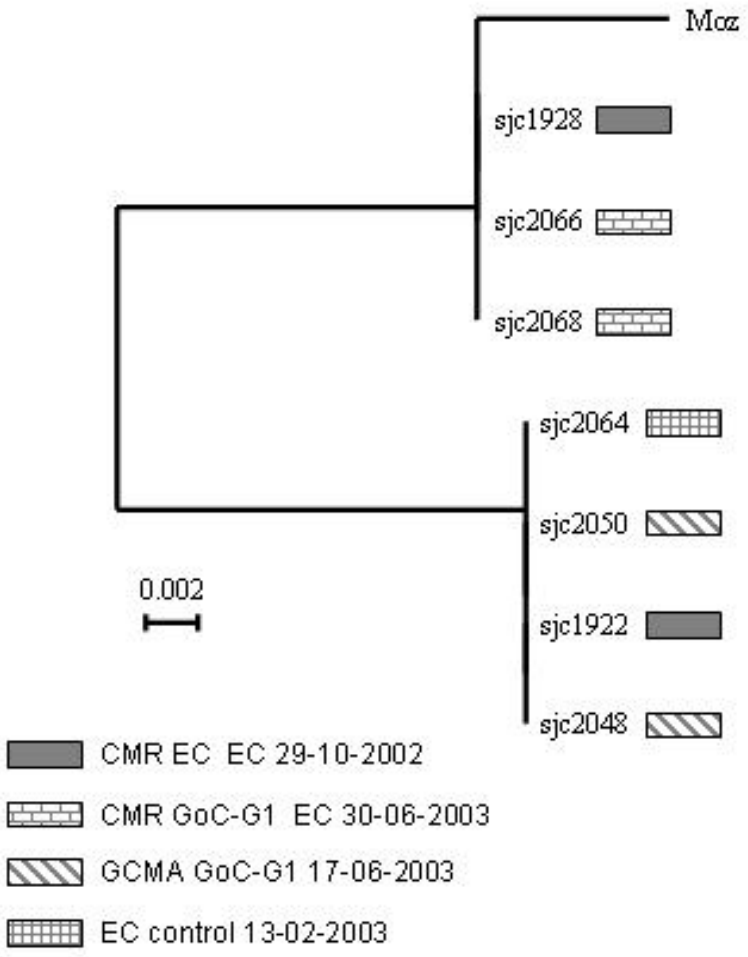


Figure 1. Phylogenetic tree constructed from a Clustal X multiple sequence alignment of the 274 nucleotide genome region of GAV spanned by the GAV1/2 2-step PCR primers. A genotypic variant of GAV (Genotype 6) identified in *P. monodon* from Mozambique (denoted as Moz) is included as it is closely related to the GAV variant identified in Australian EC and GoC *P. monodon* sampled from CMR.

There appear to be two possible explanations for this and it needs to be noted that the GoC-G0 prawns, as well as the GoC-G1 prawns tested at CMR were obtained from GCMA stocks. One possibility is that the GCMA GoC stocks (G0 and G1) were infected at a very low level with the GAV variant and that infection was expressed at higher level after the prawns were relocated. However, if the GCMA GoC-G1 prawns were harbouring very low-level infection with this variant GAV type before they were moved to CMR, we would have expected to find the variant sequence in the stocks sampled from GCMA. This was not the case if only the known EC GAV genotype was identified in GCMA GoC-G1 prawns. The other possibility is that the GoC-G1 lines at CMR and GCMA were infected independently at each site. As progeny of wild *P. monodon* would have existed in the hatchery and farm ponds at GCMA and as EC *P. monodon* existed at CMR that possessed the variant GAV genotype, the infections in the GoC-G1 prawns might have been arisen by contamination by different GAV types existing at the 2 sites.

Group 4

GAV infection at moderate levels and high prevalence was detected in the GoC-G2 families generated independently at CMR and GCMA from the first point that they were tested by PCR in early 2004. GAV sequences were determined for GAV5/6 PCR products amplified from 3 of a batch of 24 prawns that all generated 1-step PCR products for GAV. The 3 GAV sequences obtained from the GoC-G2 prawns were identical and identical with the GAV sequence obtained with the EC control prawn.

Summary for GAV

The GAV sequences generated from the different stocks of GoC prawns (G0, G1 and G2) collected from either GCMA or CMR were essentially indistinguishable from the GAV genotype that occurs at very high prevalence in EC wild and farmed *P. monodon*. This is highlighted in the phylogenetic tree constructed using 577 nucleotide sequences of 1-step PCR products amplified from GoC-G0 and GoC-G2 prawns as well as from various EC stocks shown in Figure 2. All of the GAV sequences generated from any of the GoC prawn stocks tested clustered with the GAV genotype sequence known to exist in the EC *P. monodon* population. This suggests that GAV detected in the stocks was acquired from local stocks of EC prawns being reared in the same facilities at the same time as the GoC prawns. This is supported by the fact that we were not able to detect GAV in any GoC-G0 founder prawns and that GAV was not detectable by qPCR in any of 215 *P. monodon* collected from near Weipa that were stressed by bleeding to specifically address the issue of whether the GAV-free *P. monodon* indeed exist in the GoC population (Chapter 3).

A new GAV genotype previously unrecognised in Australian *P. monodon* was detected at CMR in EC prawns sampled in October 2002 and in GoC-G1 prawns sampled in June 2003. The variant was 3.6% to 4.2% divergent at the nucleotide level from the known EC GAV genotype. Compared to other genotypic variants of which we are aware, the variant is most closely related to a distinct GAV genotype (Genotype 6) only previously detected in *P. monodon* from Mozambique. The nature of the GAV variant with respect to its pathogenicity compared to the prototype GAV strain, and what populations of *P. monodon* it exists in along the East Coast of Queensland, remain important questions for which we should seek answers.

Origin of P. monodon from which MoV sequence data were generated

The origin and location of EC and GoC *P. monodon* from which MoV 2-step PCR products were amplified and sequenced are listed in Table 2. A MoV sequence generated from PCR product from a *P. merguiensis* prawn was also included to highlight our findings that the genome of MoV does not display the level of quasi-species variation as found with GAV. Prawns analysed included founder GoC-G0 prawns sampled from GCMA in April 2002 within 2 weeks of their transfer to Southeast Queensland (Group 1), GoC-G1 prawns sampled from GCMA at ~6 months of age in June 2003 (Group 2), *P. merguiensis* prawns sampled from a farm in June 2003 (Group 3) and GoC-G1 prawns sampled from tanks at CMR in June 2003 (Group 4) as well as a wild EC *P. monodon* used as a test control.

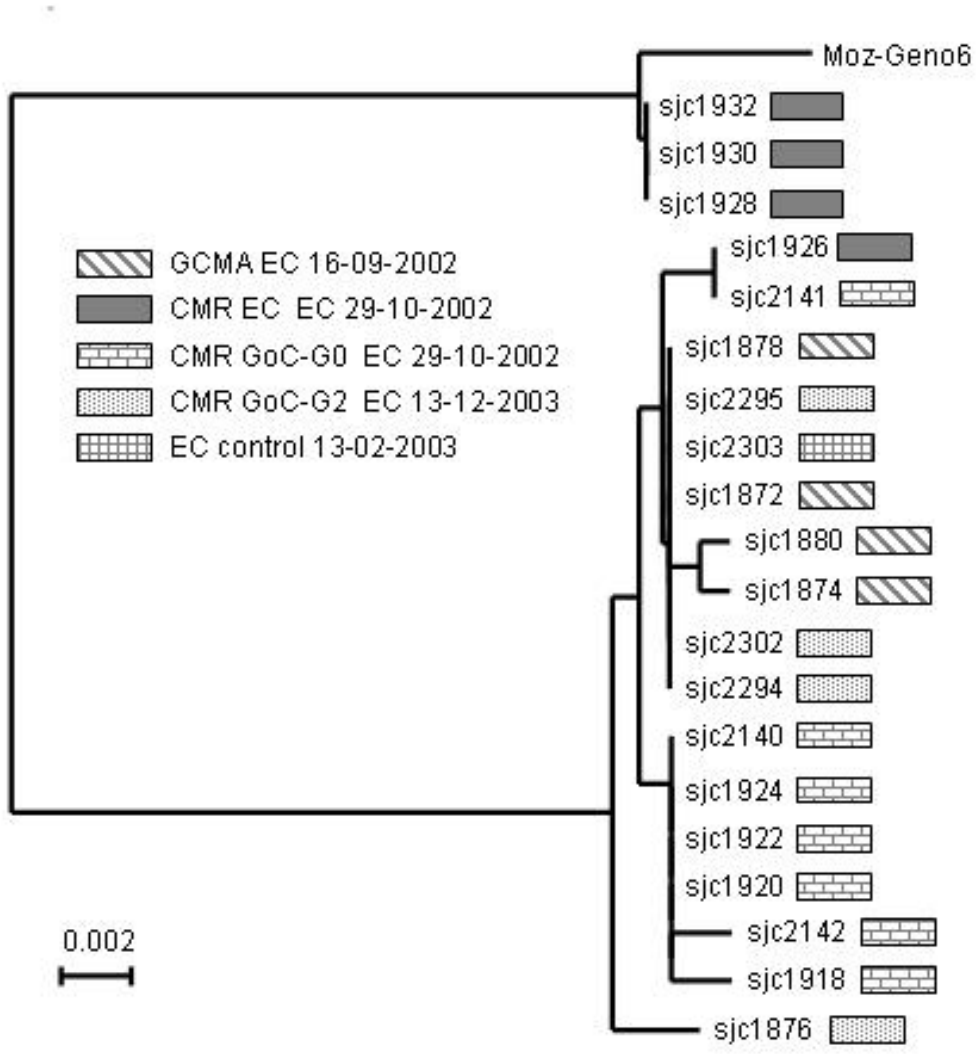


Figure 2. Phylogenetic tree constructed from a Clustal X multiple sequence alignment of the 577 nucleotide genome region of GAV spanned by the GAV5/6 1-step PCR primers. The various GoC and EC prawns from which PCR products were generated and sequenced are listed in Table 1. A genotypic variant of GAV (Genotype 6) identified in *Penaeus monodon* from Mozambique is included as it is closely related to the GAV variant identified in Australian EC and GoC *P. monodon* sampled from CMR.

Table 2. *Penaeus monodon* and *P. merguiensis* used to generate MoV sequences

Group	Date	Location	Origin	Sample	Number	Codes	MoV primers
1	03-04-2002	GCMA	GoC-G0	broodstock	4	(2562- 2569)	148/149
2	17-06-2003	GCM	GoC-G1	broodstock	3	(2052 -2057)	148/149
3	17-06-2003	Seafarm		*P. merg.*	3	(2058 - 2063)	148/149
4	30-06-2003	CMR	GoC-G1	broodstock	2	(2072 - 2075)	148/149
			EC	(test control)	1	(2070 - 2071)	148/149

MoV sequence comparisons
Sequences obtained in the 274 nucleotide region of the MoV M-RNA genome segment spanned by the 2-step PCR primers MoV148/149 generated were identical for all prawns tested, expect for a single nucleotide change obtained with one prawn, irrespective of their origin.

Summary for MoV
As MoV was detected in the GoC-G0 founder stock very soon after their arrival in Southeast Queensland, we can speculate that it was brought with the wild prawns. However, the lack of any sequence variation whatsoever gave us no clear indication of as to whether this was the case or not. MoV is known to infect or have been detected by PCR in species other than *P. monodon* including *P. japonicus* and *P. marguiensis*. This suggests that MoV might have a much broader host range compared to GAV and we hypothesize that the virus might be much more widely distributed in the environment. We have yet to undertake MoV qPCR testing of samples from 150 wild GoC *P. monodon* collected near Wepia, GoC, and that were stressed to potentially elevate the pre-existing viral infections. The results of this testing should conclusively demonstrate whether or not MoV exists in GoC *P. monodon*, and the prevalence at which infections occur.

4.4 Discussion

The GAV sequences generated from the different generations of *P. monodon* that originated from GoC prawns (G0, G1 and G2) collected from either GCMA or CMR were essentially indistinguishable from the GAV genotype that occurs at very high prevalence in EC wild and farmed *P. monodon*. The results indicate that GAV detected in the stocks sourced from the Gulf of Carpentaria was acquired from stocks sourced from the East Coast that were reared in the same facilities at the same time as the GoC prawns

Further evidence in support of the observation of the GAV status of the Gulf of Carpentaria stocks is that GAV was not detected in any GoC-G0 founder prawns (see Chapter 2). In addition to this no GAV was detected in any of the 215 *P. monodon* collected from Weipa, even when these stocks were stressed by bleeding (Chapter 3).

MoV was detected in the GoC-G0 founder stock very soon after their arrival in S.E. Queensland indicating infections in the wild prawns. However, the lack of any sequence variation meant that this observation could not be independently validated. MoV is known to infect or have been detected in species other than *P. monodon* including *P. japonicus* and *P. merguiensis*, indicating a much broader host range compared to GAV.

5. CHAPTER 5. AFFECT OF GAV AND MOV LOAD ON SPAWNING

The relationship between GAV and MoV viral load and spawning efficiency

5.1 Introduction

The objective of this component was to determine whether a quantitative relationship exists between viral load in *Penaeus monodon* broodstock and spawning efficiency. Previous research had indicated that high viral loads may impair broodstock performance (Hsu et al. 1999). Accordingly the focus of this component was on examining both GAV and MoV loads in broodstock and determining whether there was any relationship between the loads of these viruses in broodstock and the spawning efficiency of the broodstock.

5.2 Methods

Four sets of broodstock were examined:

- GCMA wild EC broodstock used for commercial hatchery production in 2002
- AIMS wild and G3 EC broodstock spawned in October 2002
- reproductive tissues, eggs and 15 day old postlarvae from wild EC broodstock spawned at AIMS in May–June 2002
- Two separate groups of G1xG4 domesticated broodstock raised in ponds at BIARC and spawned at AIMS in August and October 2003

GCMA samples

Broodstock used for this experiment were spawned between August and October 2002 as part of the normal stocking program at GCMA. Data collected for each spawning included; date of spawning, egg number, nauplii number and hatch rate. GAV load in gill snips was determined using three methods: Farming Intelligene test carried out at GCMA (conventional PCR test), nested (1- and 2-step) PCR carried out at CLI and a qPCR test carried out at AIMS. MoV load in gill snips was determined by: Conventional 1- and 2-step PCR and qPCR carried out at CLI. Many of the broodstock spawned multiple times, however, only data from first spawnings were included in the analysis.

RNA extraction from gill samples was performed on the same day as spawning took place. A total of 60 spawners were selected for analysis, and divided into three categories depending on spawning performance: high, medium and low (Table 1).

Table 1. Characterisation of high, medium and low spawning performance of *Penaeus monodon* broodstock based on number of eggs spawned, hatch rates and numbers of nauplii produced.

Spawning performance	Egg number	Hatch rate	Nauplii number
High	>250,000	>75%	>250,000
Medium	100-250,000	50-75%	100-250,000
Low	<100,000	<50%	<100,000

AIMS EC May –June 2002 Spawnings
Samples were taken from females and males used to generate progeny from a total of 42 successful spawnings. Data collected for each sample included: pedigree data i.e. which female was mated with which male, date of spawning, egg number, nauplii number, hatch rate, GAV load in male and female gill snips determined by qPCR at AIMS. Samples for GAV and MoV analysis were also taken of gonads (ovaries and testes), eggs and PL15s resulting from successful spawnings.

AIMS EC October 2002 Spawnings
Data collected for each spawning included: pedigree data (i.e. which female was mated with which male), date of spawning, egg number, nauplii number, hatch rate, GAV load in male and female gill snips determined by qPCR at AIMS.

For this experiment, matings were done by artificial insemination and the two paired spermatophores from a male were used to inseminate two separate females i.e. most of the females in this dataset received only half the usual complement of sperm. In some cases, only half spermatophores were used i.e. the females only received one quarter of the normal amount of sperm. As the matings were done by artificial insemination, the males were also recorded and gill samples taken from the males for analysis. Thus with this set of matings it was possible to analyse the viral load in the male as well as the female broodstock.

Gill snips were taken from females 1-3 days after spawning, and from males 1-2 days after they had been used for artificial insemination

BIARC EC trial G1xG4 August 2003 spawnings
A weakness in the data presented for the AIMS October 2002 spawnings was that gill snip samples were only obtained from animals that actually spawned, i.e. the prevalence and viral loads of GAV and MoV in the broader population of candidate broodstock was not tested. To test the effect of eyestalk ablation on viral load an experiment was carried out at BIARC in July-August 2003 using a mixture of wild and domesticated G1 and G4 stocks (G1= first generation domesticated stocks, G4=crosses between AIMS G3 males and wild females) in which gill snips were taken from animals shortly after ablation. The goal was to measure viral status at the onset of the spawning period (i.e. at the time of ablation), as opposed to post-spawning viral load.

The trial used natural matings in tanks. Gill snips were taken from females 4 days post ablation, to allow measurement of viral loads earlier in the spawning cycle. The animals were monitored daily, with deaths or spawnings recorded. The trial was terminated after 18 days, at which point repeat gill snips were taken from any animals that had survived the trial, including males. In total, gill snips were taken from 18 wild and 43 domesticated females and 27 domesticated and 15 wild males.

Broodstock in this trial were only tested for GAV using qPCR at AIMS.

AIMS EC G1 x G4 October 2003 spawnings
Spawnings were conducted in October 2003 with the G1 and G4 domesticated broodstock that were raised in the BIARC ponds and shipped to AIMS for spawning. Gill snips were taken from females 1-3 days after spawning, and from males 1-2 days after they had been used for artificial insemination. The following data were collected: pedigree data (i.e. which female was mated with which male), date of spawning, egg number, nauplii number, hatch rate, GAV load in male and female gill snips was determined by real-time qPCR at AIMS, MoV load in male and female gill snips was determined by qPCR at CLI.

Correlations between parameters measured for each set of broodstock were examined using pair wise correlation analysis. Different types of regression analysis were used, depending on the data type. For continuous data such as egg count, a linear regression was used. For viral load measured by qPCR assay, a log transformation was carried out prior to carrying out linear regression. Where data consisted of discrete, ordered classes, (e.g. the Farming Intelligene test which scores the results from 0 to 5), an ordinal logistic regression was used. All data were analysed using the statistical package JMP5.

In addition to this regression analysis, an analysis of variance was carried out to determine whether there was any difference between the three groups of GCMA spawners, namely those grouped as high, medium and low performers.

5.3 Results

GCMA samples and AIMS EC October 2002 Spawnings

For convenience the results from GCMA samples and AIMS EC October 2002 Spawnings are presented together. In total, data were obtained for 99 spawnings (60 from GCMA, 39 from AIMS). This included measurement of viral load in 99 female broodstock and 29 males. Viral template number was variable among all broodstock screened (Figs 1 and 2).

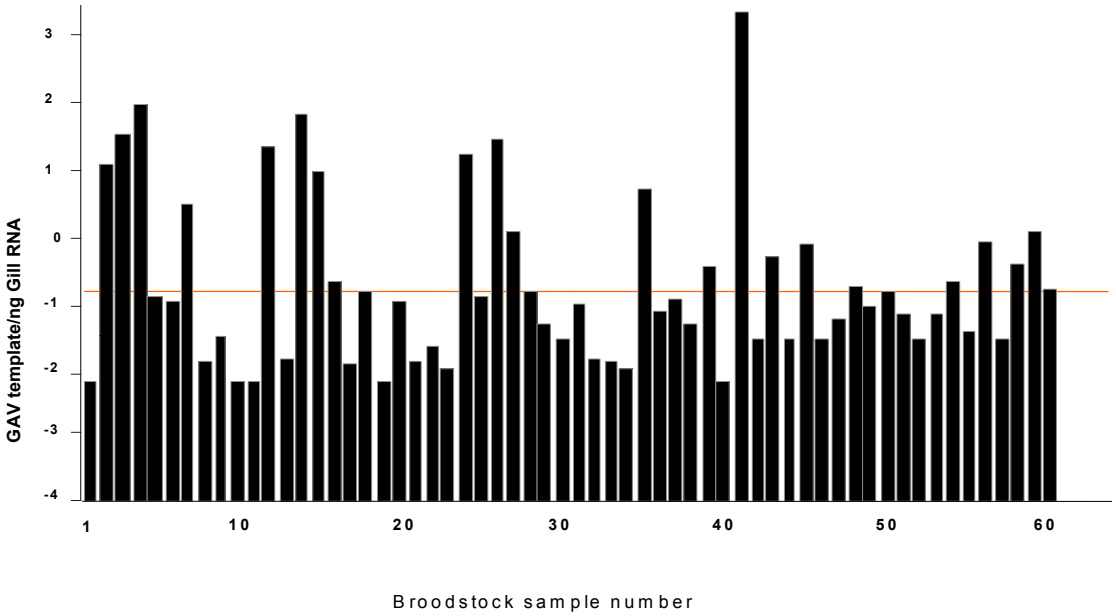


Figure 1. Distribution of GAV template number per nanogram (ng) of gill RNA in *Penaeus monodon* broodstock spawned at Gold Coast Marine Aquaculture (GCMA). Red line denotes limit of reliable detection.

The GAV load was below the limit of reliable detection by qPCR in 38 out of the 60 GCMA broodstock screened (Fig. 1), and in 10 out of the 29 males screened at AIMS. In contrast to this, GAV levels were above the reliable limit of detection by qPCR in all female broodstock from AIMS.

The MoV levels were higher than GAV levels and above the limit of reliable detection in all broodstock examined by qPCR (Fig 2). A number of samples had MoV levels greater than 10^6 per ng total RNA, suggesting that up to 1% of total cellular RNA could be viral, and two samples from the GCMA broodstock showed levels greater than 10^7, indicating that up to 10% of cellular RNA was MoV template.

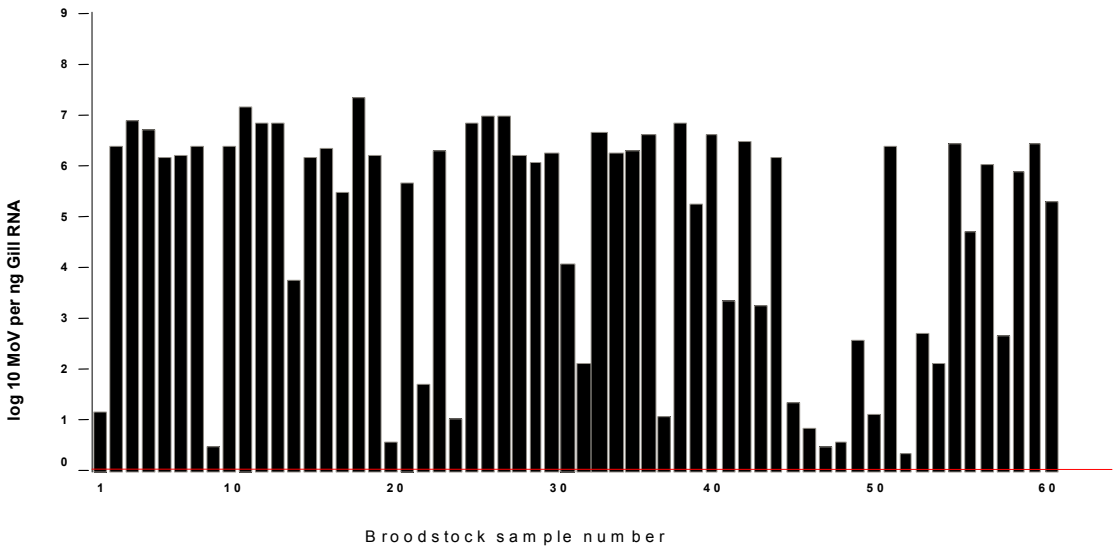


Figure 2. Distribution of MoV template number per nanogram (ng) of gill RNA in *Penaeus monodon* broodstock spawned at GCMA. Red line denotes limit of reliable detection.

Among the GCMA broodstock those numbered 1-20 (Figs 1 and 2) showed a "high" spawning performance, broodstock numbers 21-40 showed a "medium" spawning performance and broodstock numbers 41-60 showed a "low" spawning performance.

Table 2. Summary of statistically significant correlations observed in the regression analysis.

Y-factor	X-factor	Significance of correlation observed through analysis of GCMA data	Significance of correlation observed through analysis of AIMS data
MoV template number in female broodstock (qPCR)	Nauplii number	P<0.01	No correlation
Total viral load in female broodstock	Hatch rate	P<0.01	No correlation
Total viral load in female broodstock	Nauplii number	P<0.05	No correlation

In GCMA broodstock, a correlation was found between MoV load and total viral load (by qPCR) in female broodstock and viral load in their eggs and nauplii (Table 2). This was not substantiated by the results from AIMS broodstock which showed no correlation for the same parameters.

The correlation analysis showed a positive association between GAV load and MoV load in the broodstock from GCMA, but not broodstock from AIMS.

For GAV, analysis of variance revealed that there was no significant difference between viral load and spawning efficiency in the three groups of (high, medium and low performing) spawners at GCMA (Fig. 3). However, for MoV there was a significant difference between the three groups (p=0.02), with a higher level of MoV correlated with higher broodstock performance (Fig. 4).

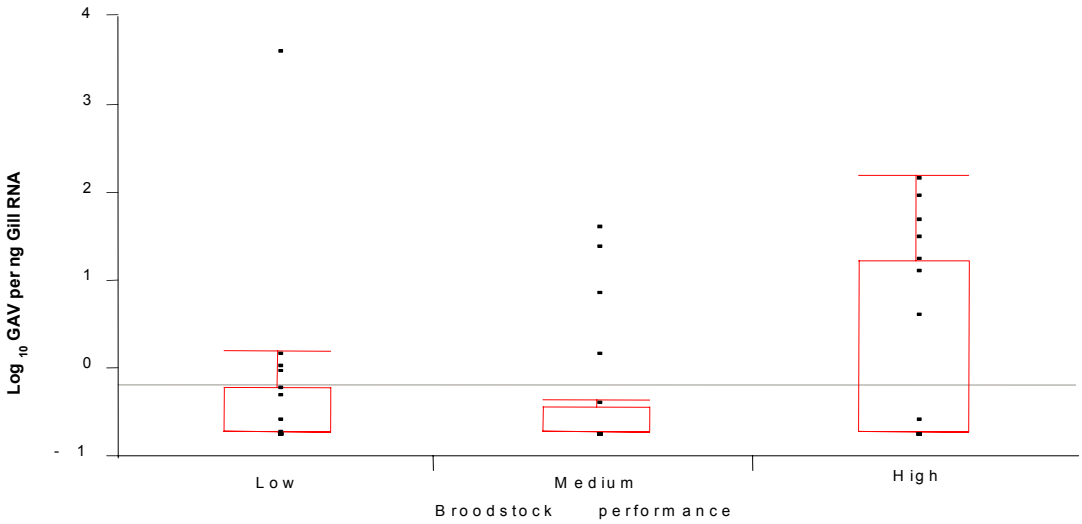


Figure 3. Relationship between *Penaeus monodon* broodstock reproductive performance (low, medium, high) and levels of GAV detected in gill snips of stocks from GCMA.

AIMS May to June 2002 Spawnings

GAV was undetectable in 22 out of 23 egg samples tested, so no correlation analysis was possible with egg GAV load data. In contrast, a range of MoV values were obtained from the eggs. GAV and MoV were both detected in ovaries, testes, nauplii and PL15 samples.

For GAV and MoV correlations existed between viral load in broodstock gill snips and viral load in testes and ovarian tissues ($p<0.01$). No correlation was found between GAV and MoV load in broodstock gill snips and viral load in their offspring (PL15).

BIARC trial EC G1 x G4 August 2003

There was a decrease in GAV levels over the course of the trial in broodstock that survived (Table 3). The GAV load in surviving female broodstock at the start of the trial and at the end of the trial showed no consistent pattern of change; viral load increased in 6/14 females, remained the same in 4/14 and decreased in 10/14 (Fig. 5). In broodstock that failed to spawn during the trial but survived the trial, GAV levels increased. However, other than this, there was no relationship between GAV load in prawns at the start of the trial and whether or not they survived.

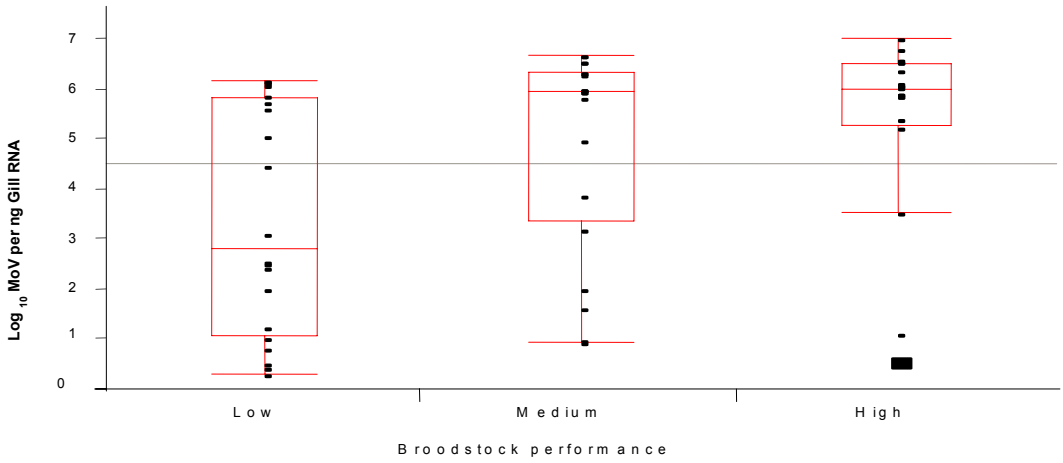


Figure 4. Relationship between *Penaeus monodon* broodstock reproductive performance (low, medium, high) and levels of MoV detected in gill snips of stocks from GCMA.

Table 3. Gill Associated Virus (GAV) loads measured in samples from the spawning trial at BIARC in July 2003

Sample	n	GAV load (templates/ng RNA)
G4/G1 domesticated broodstock, start of trial	34	$3.30 \times 10^4 \pm 2.93 \times 10^4$
G4/G1 domesticated broodstock, end of trial	30	$6.90 \times 10^3 \pm 3.14 \times 10^3$
Wild broodstock, start of trial	14	$7.78 \times 10^4 \pm 7.22 \times 10^4$
Wild broodstock, end of trial	20	$1.60 \times 10^4 \pm 8.22 \times 10^3$
Broodstock that spawned during trial, start of trial	17	$6.41 \times 10^4 \pm 5.95 \times 10^4$
Broodstock that spawned during trial, end of trial	6	$1.95 \times 10^4 \pm 9.55 \times 10^3$
Broodstock that failed to spawn during the trial but survived to end of trial, start of trial	10	$3.11 \times 10^2 \pm 2.88 \times 10^2$
Broodstock that failed to spawn during the trial but survived to end of trial, end of trial	14	$1.35 \times 10^4 \pm 6.27 \times 10^3$

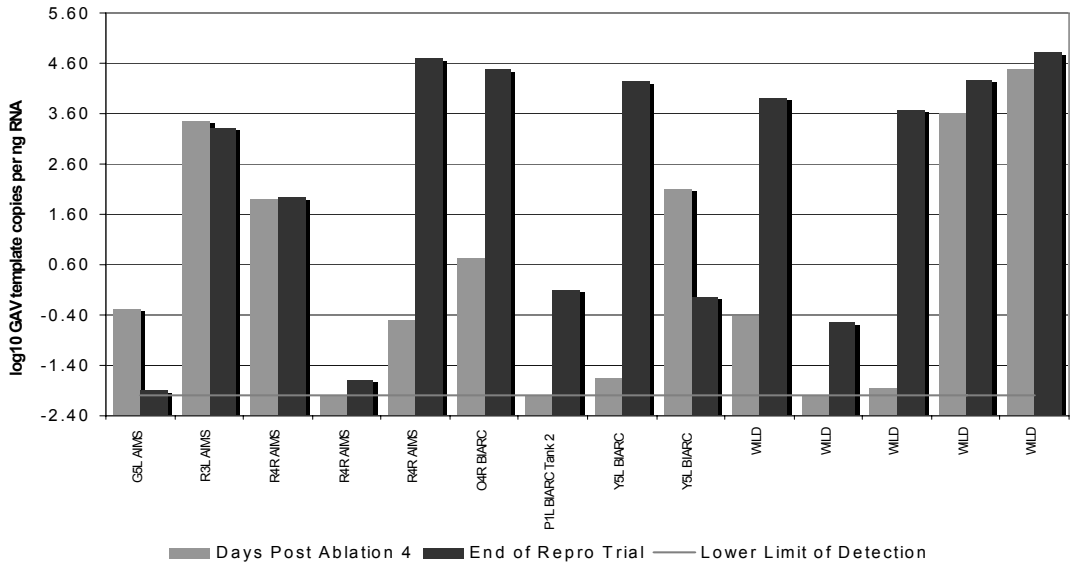


Figure 5. Paired GAV loads in surviving female EC broodstock at the beginning and end of the reproduction trial at AIMS.

AIMS EC G1/G4 October 2003

GAV was detected in 20/22 females and 15/23 males. MoV was detected in 13/22 females and 5/23 males that spawned. For GAV and MoV no correlations were found between parental gill virus load and egg and nauplii number, or hatch rate.

5.4 Discussion

The results if this study showed that there was no consistent relationship between viral load of GAV or MoV and spawning efficiency in *P. monodon*. This was the case whether GAV load or MoV load alone were considered, or when total GAV plus MoV load was considered. Broodstock from GCMA with high MoV levels were among the most efficient spawners. Although GAV levels were variable and generally low, some of the highest GAV levels were from high performing broodstock at GCMA.

From the matings trials at AIMS, both male and female gill snip samples were available. The results for these trials indicated that male viral load also had no effect on spawning efficiency.

Overall the results indicate that high levels of MoV and GAV are not an impediment to high spawning efficiency.

The results also showed that there was no consistent pattern in changes in viral loads following broodstock ablation and spawning. In some broodstock viral load decreased, in others the load increased or remained stable.

The results of this study contrast with those of previous studies in which high viral loads were correlated with impaired spawning performance of prawn broodstock. In stocks of *P. monodon* infected with White Spot Syndrome Virus (WSSV) a high proportion (12/15) PCR positive broodstock failed to spawn (Hsu et al. 1999). Similarly, female *L. vannamei* broodstock infected with high levels of IHHNV either failed to spawn or their eggs did not hatch (Hsu et al. 2003). Although the precise mechanism by which WSSV or IHHNV infections impaired spawning performance is unknown, it is likely that this was due in part to these viruses progressing beyond infection to disease, including the degradation of reproductive tissues.

In the current study, it is conceivable that despite high viral levels of GAV and/or MoV these infections were benign, at least with respect to reproductive tissues. In this context it should be noted that for the trials conducted with stocks that had been shipped from BIARC to AIMS there had been *de facto* selection for tolerance to stress. For example a total of 151 G1 females were shipped by air from BIARC to AIMS. Of these, only 81 survived to ablation, representing 47% mortality either during transport or shortly thereafter (6% mortality was in transit, 41% post-stocking). Subsequently, of the 81 broodstock ablated, 40 more died (i.e. further 50% mortality), most within 10 days post-ablation. Hence the broodstock that survived transport and ablation, and went on to spawn, showed considerable resilience compared to other broodstock from the same cohort. The fact that they also showed an overall lower GAV load may indicate that they have greater overall tolerance to stress.

The results of this study again highlight the importance of further studies to determine the relationship between GAV and MoV and *P. monodon*. Of particular concern and interest are the factors that result in the progression of infections with these viruses to disease in tissues or organs including testes and ovaries

CHAPTER 6. EGG WASHING EXPERIMENTS

The effects of egg-washing treatments on egg viability and on levels of Gill Associated Virus (GAV) in *Penaeus monodon* eggs, larvae and postlarvae.

5.5 Introduction

The objective of this component of the study was to determine whether washing newly spawned *Penaeus monodon*, eggs with or without virucidal agents could either eliminate or significantly reduce levels of Gill Associated Virus (GAV) without adversely affecting hatch rates.

At the start of the project the intended experimental approach was to spawn broodstock, wash the eggs with or without virucidal treatments then compare GAV loads and hatch rates in treated and untreated samples. These results would then be used in an iterative process to refine the treatments with virucidal agents to achieve complete or partial removal of GAV with minimal harm to the eggs. However, once the project team had developed a sensitive, quantitative assay for GAV (see Chapter 2), it was soon discovered that GAV levels in egg samples were almost always below the level of detection. Several assays of different larval stages revealed that GAV was generally un-detectable in any stages younger than postlarvae. In response to this finding the project team completed some preliminary egg washing experiments then designed an alternative approach that tested the potential of virucidal compounds using a GAV viral inoculum. Finally, the four compounds that were most effective in destroying GAV in a viral inoculum were tested for their effects on *P. monodon* hatch rates.

5.6 Preliminary egg washing experiments

5.6.1 Methods

The wild EC female broodstock used in the experiments were sourced from the Cairns region and transferred to broodstock tanks at AIMS. The broodstock were checked on a daily basis to assess the state of maturation of their gonads. The night before they were likely to spawn, they were placed in individual spawning tanks. These tanks are designed so that eggs from individual spawnings collect in the overflow from the spawning tank. The morning after spawning, the eggs were washed through a 355 μm mesh to remove proteinaceous and faecal material. They were then transferred to a 4L container, and 5 x 2 ml sub samples taken for total egg counts.

After sub-sampling for egg counts, the remaining eggs were divided into six containers (i.e. approximately 700 ml aliquots). Each sample was then separately concentrated on a 150 μm screen; the eggs were harvested into a 250 ml Cospak jar and resuspended in 10 ml seawater. This 10 ml was transferred to a 50 ml Falcon tube, and 40 ml additional solution was added as appropriate for the treatment concerned. One of the six samples was placed directly into the AIMS hatching system to act as a "no wash" control.

In the experimental design for each of three separate spawnings there were nine treatments; four different levels of salinity (8, 18, 28, 32.5 ppt), one level of formalin (100 ppm) and three different levels of Virkon S (0.5, 3, 6, 9 ppm). Each treatment had three replicates and there were two controls (eggs that were not washed and eggs that were washed with 35 ppt seawater). Chemicals were prepared either in sea water that had been filtered through a 0.45 μ membrane

to remove as much organic matter as possible, or in artificial seawater. This was particularly important for Virkon-S which may be inactivated by organic matter. The eggs were treated in the Falcon tubes for 60 minutes with gentle aeration. Then they were transferred to the AIMS hatching system where they were left to hatch overnight. Nauplii were collected in separate containers. Total nauplii hatched were counted for each treatment the next day to determine hatch rate.

5.6.2 Results

The percentage hatch rate of the eggs in each treatment was compared to the percentage hatch rate in the untreated controls. The results showed that washing in seawater at 35 ppt or at reduced salinities had a detrimental effect on hatch rate, though this effect was relatively small, in the range of 5 % lower then the control hatch rates (Fig. 1).

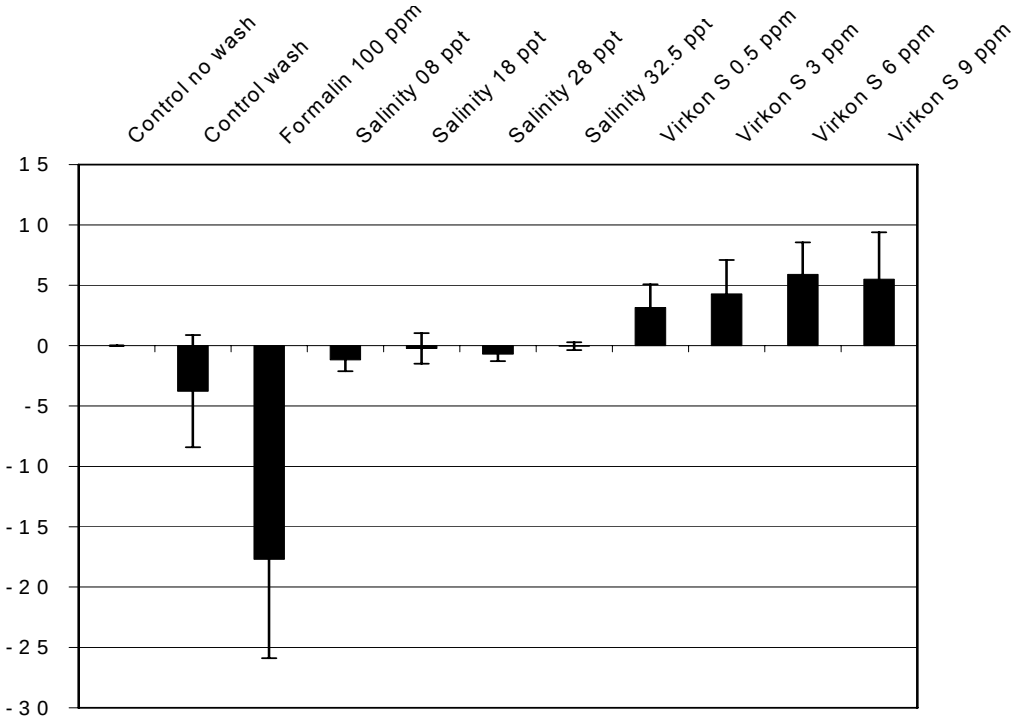


Figure 1. Percentage change in hatch rate compared to the “no-wash” control for the different washing agents tested in the preliminary trials. The number of replicates was n=12 for control wash, n=3 for salinity and formalin treatments and n=6 for Virkon S treatments.

Treatment with 100 ppm formalin had the most pronounced detrimental effects on hatch rates (~ 17% lower than control hatch rates). Treatment with VirkonS had a positive effect, but this was relatively small, within the range of 5% higher than the control hatch rates.

5.7 The effectiveness of different chemical disinfectants on in-vitro inactivation of GAV.

5.7.1 Methods

Production of inoculum
The viral inoculum used in this study was produced from farm reared *P. monodon* that had received a viral inoculum (Book 149 NY p29). The gills and lymphoid organs were collected separately, snap frozen in liquid nitrogen and stored at –80ºC. Tissues were thawed by diluting (5 fold) in cold 0.9% NaCl at 4ºC. Tissues were homogenized using a PRO250-Sawtooth Blade Homogeniser at 4ºC. The tissue homogenate was centrifuged at 6,000x*g* (JA-20 Rotor, 7,000 rpm) for 15 min at 4ºC. The supernatant was transferred to a clean tube and spun at 10,000x*g* (JA-20 Rotor 9,250 rpm) for 20 min at 4ºC. The supernatant was then filtered through a membrane filter series (5µm, 0.45 µm and 0.2µm), snap frozen in liquid nitrogen in 1.5 ml aliquots and stored at –80ºC until use.

RNA extraction
The procedure for RNA extraction was standardised by placing 200 µl aliquots of inoculum into 2 ml screw capped, o-ring sealed tubes with 2 x 3mm and 1 x 2 mm glass beads (acid washed and baked) and 900 µl TRIzol-LS™ (Life Technologies). The sample was beaten in a bead-beater (Biospec Products) for 2 minutes in an aluminium rack pre-cooled to -20°C. RNA was extracted following homogenisation in TRIzol-LS™ according to the manufacturer's instructions using the high salt precipitation step. RNA was quantified spectrophotometrically at A260nm. Up to 1.0 µg of total RNA was reverse transcribed using 200 Units (U) of Superscript III Reverse-Transcriptase (Invitrogen), 40 U of RNAse out (Invitrogen) and 2.5 µM random hexamer primers (pd(N)6) (Amersham) following manufacturer's instructions in a 20 µl reaction volume. When reverse transcribing RNA at least one negative reverse transcriptase control (neg RT) was processed.

The GAV load was quantitatively determined using a dual labeled probe with qPCR. A TaqMan probe was designed from the GAV (pG12) clone which contains the sequence of the ORF1 region in the GAV genome (Cowley et al. 2000a; de la Vega et al. 2004). Absolute quantification of the number of GAV RNA template copies ng-1 total RNA was determined by parallel qPCR amplification of the cDNA from the inoculum with the pG12 RNA standard curve described in de la Vega et al. 2004.

In vitro expression of "free" RNA
In order to determine the effects of disinfectants on RNA, "free" RNA was added to the prawn viral inoculum. To ensure that the free RNA that was not detectable in prawn tissue via PCR RNA from *Panulirus ornatus* was used. The "free" RNA was diluted to determine the amount to add to *in vitro* virus inactivation incubations for optimal detection via qPCR. 1 µg from two batches of "free" RNA was reverse transcribed with neg RT controls. The cDNA was then diluted 10 fold and detected via qPCR.

Stability of viral and "free" RNA
The stability of GAV and "free" RNA over optimal incubation periods was examined. In triplicate, 450 µl of the viral inoculum was diluted 1:2 with Nuclease free water (Ambion) for a final volume of 900 µl spiked with "free" RNA at 0.75 ng/µl inoculum. Samples were incubated at 25°C and 200 µl was removed at 0, 10, 20 and 60 mins, snap frozen in liquid nitrogen and stored at -80 °C. RNA was extracted and reverse transcribed and PCR was run for GAV and "free" RNA on the inoculum.

Inactivation of viral inoculum using disinfectants

The different chemical disinfectants and concentrations tested are listed in Table 1. The viral inoculum was diluted 1:2 with nuclease free water (Ambion) and spiked with "free" RNA at 0.75 ng/µl inoculum. To each of four replicate tubes of spiked inoculum a treatment at two concentrations (Table 1) was added including a control no treatment group. 300 µl of spiked inoculum from the control tube was removed at the start of the experiment, 200 µl for later RNA extraction and 100 µl for confirmatory whole animal bioassays, these were immediately snap frozen in liquid nitrogen and stored at -80°C. Samples were vortexed for 10 seconds then incubated at 37°C for 30 mins.

Table 1. List of disinfectant treatments and concentrations that were investigated against a prawn viral inoculum containing GAV

Treatment	Concentration	Mode of Action
Iodine	0.1 ppm, 0.3 ppm	Halogen
Povidone Iodine (Betadine)	0.1 ppm, 0.3 ppm	Halogen
Formalin	100 ppm, 200 ppm	Aldehyde
VirkonS	10 ppm, 40 ppm	Oxidising Agent
Triton X 100	1%, 2%	Detergent
tri-n-butyl phosphate (TNBP)	0.3%, 0.6%	Solvent
Sodium Dodecyl Sulfate (SDS)	0.025%, 0.050%	Detergent
Lipase (*Candida rugosa*)	0.5 mg/ml, 1 mg/ml	Enzyme
Trypsin	0.1 mg/ml, 1mg/ml	Enzyme
Proteases (Porcine Pancreatic)	0.1 mg/ml, 1mg/ml	Enzyme

Following the disinfection treatments the number of GAV template copies detected per microlitre of inoculum treated was recorded.

5.7.2 Results

The quantity of GAV and MoV in the viral inoculum produced is shown (Table 2).

Table 2. Viral inoculum produced from *Penaeus monodon* infected with a GAV/MoV inoculum. Volumes and qPCR quantification results for both GAV and MoV.

Tissue source	Volume	GAV load Per ng RNA	GAV load per µl	MoV load per ng RNA	MoV load per µl
Gills	70 ml	4.65E+03	2.51E+05	1.64E+07	8.85E+08
Lymphoid organ	3 ml	2.63E+04	4.71E+06	1.36E+07	2.03E+09

The results revealed a steady decline in the quantity of "free" RNA remaining in the inoculum over the course of an hour (Fig 2).

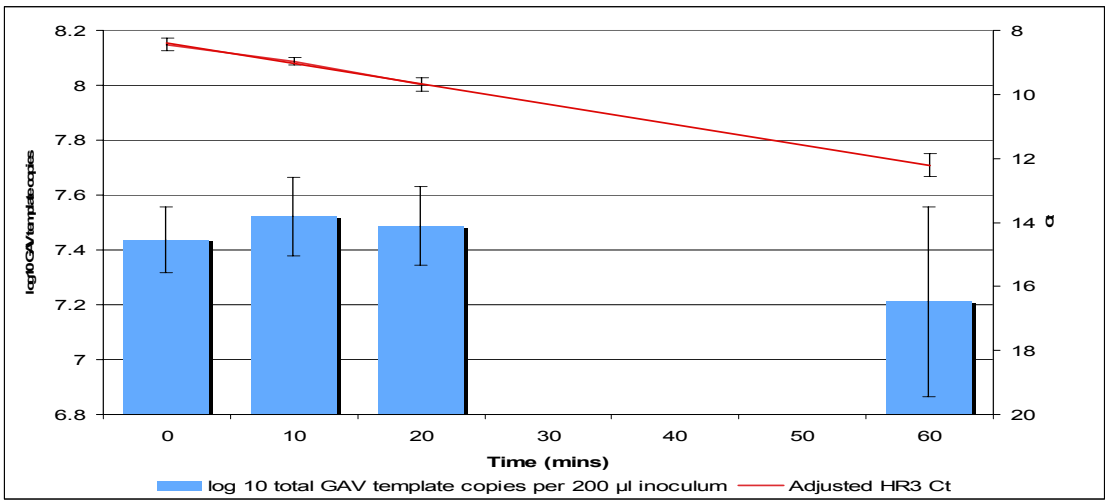


Figure 2. Mean and standard deviation of GAV (blue histograms) and "free" RNA (red line) in an inoculum incubated at 25°C for 60 minutes. Left axis represents the GAV copies per µl inoculum. The right axis represents the Ct value for the "free" RNA (HR3).

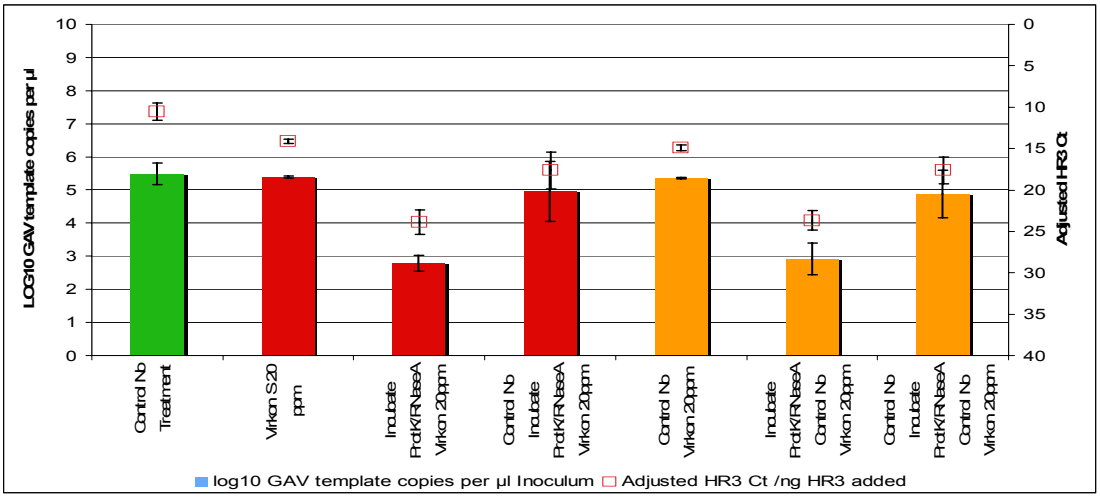


Figure 3. Mean and standard deviation of GAV and "free" RNA in viral inoculum following treatment with VirkonS at 20 ppm and a ProteinaseK/RNaseA treatment.

Combinations of incubation with VirkonS and an RNase A/Proteinase K treatment are shown after incubation for 60 minutes at 25°C (Fig. 3). Results show that treatment with VirkonS at 20 ppm did not destroy the GAV RNA in the inoculum. Incubation with ProteinaseK/RNaseA did have an effect however, regardless of whether it was pre-treated with VirkonS or not.

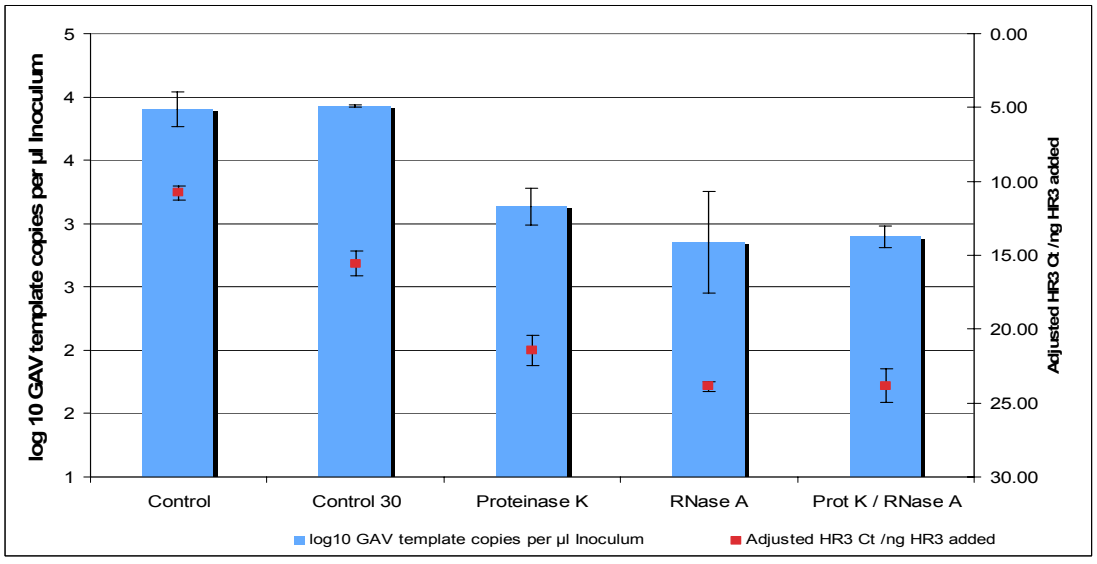


Figure 4. GAV and "free" RNA (mean and standard deviation) after 30 minutes incubation at 25°C following treatment with Proteinase K, RNase A or a combination of both.

Treatment with Proteinase K and RNase A incubated for 30 minutes without treating with a disinfectant showed that the "free" RNA was removed following RNase A treatment (Fig. 4). There was also destruction when only treated with Proteinase K, similar to a combined Proteinase K/RNase A treatment. GAV levels were lower although the viral RNA was not destroyed following incubation with Proteinase K or RNase A or a combination of both.

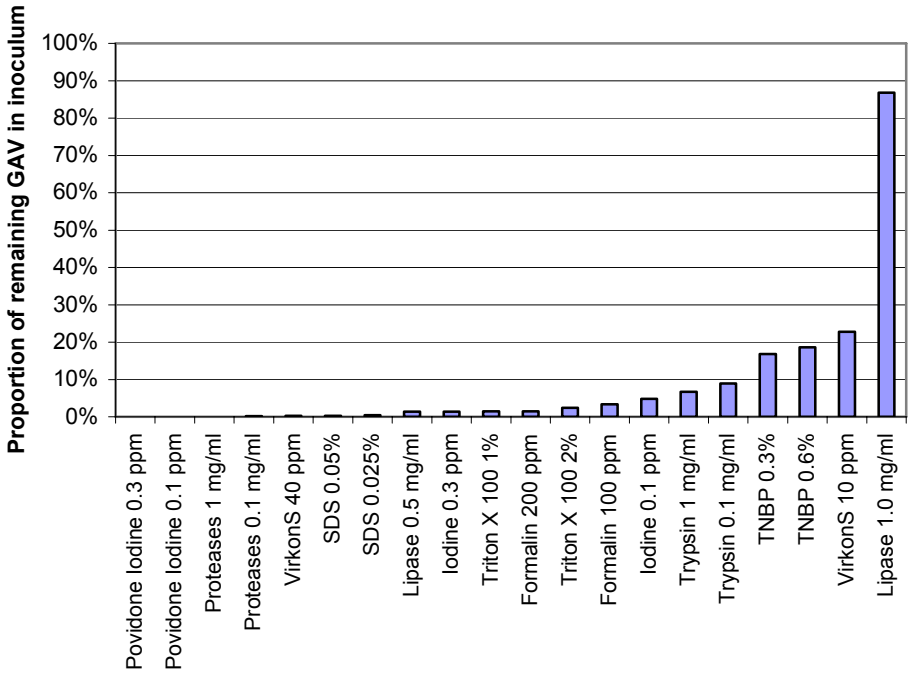


Figure 5. Ranked proportion of remaining GAV in inoculum compared to control tubes incubated under the same conditions, 4 replicates per treatment and concentration combination

There was pronounced variation in the amount of GAV remaining in the inoculum following incubation with the different chemicals. Povidone iodine and general proteases were the most effective at removing GAV from solution. Other products that worked well at either concentration tested were SDS and TritonX100. There was a large difference between the use of lipases at 0.5 and 1 mg/ml. At the higher concentration it appeared to preserve the GAV in the inoculum and offered no virucidal effect. VirkonS was effective at 40 ppm although reduced in its efficiency when tested at 10 ppm. The use of iodine in the unbound form was not as effective even though we used equivalent concentrations of active ingredient.

5.7.3 Discussion

The results indicate that Iodine, particularly Povidone iodine (Betadine) is effective for use in removing GAV from eggs. Iodine is a broad spectrum disinfectant that is also likely to reduce risks of infection from bacteria, protozoa and fungi. The concentration of iodine used in the experiment (0.1 and 0.3 ppm active iodine) was lower than concentrations that we found to be toxic to prawns eggs and larvae (3 ppm).

Sodium Dodecyl Sulfate (SDS) also worked well at the low concentrations tested. Previous studies have shown that, at these concentrations, SDS has low toxicity in cell culture (Howett et al. 1999). TritonX100 also worked, although it was not as effective as SDS. Both of these chemicals appear to have potential use for inactivating GAV.

Lipase treatment was effective at a low concentration (0.5 mg/ml), possibly via degradation of the viral envelope in GAV. However, increasing the lipase concentration two-fold to 1 mg/ml almost completely negated the effectiveness of the treatment. The reason for this effect is yet to be determined.

Trypsin was effective in inactivating GAV, with little difference in the effects of lower or higher concentration. A caution with using proteases would be to ensure that there is no risk of

increasing the infectivity of virus particles. For example, it was found that trypsin increased rotavirus infectivity by imparting order to spikes associated with the nucleocapsid that make virus entry into cells more efficient (Crawford et al. 2001).

The poor result with VirkonS against GAV was unexpected given the published results against a suite of viruses at much lower concentrations (Suphamat, 2000). Unpublished information from industry trials has indicated that 10 ppm VirkonS is toxic to prawn eggs. This would restrict its use at 10 ppm let alone 40 ppm, the only concentration that appeared to work well.

The reason for the poor performance of 10 ppm VirkonS is possibly due to the amount of organic matter in the inoculum. Cellular and bacterial contamination was removed via centrifugation but the inoculum was still rich in suspended organic matter. VirkonS oxidizes organic matter and under high organic loads the effectiveness of this treatment is severely reduced. The results indicate that for VirkonS to be effective it would be essential to remove organic matter by washing eggs prior to their treatment.

Tri-n-butyl phosphate (TNBP) was less effective than other treatments, apart from VikonS at 10 ppm and lipase at 1 mg/ml.

In summary, with the exception of lipase at 1 mg/ml, all of the treatments tested had significant effects on inactivating GAV *in-vitro*. Among the treatments tested Betadine at 0.1 ppm appeared to offer the best potential. This finding is consistent with the recommendations of the OIE International Aquatic Health Code for washing eggs of Penaeid prawns to reduce the transmission of pathogenic organisms in hatchery systems (see web page: http://www.oie.int/eng/normes/fmanual/A_00014.htm, Methods For Disinfection Of Crustacean Farms). The protocol relies on the use of 100 ppm (0.01%) formalin for 1 min and 0.1 ppm (0.00001%) of an iodophore (e.g. Betadine) for 1 min and is based on results from (Brock & Bullis, 2001; Chen et al. 1992).

5.8 The effects of four different virucidal chemicals on hatch rates of eggs determined

5.8.1 Methods

Broodstock

Female (10) and male (10) wild *Penaeus monodon* broodstock were captured off the coast of Cairns (Bill Izzard). Animals were maintained in the AIMS Maturation Facility and held at 28°C. Females were unilaterally eyestalk ablated and mating was allowed to occur naturally. In total four different spawnings were used in these experiments.

Hatching and Counting of Eggs

On the morning after a spawn event, the AIMS hatching system was filled with fresh seawater maintained at 28°C for AIMS hatching trays or as a water bath for experimental hatching containers. To obtain sufficient replicates, these experimental hatching containers were 600 ml circular take-away containers (Cast Away, CA-MR). In each experiment prior to use, 450 ml of 0.2 µm filtered fresh seawater was added to hatching containers (Fig. 6).

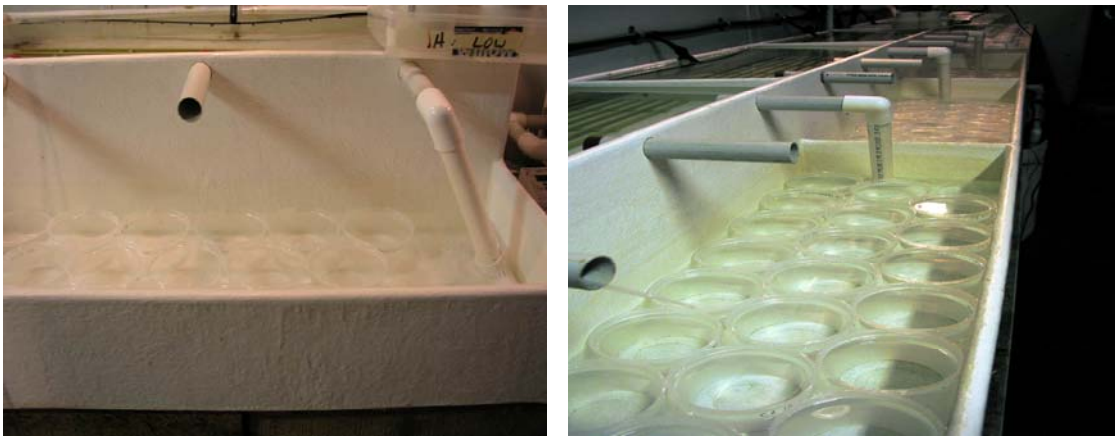


Figure 6. Circular 600 ml take-away containers (hatching containers) in the heated water bath for hatching vessel trials and egg washing experiments.

Each egg batch from females that spawned the previous night were collected in the morning and gently washed with seawater in a 5L beaker made up to 4L with fresh seawater. Moderate aeration was provided to gently mix the eggs and 5 replicate 2 ml egg samples were collected and immediately counted.

Egg Toxicity Washing Experiment

A total of 60 hatching containers were prepared with 450 ml of 0.2 μm filtered fresh seawater for each treatment replicate and wash times (Fig. 7).

The trial ran twice. Eggs were collected the morning after spawning, prepared and counted as described above. A total of 5 treatments were used (including control) with volumes required for 1L treatment shown (Table 3). A stock concentration of Sodium Dodecyl Sulphate (SDS) (ICN, Biomedicals) of 5% was made by adding 2.5 g of SDS to 50 ml MilliQ water. A stock concentration of pancreatin (from porcine pancreas) (Sigma) of 5% was made up by adding 2 g of pancreatin to 40 ml phosphate buffered saline (PBS) (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4 and 0.24g KH_2PO_4 made up to 1L with MilliQ H_2O, pH to 7.4). A stock solution of Betadine (100 ppm) (Mundipharma) was made by adding 100μl of Betadine (1% w/v available iodine or 10,000 ppm available iodine) in 9.9 ml of MilliQ H_2O. TritonX100 (t-octylphenoxypolyethoxyethanol, Sigma) and tri-n-butyl phosphate (TNBP) (Sigma) were used at original concentrations.

Table 3. List of treatments and concentrations with volume required in 1000 ml treatment volume.

Treatment	Final volume	Stock concentration	Volume to add
Control	1000 ml	-	-
TritonX100 1%	1000 ml	100%	10 ml
and TNBP 0.3%		100%	3 ml
Sodium Dodecyl Sulphate (SDS) 0.025%	1000 ml	5%	5 ml
Pancreatin (0.1 mg/ml)	1000 ml	5%	2 ml
Betadine 0.1 ppm	1000 ml	100 ppm	1 ml

A total of 4 replicates for each treatment were used for each egg batch. Twenty treatment containers (Cospak jars 2.5 litre) were filled with 818 ml of 0.2 μm filtered fresh seawater (Fig.

7). The treatment was added to each container in the concentrations/volumes described in Table 2. Containers were mixed well in preparation for adding the eggs.

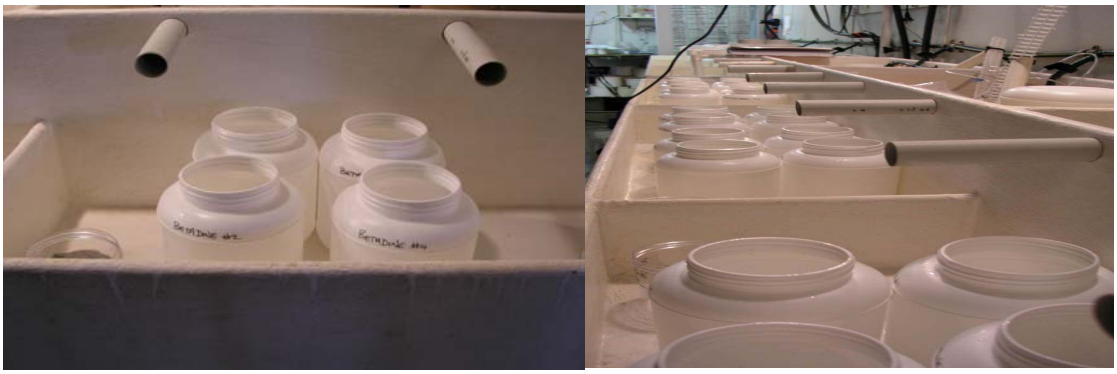


Figure 7. Treatment container (2.5 L Cospak jar) lay out with 4 replicates per treatment.

Eggs from each treatment were removed and washed at 5, 10 and 30 minutes. After mixing the samples well by swirling for 5 seconds, 300 ml from each treatment replicate were collected at each time point (5, 10 and 30 min) onto a 150 µm mesh-screen. All eggs (including control treatment) were washed thoroughly for 10 to 20 seconds in flowing fresh seawater (28°C) by swirling and lifting the screened container up and down until all signs of the treatment were gone (for example, the detergent treatments were washed until the bubbles dissipated). The eggs were then transferred to the 600 ml hatching containers in the water bath.

The eggs were left to hatch in the system with each hatching container mixed gently every 2 hours to ensure adequate oxygen. Approximately 17 hours post spawning the hatched nauplii and remaining eggs in each container were concentrated on a 150 µm mesh-screen and stored for later counting as previously described.

Egg and nauplii counting

Five replicate 1 or 2 ml samples were taken from each 50 ml sample volume for replicate total egg and nauplii counts. These were used to estimate a true hatch rate per sample based on the number of nauplii and remaining unhatched eggs. Predicted hatch rate based on the ratio of counted nauplii against volumetric extrapolation of sample egg count from the original total egg count were measured (Fig. 8).

$$predicted\ egg\ count = volume\ of\ sample\ (ml) \times \left(\frac{total\ egg\ count}{total\ egg\ volume\ (ml)} \right)$$

Figure 8. Equation describing the predicted egg counts per sample

The predicted and true hatch rates from each hatching vessel were compared using a paired *t* test (JMP software). The counts and hatch rates from all hatching containers and AIMS hatching trays were compared using analysis of variance followed by a pair wise comparison (Tukey-Kramer) when a difference ($p<0.05$) existed (JMP software). The effects of egg washing treatments, wash time and count replicates on true hatch rate were compared.

5.8.2 Results

Egg treatment experiment

There was no effect of wash time on the control treatments (Figs 9 and 10). Some of the treatments had a negative effect on the hatch rate when compared to the control treatments for both egg batches (Figs 9 and 10). The fertility of eggs from the second batch of washed eggs was much lower, reducing hatch rate in all treatments including the control. When the overall hatch rate was low the effects of the treatments were more subtle.

Washing the eggs with SDS caused an almost linear reduction in hatch rate with increasing wash time. Washing with TritonX100 and TNBP for more than 10 minutes caused a significant reduction in hatch rate. Washing the eggs with pancreatin (0.1 mg/ml) or Betadine (0.1 ppm) for up to 30 minutes did not significantly change the hatch rate from the control treatments.

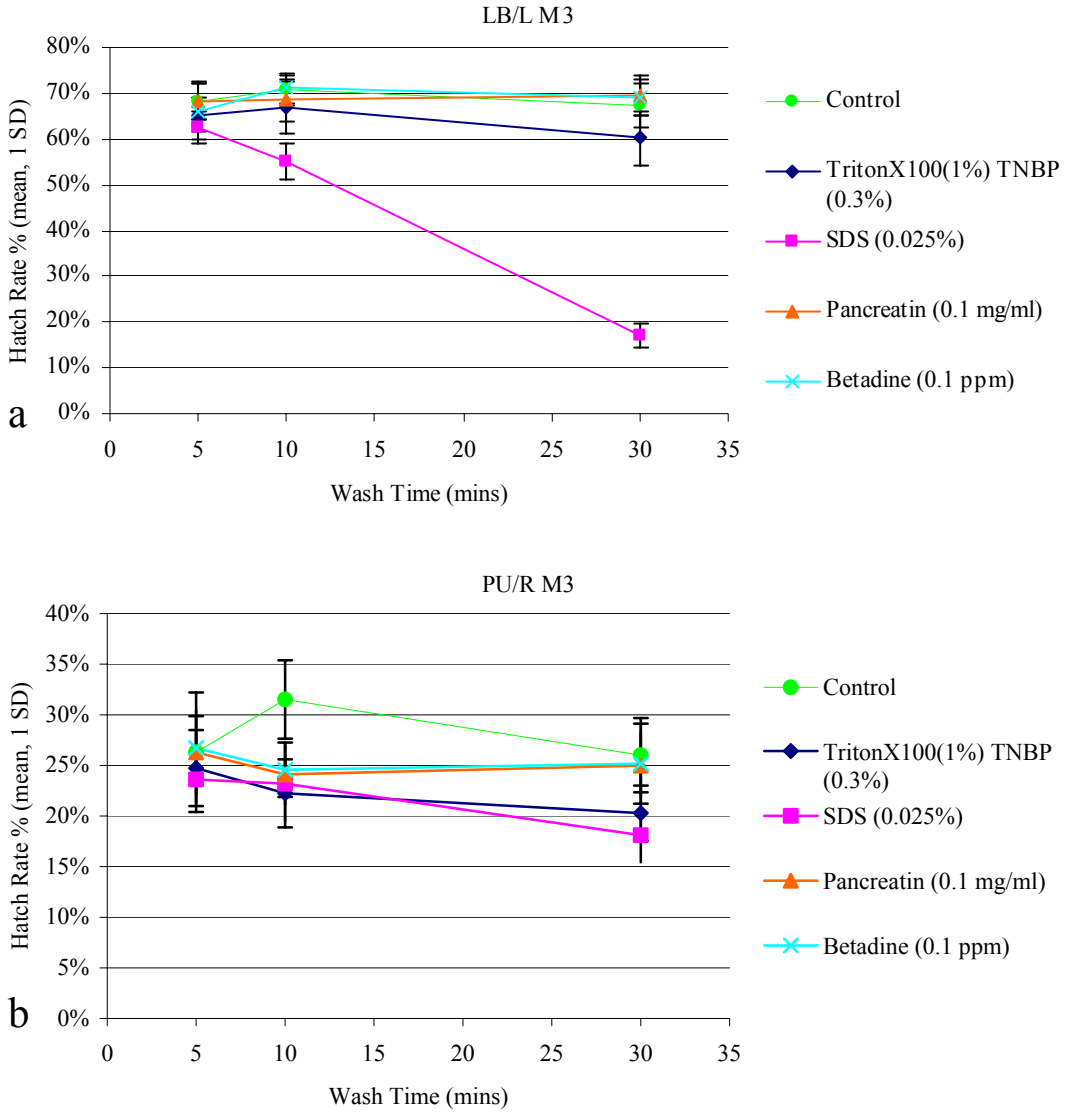


Figure 9 a,b. Mean hatch rates (±1 standard deviation) by treatment from two egg batches (a, LB/L M3 and b, PU/R M3) shown against wash time (5, 10 and 30 minutes).

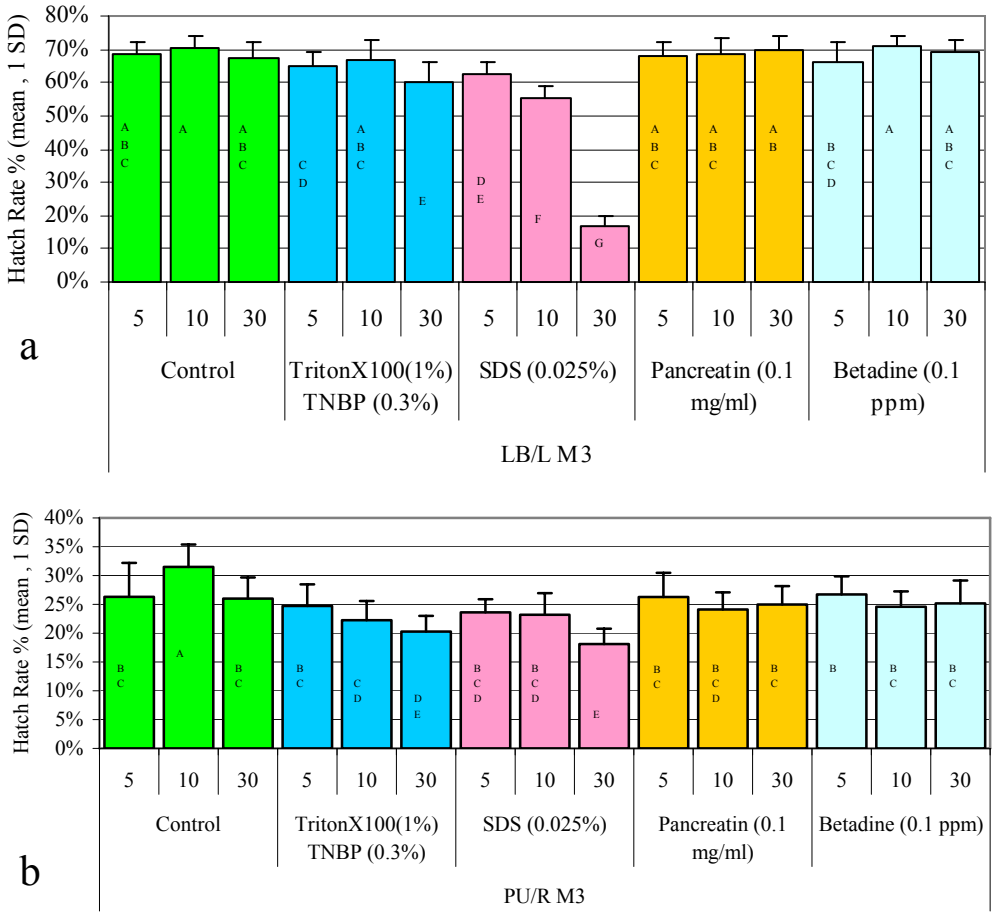


Figure 10 a,b. Mean hatch rates (±1 standard deviation) from two egg batches (LB/L M3 and PU/R M3) grouped within treatment and wash time (minutes) comparing treatments. Three way analysis of variance and paired mean comparison (Tukey-Kramer) showing significantly ($p<0.05$) different treatment/wash times not connected by a letter.

As there is substantial batch to batch variation in egg hatch rates in *P. monodon*, single batches of eggs were used to test all treatments and wash times. The large scale of the experiment meant that a large number of hatching vessels (60) were required, more than the capacity of the AIMS hatching system. Small 600 ml takeaway containers were used to hatch the eggs following wash treatment. The smaller volume of water used for hatching prawn eggs raised concerns over the amount of oxygen required in each container. Previously, large salmonid eggs were maintained in Petri dishes, up to 42 in 50 ml for 64 days without oxygen levels in the water reaching critically low levels (Wedekind & Mueller, 2004). In comparison to the 600 ml containers this is equivalent to keeping 504 eggs in 600 ml. We looked at a much higher egg load although our incubation period was much lower (around 7 hours). Hatch rates from small hatching containers were slightly lower than the conventional AIMS hatching trays. The high water circulation and large screen surface area may have resulted in the higher hatching rate. As the controls were kept under the same conditions it was acceptable that hatch rates were marginally lower. Experiments using large systems are impracticable when testing large numbers of treatments at once. The sacrifice of a slightly lower hatch rate is worth the high throughput experimental design.

Leaving eggs in the treatment containers for between 5 and 30 minutes did not appear to significantly reduce hatch rates and any reduction in hatch rate seen through time were due to the treatments alone.

When comparing hatch rate estimates the variation associated with predicted values was due the volumetric measuring of egg samples. Although, an effort was made to mix samples well and aliquot the same volume to each treatment replicate the amount of error was still high. For this reason hatch rates based on the extrapolating egg counts from the volume and original total egg counts will always be more variable.

5.8.3 Discussion

Previous *in-vitro* assays demonstrated that Betadine (Povidone iodine) and pancreatin were the most effective chemical means of inactivating GAV and MoV. The results of the egg washing experiments demonstrated that Betadine and pancreatin also had the most benign effect on hatch rates. Betadine is already described by OIE as a treatment for washing prawn eggs. The treatment time is short, only 1 minute. SDS was effective in destroying GAV, however during the later incubation periods (10 and 30 minutes) the hatch rate was significantly reduced. Although TritonX100/TNBP performed well there was a reduction in hatch rate after 30 minutes of treatment.

The difference in the results between the two egg batches used showed us that the overall hatch rate changed the effect of the treatment. When the hatch rate was high the negative effect of the treatment was more exaggerated. This identifies one of the original concerns about testing treatments with different batches of eggs. If the lower hatch rate had been used to test SDS, the negative effect on hatch rate after treatment for 30 minutes would not have been seen.

If costs of treatment are compared, the lease expensive is Betadine (AUS$0.13 per 100 litres) and the most expensive Triton X100 TNBP (AUS$133 per 100 litres).

Pancreatin is less expensive than TritonX100/TNBP (AUS$3.50 to 7.00 per 100 litres) and performed almost as well as Betadine. During the *in vitro* inactivation trial an incubation temperature of 34°C was used but in the egg washing experiment, water temperature was maintained at 28°C. How the difference in temperature and buffering capacity of seawater altered the efficiency of the enzymes has not been investigated.

Further studies are needed to determine the optimal treatment times and water temperatures for viral inactivation using Betadine and pancreatin. Once minimum treatment times and optimal temperatures have been determined these chemicals can be used by hatcheries to routinely wash eggs and reduce the risks of GAV being vertically transmitted.

The effects of egg washing treatments have not been tested on later larval stages and this needs to be considered before integrating these treatments into hatcheries.

The effectiveness of different egg washing treatments vary according to the organic load in the water. VirkonS and ozone may be suitable for use as a virucidal agents at concentrations that are not toxic to developing larvae, if the organic load in the water is managed.

6. CHAPTER 7. PRECISE LOCATION OF GAV AND MOV

Precise location of GAV and MoV in *Penaeus monodon* gonads, eggs, larvae and postlarvae using fluorescence techniques.

6.1 Introduction

The objective of this component was to use fluorescent *in situ* hybridisation (FISH) as a technique to determine the precise location of GAV and MoV virus particles in *Penaeus monodon* gonads, eggs, larvae and postlarvae. Determining the location of these viruses is important to understanding their modes of transmission and the potential effectiveness of egg washing with or without virucidal compounds. Egg washing can only be effective if the source of the infection is external to the gametes, for example in the spermatophore, seminal fluid or ovarian fluids.

Following a review of available viral detection techniques, the use of fluorescent *in situ* hybridisation using an RNA probe for the identification of viral particles on or within *P. monodon* reproductive tissues, developing embryos, larvae and postlarvae was examined.

In situ hybridisation with RNA probes has been used to detect specific RNA within cells down to low copy numbers (eg. 20-30 copies) (Braissant & Wahli, 1998; Chen et al. 2002; Munoz et al. 2002; Seidman, 2003). Fluorescent labelled probes are used to amplify a signal detected using *in situ* hybridisation with a reported two fold increase in sensitivity compared to bright field microscopy and colorimetric labelling techniques (Egger et al. 1999). Fluorescent *in situ* hybridisation (FISH) involves hybridisation of biotin or digoxigenin (DIG) labelled probes to prepared specimens and visualisation of those probes with fluorochrome-conjugated reagents. This type of non-radioactive method is popular because it is safer for the user, produces faster results, labelled probes are more stable, and the results are more consistent (Relf et al. 2002).

GAV and MoV are both RNA viruses, GAV is positive stranded RNA or sense strand. MoV is a negative stranded RNA virus or anti-sense strand. RNA probes against GAV and MoV form RNA-RNA hybrids which are more stable than DNA-RNA hybrids. In addition, non-specific tissue signal can be removed after hybridisation with ribonucleases (RNAse) since RNA duplexes are resistant to degradation (Jones et al. 2003; Seidman, 2003). To further increase sensitivity long probes (~600 bp) were designed that incorporate more label (e.g. DIG).

6.2 Methods

Samples

Initial samples of eggs and nauplii were from naturally mated female *P. monodon* collected from the Cairns region. Eggs and nauplii were also collected from ***P. monodon*** breeding lines that contributed to the families produced as part of this project (Chapter 1). Females were spawned using standard AIMS maturation and spawning protocols. Fifteen day old postlarvae (PL15) were collected from AIMS as a component of the reproductive assessment trials (Chapter 1).

Sampling

The tissues and developmental stages that were fixed and processed for PCR and *in situ* hybridisation are listed in Table 2. Whole cephalothorax of broodstock were fixed using Davidson's fixative, injecting and immersing heads prior to further processing (Bell & Lightner, 1988). A small sample of gill tissue was snap frozen in liquid nitrogen. Eggs and nauplii were collected from AIMS spawnings. Subsamples were preserved for later RNA extraction using RNA*later* (Ambion) and stored at -20°C. Samples were fixed for transmission electron microscopy using 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at 4ºC for 20 hours. Following fixation the samples were washed three times in cold 0.1M sodium cacodylate buffer before storage at -20ºC in 0.1M cacodylate buffer and 100% ethanol (1:1) until further processing. The remaining sample was fixed for FISH in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4ºC for 4 hours. Following fixation the sample was washed three times in cold 1xPBS before storage at -20ºC in 1xPBS and 100% ethanol (1:1) until further processing. Postlarval samples were also collected in a similar fashion using 10 larvae per sample per fixation method.

Table 1. Tissues that were collected for *in situ* hybridisation including tissues that were screened using PCR for GAV and MoV.

Tissue	Tissue in RNAlater	N	RNA	cDNA	Glutaraldehyde	Paraform-aldehyde	Davidson's Fixative
Egg	50	-	50	50	24	50	-
Nauplii	27	-	27	27	19	27	-
Protozoea	8	-	8	8	7	8	-
Mysis	4	-	4	4	3	4	-
PL 15	36	69	105	60	4	5	12
Gills	5	30	35	35	-	-	
Cephalothorax	-	-	-	-	-	-	35

N= tissue in liquid nitrogen

RNA extraction

Tissues were removed from RNA*later* and placed into 2 ml screw capped, o-ring sealed tubes with 2 x 3mm and 1 x 2 mm glass beads (acid washed and baked) and 900 µl TRIzol-LS™ (Life Technologies). The sample was beaten in a bead-beater (Biospec Products) for 2 to 5 minutes in an aluminium rack pre-cooled to -20°C. RNA was extracted following homogenisation in TRIzol-LS™ according to the manufacturer's instructions using the high salt precipitation step. RNA was quantified spectrophotometrically at A260nm. Up to 1000 ng of total RNA was reverse transcribed using 200 Units (U) of Superscript III Reverse-Transcriptase (Invitrogen), 40 U of RNAse out (Invitrogen) and 2.5 µM random hexamer primers (pd(N)6) (Amersham) following manufacturer's instructions in a 20 µl reaction volume. When reverse transcribing RNA at least one negative reverse transcriptase control (neg RT) was processed.

The GAV load was quantitatively determined using a dual labelled probe with real qPCR. Absolute quantification of the number of GAV RNA template copies ng^{-1} total RNA was determined by parallel qPCR amplification of the cDNA from the inoculum with the pG12 RNA standard curve described in (de la Vega et al. 2004). The MoV load was quantitatively determined using a dual labelled probe with qPCR. MoV conventional 1- and 2-step nested PCR was performed on cDNA as described by Cowley et al. (2005).

Histological Processing

Initially, 5 µm serial sections were received (Russell McCulloch, CLI) of a prawn head with hepatopancreas, lymphoid organ, nerve tissue and associated tissues. The embedding of all

tissues followed routine histological procedures. Egg and nauplii samples were initially embedded in 1% agarose in DEPC treated water (Sigma) and dehydrated in 100% methanol for 12-24 hours prior to further histological processing.

Probe synthesis
A template for transcription for RNA probes was synthesised by re-designing conventional primers for GAV (Cowley et al. 2000) and MoV (Cowley et al. 2005). The resulting DNA template was used to transcribe RNA probes, both sense and anti-sense strands. The RNA probes were validated using RNA extracted from viral inoculum prepared from the lymphoid organ of moribund *P.monodon* held at AIMS containing 2.36E+07 GAV viral copies and 1.22E+10 MoV viral copies per µl of inoculum.

Following incubation of the samples, the hybridisation solution was removed and the membrane was immediately washed in 2xSSC containing 0.1% SDS. The membrane was incubated at room temperature for 5 minutes with shaking. Washing in 2xSSC containing 0.1% SDS was repeated. The membrane was then washed twice for 15 minutes in 0.1xSSC containing 0.1% SDS preheated to 68°C. Colorimetric detection using NBT/BCIP (Roche) was as per manufacturer's instructions. Fluorescent detection using HNPP/Fast Red (Roche) was as per manufacturer's instructions.

Hybridisation
Slides were washed and incubated in hybridisation solution (2X SSC (Sambrook et al. 1989), 50% formamide, 10% dextran sulphate, 0.01% sheared salmon sperm DNA (Sambrook et al. 1989), and 0.02% SDS.

The Dig-labelled RNA probes were resuspended to a final concentration of 350 ng/ml in hybridisation solution, applied to sections and denatured at 95 °C for 4 minutes followed by a 4 hour incubation at 55 °C in a humid chamber. Sections were washed overnight at 55 °C in 2x SSC, followed by three washes at 55 °C with 50% deionized formamide in 1x SSC for 20 minutes, and two washes in 1x SSC at room temperature for 15 minutes. Fluorescent detection with anti-DIG alkaline phosphatase antibody conjugate (Roche) was carried out with HNPP (2-hydroxy-3-naphtoic acid-2'-phenylanilide phosphate) Fluorescent Detection System (Roche) and colorimetric detection with NBT/BCIP (Roche) according to the manufacturer's instructions. Tissue sections were counterstained with DAPI (4′,6-diamidino-2-phenylindole,dihydrochloride) (0.1µg/ml in PBS for 5 minutes) nucleic acid stain for the fluorescent detection. A coverslip was mounted using an aqueous mounting medium with anti-fading agents (ProSciTech). Tissues were counterstained with 1% Bismarck Brown (2.5 mins) for the colorimetric detection dehydrated through an ethanol series and mounted in a solvent based resin, Mount Quick (ProSciTech). Slides were examined with an Axioskop 2 mot plus microscope (Zeiss) fitted with and Axiocam MrC5 digital camera and Axiovision 4.1 imaging software (Carl Zeiss Vission GmbH). HNPP/Fast Red was detected by fluorescence microscopy using a far blue filter (FITC filter) which emitted a signal in a wide range between 540–590 nm with a maximum at 562 nm.

6.3 Results

Initial screening for GAV and MoV of tissue samples resulted in very few strongly positive egg and larval samples. Tissues that were strongly positive for GAV or MoV were used for viral detection using *in situ* hybridisation.

The initial blot hybridisation with MoV and GAV with colorimetric and fluorescence detection methods showed that the RNA probes were able to detect both GAV and MoV in the prepared viral inoculum down to 4.72E+04 GAV viral copies and 2.44E+07 MoV viral copies (Table 3).

Fluorescence detection of the probes was more sensitive than colorimetric through the dilution series for GAV but equivalent for MoV.

Table 2. Detection of MoV and GAV using single stranded RNA probes on a serial dilution of a viral inoculum prepared from lymphoid organ tissues of *Penaeus monodon*. Comparison of colorimetric (NBT/BCIP) versus fluorescence (HNPP/Fast Red) detection methods. Shaded cells indicate positive signal.

		Dilution series 10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
	GAV viral copies	4.72E+07	4.72E+06	4.72E+05	4.72E+04	4.72E+03
	MoV Viral copies	2.44E+10	2.44E+09	2.44E+08	2.44E+07	2.44E+06
NBT/BCIP	GAV5 sense probe					
	GAV6 anti-sense probe					
	MoV25 anti-sense probe					
	MoV24 sense probe					
HNPP/Fast Red	GAV5 sense probe					
	GAV6 anti-sense probe					
	MoV25 anti-sense probe					
	MoV24 sense probe					

The complementary strand RNA probes that were designed for GAV and MoV showed differential binding to the viral RNA in tissues. When the viral inoculum was initially probed the anti-sense strand GAV6 probe and sense strand MoV24 probe bound. For GAV the anti-sense probe is the complementary strand as it is a single stranded positive (sense) strand virus (Cowley et al. 2002). MoV is also single stranded, however the mature virus particle is a negative (anti-sense) strand and therefore a sense probe is the complementary strand.

Staining methods were compared using control positive slides from using each RNA probe with colorimetric and fluorescence detection systems.

Colorimetic staining

With colorimetric staining (NBT/BCIP) tissues probed positive for GAV anti-sense probe and MoV sense probes only (Figs 1 d,f,j and m). Using the GAV6 anti-sense probe a strong positive reaction was seen in lymphoid organ tissues almost exclusively within spheroids (Figure 1 d and f). Across the sectioned hepatopancreas only one cell probed faintly positive. Using the MoV24 sense probe lymphoid organ tissues stained strongly positive (Figs 1 j and m). Unlike the GAV infection, tubule lumen and interstitial tissues also probed positive. Other tissues that probed positive were the epithelial tissues associated with the exoskeleton and connective tissues.

Fluorescence staining

Fluorescence detection (HNPP/Fast Red with DAPI) resulted in the same tissues probing positive as described for colorimetric detection. In most cases a more intense signal was seen using fluorescence detection as well as additional tissues probing positive for GAV and MoV (Figs 1 a,b,c,e,g,h,i,k,n).

GAV5T7 (sense) and MoV25 (antisense) probes faintly stained tissues in locations where their alternate probes were strongly positive (Fig. 1 l). Using the GAV5 sense probe there was feint signal in the spheroids of the lymphoid organs. The GAV6 anti-sense probe staining was similar to colorimetric staining however there were isolated sites of positive staining associated with tubule matrix as well as within the lumen. A strong reaction was also seen in the hepatopancreas associated with hepatopancreacytes. The MoV25 anti-sense probe reacted faintly with lymphoid organ tissues associated with nuclei more than other tissues (Fig. 1 l). The MoV24 sense probe displayed a strong reaction in lymphoid organ as described for colorimetric staining. With MoV, the probe is not reacting to every spheroid as intensely as described with

GAV, instead there is more uniform staining of all tissues of the lymphoid organ though. This gives the tissue the appearance of being dirty. Other tissues that stained positive were the epidermal tissues associated with the exoskeleton, tissues surrounding an arterial vessel and probe positive interstitial tissues surrounding hepatopancreas tubules. There was also signal associated with connective tissues underlying strongly positive epidermal tissues. No non-specific staining of tissues was seen using a no probe control.

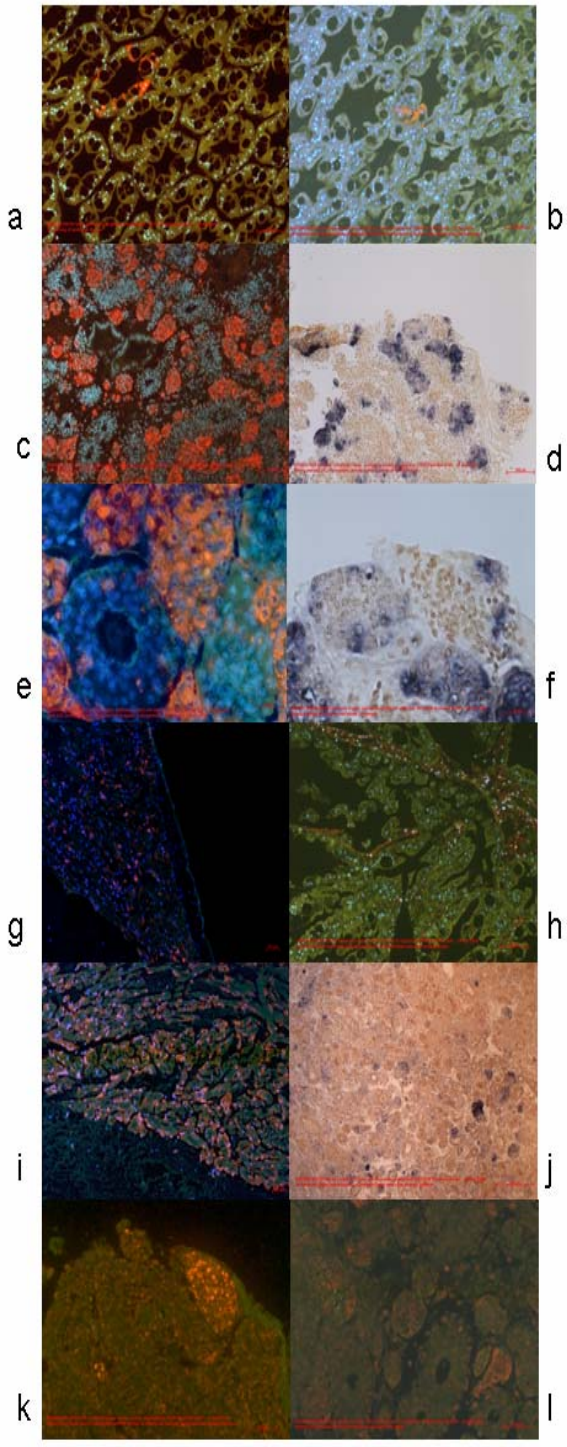
The egg samples were screened and FISH was done on PCR positive GAV samples. The weak signal that was detected using the GAV probes was very feint and observed outside the eggs associated with what looked more like debris than egg tissue (Figs 1 o to v). Confirmatory NBT BCIP staining of antibodies to GAV coat protein using the protocols at CSIRO Livestock Industries (Russell McCulloch) showed no signal in the eggs, even after staining tissues overnight with NBT/BCIP.

The postlarvae probed were from the *P.monodon* postlarval bioassay trial which were infected with GAV/MoV by feeding Artemia soaked in inoculum (see bioassay report). There was an infection in the lymphoid organ which was the most dominant site of infection (Figs 1 w, x). There was also very strong GAV signal along the midgut epithelial cells and in the hepatopancreacytes (Figs 1 y to dd).

In the male reproductive tissues GAV6 (antisense) probe for GAV was visible in the vas deferens tissues in focal regions only and at low intensity (Figs 1 jj, ll). The sense probe for GAV (GAV5) was visible at even lower intensity and in the same regions (Figs 1 ii, kk). Both sense and antisense MoV (MoV24 , MoV25) probes strongly reacted in the vas deferens (Figs 1 mm, nn, oo, pp,qq and rr). The sense probe was much more intense while the anti-sense probe was found in patches similar to the GAV probe.

In the females where a high MoV infection was recorded in the gills, the ovarian tissues showed only a weak signal for MoV using the probe (MoV24) (Figs 1 eee to qqq). The MoV probe stained tissues positive in the central ovarian cavity or connective tissues surrounding the ovaries.

The male reproductive tissues showed a low level MoV infection in the testicular lobe associated with connective tissue and not directly associated with the luminal tubule or testicular cords (Figs 1 ee to hh). The medial vas deferens in all but one of the male tissue samples, was very strongly positive for MoV (Figs 1 ss to ddd). The tissues that were most affected were the columnar epithelial tissues. The cytoplasm is uniformly stained for MoV. MoV was also detected in the primary lumen of the vas deferens, the mature spermatozoa in the lumen and developing spermatophore were surrounded with MoV (Figs 1 bbb,ccc and ddd).



a & b. Hepatopancreacytes infected with GAV. (GAV6 antisense probe, HNPP/Fast Red and DAPI (x10)

c. Lymphoid organ spheroids infected with GAV. (GAV6 anti-sense probe, HNPP/Fast Red and DAPI) (x10)
d. Lymphoid organ spheroids infected with GAV. (GAV6 anti-sense probe NBT/BCIP and Bismarck Brown) (x10)

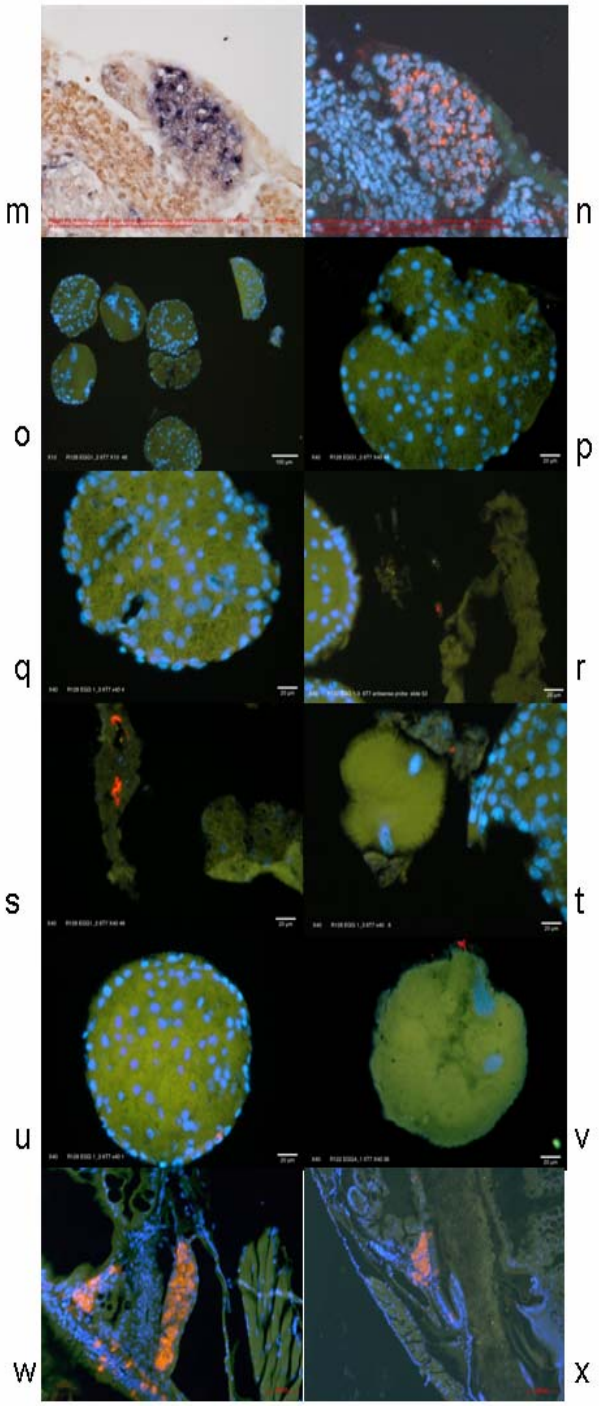
e. Lymphoid organ spheroids infected with GAV. (GAV6 anti-sense probe, HNPP/Fast Red and DAPI) (x40)
f. Lymphoid organ spheroids infected with GAV. (GAV6 anti-sense probe NBT/BCIP and Bismarck Brown) (x40)

g. heart muscle infected with GAV. (GAV6 anti-sense probe HNPP/Fast Red and DAPI)
(x10)
h. hepatopancreas interstitial tissues infected with MoV. (MoV24 sense probe HNPP/Fast Red and DAPI (x 10)

i. Heart muscle bundles infected with MoV. (MoV24 sense probe (HNPP/Fast Red and DAPI) (x10)
j. Lymphoid organ infected with MoV. (MoV24 sense probe NBT/BCIP and Bismarck Brown) (x5)

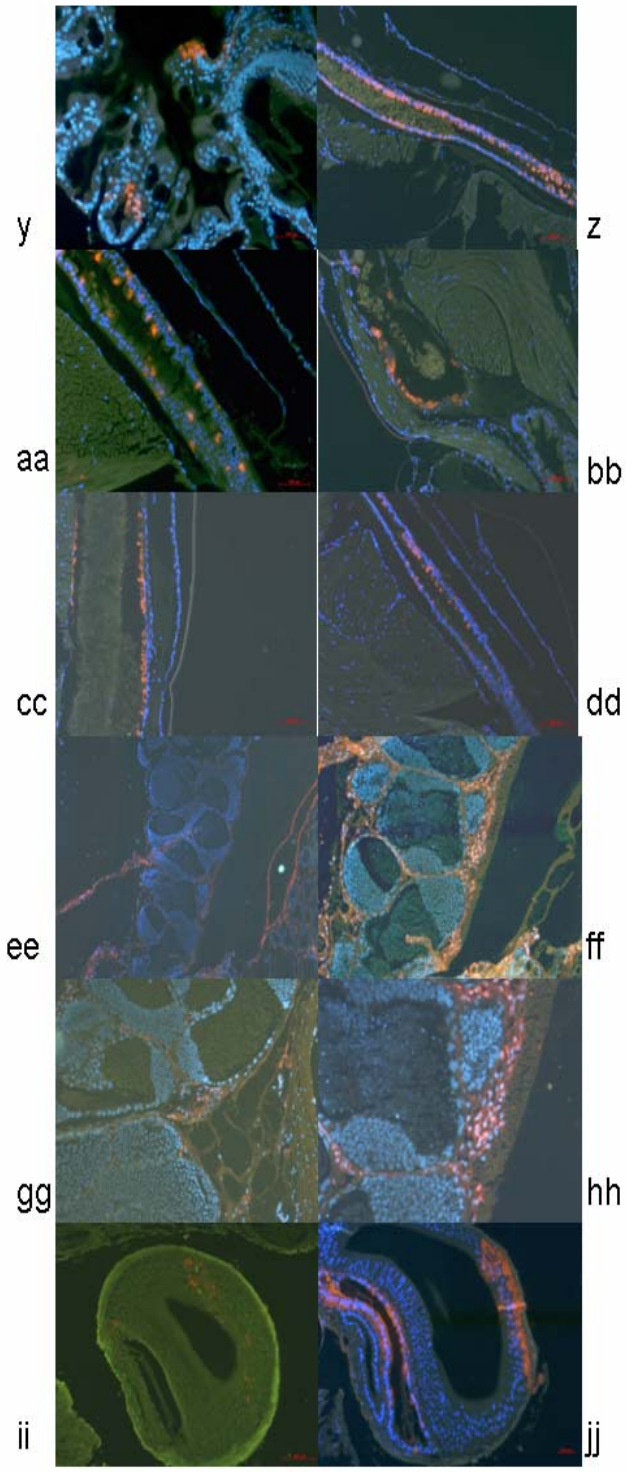
k. lymphoid organ infected with MoV. (MoV24 sense probe HNPP/Fast Red
R02-99) (x20)
l. Lymphoid organ infected with MoV. (MoV25 anti-sense probe HNPP/Fast Red(x20)

Figure 1 (a-l). *In situ* hybridisation (IHS) using RNA probes on *Penaeus monodon* broodstock and early life stages to detect GAV or MOV infections.



m. Lymphoid organ spheroids infected with MoV (MoV24 sense probe, NBT/BCIP and Bismarck Brown) (x40)
n. Lymphoid organ spheroids infected with MoV (same location as m). (MoV24 sense probe, HNPP/Fast Red and DAPI (x40)
o. Eggs with no signal (PCR +ve for GAV) (GAV6 anti-sense probe HNPP/Fast Red and DAPI) (x10)
p. Eggs with no signal (PCR +ve for GAV) (GAV6 anti-sense probe HNPP/Fast Red and DAPI) (x40)
q. Eggs with no signal (GAV6 anti-sense probe HNPP/Fast Red and DAPI) (x40)
r. Eggs (PCR +ve for GAV) with signal in debris (GAV6 anti-sense probe, HNPP/Fast Red and DAPI) (x40)
s. Eggs (PCR positive for GAV) with signal in debris (GAV6 anti-sense probe, HNPP/Fast
Red and DAPI) (x40)
t. Eggs (PCR +ve for GAV) with signal in debris and undeveloped egg. (GAV6 anti-sense probe, HNPP/Fast Red and DAPI) (x40)
u. Eggs (PCR +ve for GAV) with feint signal on the periphery of the egg (GAV6 anti-sense probe, HNPP/Fast Red and DAPI) (x40)
v. Eggs (PCR +ve for GAV) with feint signal in debris and undeveloped egg (GAV5 anti-sense probe, HNPP/Fast Red and DAPI) (x40)
w. Lymphoid organ and midgut epithelial tissue of postlarvae infected GAV. (GAV6 anti-sense probe, HNPP/Fast Red and DAPI) (x20)
x. Lymphoid organ of postlarvae infected with GAV. (GAV6 anti-sense probe, HNPP/Fast Red and DAPI) (x10)

Figure 1 (m-x). *In situ* hybridisation (IHS) using RNA probes on *Penaeus monodon* broodstock and early life stages to detect GAV or MOV infections.



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y. Hepatopancreacytes of postlarvae infected with GAV. (GAV6 anti-sense probe, HNPP/Fast Red and DAPI) (x20)
z. Midgut epithelial cells of postlarvae infected with GAV. (GAV6 anti-sense probe HNPP/Fast Red and DAPI) (x10)

aa. Midgut epithelial cells of postlarvae infected with GAV. (GAV6 anti-sense probe, HNPP/Fast Red and DAPI) (x20)
bb. Midgut epithelial cells of postlarvae infected with GAV. (GAV6 anti-sense probe, HNPP/Fast Red and DAPI) (x10)

cc. Midgut epithelial cells of postlarvae infected with GAV. (GAV6 anti-sense probe, HNPP/Fast Red and DAPI) (x20)
dd. Midgut epithelial cells of postlarvae infected with GAV. (GAV6 anti-sense probe, HNPP/Fast Red and DAPI) (x10)

ee. Connective tissue of testes infected with MoV. (MoV24 sense probe, HNPP/Fast Red and DAPI) (x5)
ff. Connective tissue of testes infected with MoV. (MoV24 sense probe, HNPP/Fast Red and DAPI) (x10)

gg. Connective tissue of testes infected with MoV. (MoV24 sense probe, HNPP/Fast Red and DAPI) (x10)
hh. Connective tissue of testes infected with MoV. (MoV24 sense probe, HNPP/Fast Red and DAPI) (x20)

ii. Epithelial tissue of vas deferens infected with GAV showing feint signal. (GAV5 sense probe, HNPP/Fast Red) (x10)
jj. Epithelial tissue of vas deferens infected with GAV showing feint signal (GAV6, HNPP/Fast Red and DAPI) (x10)

Figure1(y-jj). In situ hybridisation (IHS) using RNA probes on *Penaeus monodon* broodstock and early life stages to detect GAV or MOV infections

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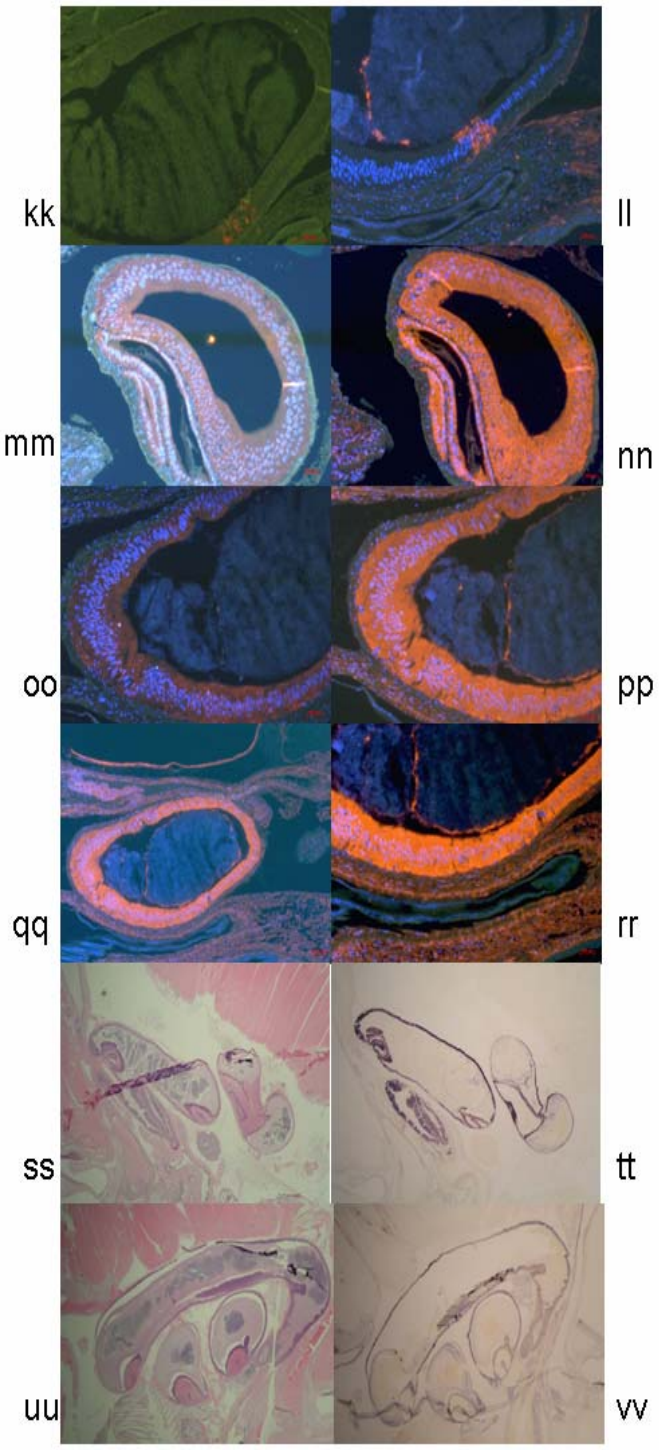


kk. Epithelia of vas deferens infected with GAV, feint signal (GAV5 sense probe, HNPP/Fast Red and DAPI x10)
ll. Epithelia, lumen, connective tissue of vas deferens infected with GAV. (GAV6 anti-, HNPP/Fast Red and DAPI x 10

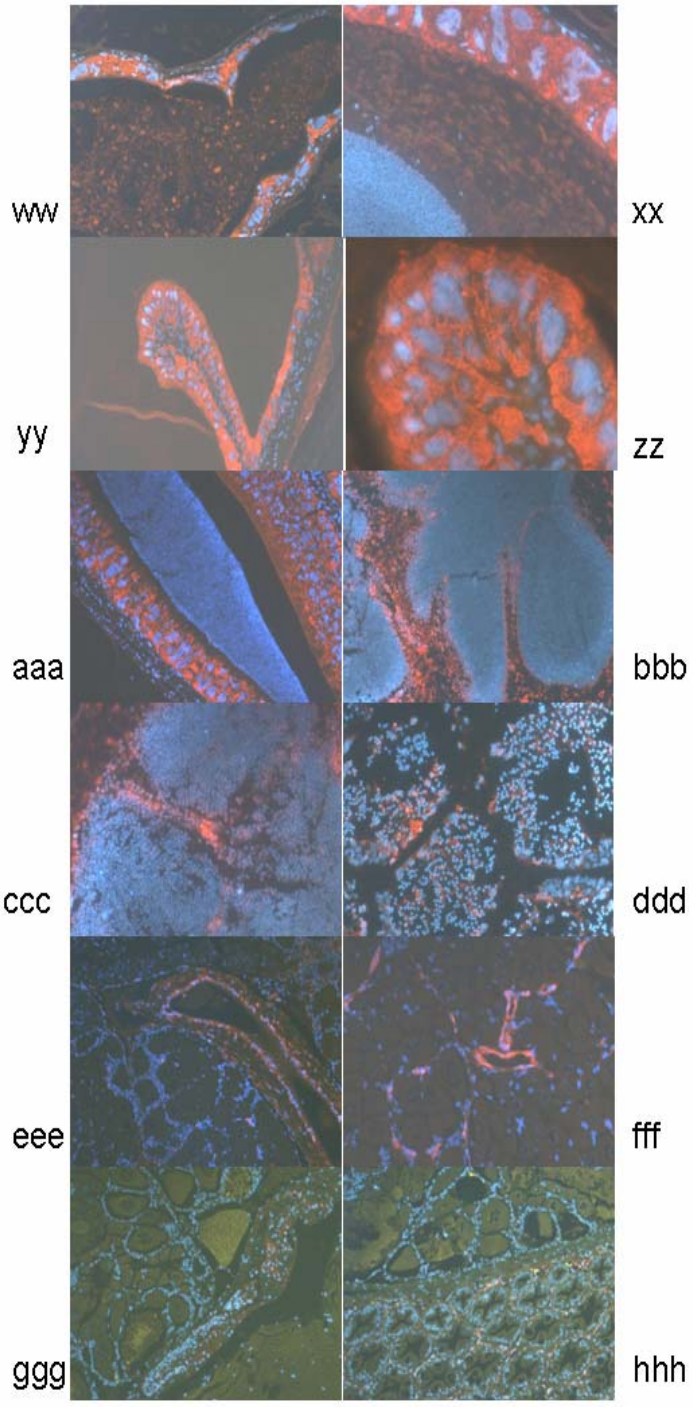
mm. Epithelia of vas deferens infected with MoV. (MoV25 anti-sense probe HNPP/Fast Red and DAPI x10)
nn. Epithelia of vas deferens infected with MoV. (MoV24 sense probeHNPP/Fast Red and DAPI x10)

oo. Epithelia of vas deferens infected with MoV. (MoV25 anti-sense probe HNPP/Fast Red and DAPI x10)
pp. Epithelia, lumen and connective tissues of vas deferens infected with MoV. (MoV24sense probe HNPP/Fast Red and DAPI) (x10)
qq. Epithelia, lumen and connective tissues of vas deferens infected with MoV. (MoV24sense probe HNPP/Fast Red and DAPI) (x5)
rr. Epithelia, lumen and connective tissues of vas deferens infected with MoV. (MoV24 sense probe HNPP/Fast Red and DAPI) (x20)

ss. H&E section of vas deferens **tt**. Epithelia of vas deferens infected with MoV showing signal to ss. (MoV24 sense probe NBT/BCIP and Bismarck Brown) (x5)

uu. H & E section of vas deferens (x5)
vv. Epithelia of vas deferens infected with MoV showing signal in corresponding to uu. (MoV24sense probe NBT/BCIP and Bismarck Brown) (x5)

Figure1(kk-vv). In situ hybridisation (IHS) using RNA probes on *Penaeus monodon* broodstock and early life stages to detect GAV or MOV infections

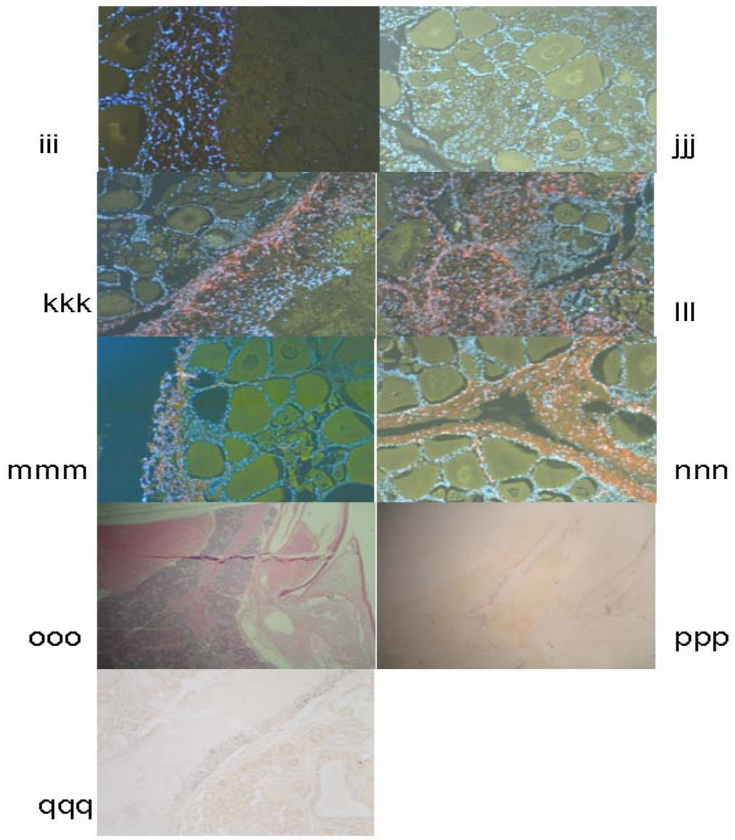


ww. Epithelia & lumen of vas deferens infected with MoV. (MoV24 sense probe, HNPP Fast Red and DAPI) (x10)
xx. Epithelia & lumen of vas deferens infected with MoV. (MoV24 sense probe, HNPP Fast Red and DAPI) (x20)

yy. Epithelia of vas deferens infected with MoV. (MoV24 sense probe HNPP/Fast Red and DAPI) (x10)
zz. Epithelia of vas deferens infected with MoV. (MoV24 sense probe HNPP/Fast Red and DAPI) (x40)

aaa. Epithelia of vas deferens infected with MoV. (MoV24 sense probe HNPP/Fast Red and DAPI) (x10) and DAPI
bbb. Lumen surrounding the spermatozoa in the vas deferens infected with MoV. (MoV24 sense probe HNPP/Fast Red and DAPI) (x10)
ccc. Lumen surrounding the spermatozoa in the vas deferens infected with MoV. (MoV24 sense probe HNPP/Fast Red and DAPI) (x10)
ddd. Lumen surrounding the spermatozoa in the vas deferens infected with MoV. (MoV24 sense probe HNPP Fast Red and DAPI) (x20)
eee and **fff**. Connective tissues surrounding vessels and oocytes in ovary infected with MoV. (MoV24 sense probe, HNPP/Fast Red and DAPI) (x10)
ggg and **hhh**. Connective tissues surrounding the ovaries and hepatopancreas tubules infected with MoV. (MoV24 sense probe HNPP Fast Red and DAPI) (x10)

Figure1(ww-hhh). In situ hybridisation (IHS) using RNA probes on *Penaeus monodon* broodstock and early life stages to detect GAV or MOV infections.



iii. Connective tissue between ovaries and muscle infected with MoV. (MoV24 sense probe, HNPP/Fast Red and DAPI(x10)

jjj. Uninfected ovarian tissue. (MoV24 sense probe, (HNPP Fast Red and DAPI) (x10)

kkk and **lll** . Ovarian tissue showing possible infection with MoV in apparently atresic oocytes (MoV24 sense probe HNPP/Fast Red and DAPI) (x10)

mmm. Connective tissues surrounding the ovaries infected with MoV. (MoV24 sense probe, HNPP/Fast Red and DAPI) (x10)

nnn. Connective tissues of the central ovarian cavity infected with MoV. (MoV24 sense probe, HNPP/Fast Red and DAPI) (x10)

ooo. H & E section of the central ovarian cavity (x5)

ppp and **qqq**. Connective tissues of the central ovarian cavity infected with MoV. (MoV24 sense probe, NBT/BCIP and Bismarck Brown) (x5)

Figure1(iii-qqq). In situ hybridisation (IHS) using RNA probes on *Penaeus monodon* broodstock and early life stages to detect GAV or MOV infections

6.4 Discussion

The majority of the egg samples that were collected over the course of the project tested negative for MoV and GAV using qPCR. This confirms previous observations that viral loads are low in early life history stages (Cowley et al. 2002). Of the samples that were PCR positive for GAV no evidence of infection within the eggs was discovered, only a weak signal in tissue debris surrounding the eggs. This result was verified using immuno-histochemistry with monoclonal antibodies against GAV (CSIRO Livestock Industries). In addition, MoV infection in ovarian tissues was limited to the surrounding connective tissues or within the central ovarian cavity associated with tissues that appeared to be undergoing atresia (Fig. 1 kkk, lll) as described previously (Peixoto et al. 2005). Well developed mature oocytes did not probe positive for MoV infection suggesting that mature oocytes were not infected prior to spawning. No GAV or MoV was detected in the oocytes prior to spawning or following spawning in the early stages of embryogenesis.

The probes for GAV and MoV were designed so that there was a sense (positive stranded RNA) and anti-sense (negative stranded RNA) probe. The opposite strand would be identical to the viral RNA sequence and should not hybridise with mature viral particles. The sense strand for GAV and anti-sense strand for MoV consistently probed positive in lymphoid organs and for MoV in vas deferens tissues. The positive probing of these tissues suggests that opposite strands of RNA are present in these areas. Strand specific probes have been used to study the reproductive biology of viruses (Bolten et al. 1998). At the site of viral synthesis a template is used, a complement of progeny strands. This indicates that the lymphoid organ and vas deferens are sites of viral replication of GAV and MoV. Only the tissues of the lymphoid organ have been previously reported as a known site of GAV replication (Spann et al. 2003).

The abundance of MoV in the vas deferens was not in proportion to other tissues and does not represent a systemic infection. The presence of probe positive tissues within the lumen and surrounding developing spermatozoa would suggest that MoV is present within mature spermatophores and released during spawning. An investigation into the presence of MoV and GAV in released spermatophores and spermatophores stored within the thelycum would provide further detail regarding vertical transmission of these viruses. This finding also supports the role of male health in the success of breeding programs however no pathology was associated with MoV infection in these tissues.

The postlarvae that were probed for GAV were strongly positive in the lymphoid organs and midgut epithelium as expected with a normal infection with GAV. The strong signal that was seen within epithelial tissues lining the midgut and within hepatopancreacytes suggests that ingestion is a direct method of infection of GAV. Infection with GAV in the cuticular epithelium of the midgut of adult *P.monodon* has previously been reported (Spann et al. 2003). The loss of GAV in early larval stages following hatching, the absence of viral particles in the eggs and the heavy infection with GAV along the midgut suggests that a major route of infection is horizontal transmission in early larval stages.

The results support the need to apply egg washing protocols to reduce the transmission of GAV and MoV. No evidence of viral infection in eggs prior to or immediately after spawning was evident. The role of vertical transmission in the life history of MoV in *P.monodon* appears to be associated with the male reproductive tissues and it is possible that virus particles are released with seminal fluids during spawning. Horizontal transmission of GAV appears to be an effective route of infection in early life history stages supporting the need to reduce viral loads in hatcheries prior to larvae reaching an active feeding stage.

7. CHAPTER 8. GENETIC PARAMETERS AND TRAIT HERITABILITY

Heritability and genetic correlations of growth, survival and reproductive traits in *Penaeus monodon* reared in tanks.

7.1 Introduction

The objective of this component of the study was to estimate the genetic parameters for growth, survival and reproductive traits in *Penaeus monodon*. These estimates were obtained using data available at the start of this project from three successive generations of domesticated stocks that had previously been reared in tanks at AIMS.

7.2 Genetic parameters for growth and survival

7.2.1 Methods

The materials and methods used for the production of the stocks are described in detail in Benzie et al. (1997) and the methods used to estimate the genetic parameters for growth and survival are described in detail in Kenway et al. (2006). The founder stocks (G0) were wild east coast (EC) males and females from the Cairns region. Reproductive, pedigree and weight data were derived from three generations (G1, G2 and G3). The G1 stocks consisted of nine full-sib families; the G2 stocks comprised seven paternal half-sib, three maternal half-sib and 19 full sib families; and the G3 stocks comprised two paternal half-sib families and 11 full-sib families.

Mating protocols

Wild caught or captive-reared broodstock were separated by sex in 4.5×1m circular tanks, maintained at 28°C with a 14:10 h light:dark cycle. At stocking, all females received a waterproof moult tag so that subsequent moulting events could be tracked. Broodstock were fed a conditioning diet of fresh frozen squid, New Zealand mussels, pipis (*Plebidonax deltoides*) and an in-house maturation paté containing astaxanthin. Female broodstock were artificially inseminated 2 days post-moult using spermatophores extracted from male broodstock by electro-ejaculation. Where the aim was to create half-sib families, spermatophores from a single male were used to inseminate more than one female. Immediately after artificial insemination each female was unilaterally eyestalk ablated (Primavera, 1978) using heated wire snips, eye-tagged for individual identification and then returned to its tank. Ablated females were monitored each afternoon, using a submerged torch, for signs of ovarian development. Females with fully mature ovaries were transferred to individual 120L spawning tanks. The following morning eggs were collected and transferred to a hatching tray for hatching into nauplii. Nauplii were collected as separate family batches.

Rearing of larvae and postlarvae

Hatched nauplii were transferred into separate 50L larval rearing tanks (LRT) at a density of 4000 nauplii per tank. Three replicate LRTs were run per family. Larvae were reared as described by Benzie et al. (1997). At postlarvae PL15 (26 days post spawning) families were relocated into one or more plankton mesh cages (80cm deep×30cm× 30cm) suspended within 4.5m diameter nursery tanks (NT) filled to 1m, with a maximum of 14 cages per tank. Postlarvae in cages were fed as described by Benzie et al. (1997). On average there were five replicate cages per family with three families having no replicates. At 16 weeks of age prawns

were individually tagged at an average weight of 3 g using streamer tags (Benzie et al. 1995) and families were then on-reared communally in 4.5m growout tanks (GT). Families were evenly distributed across tanks, and tanks were stocked at 150–200 juveniles per growout tank. Mortalities were monitored and additional unmarked animals were added into the growout tanks as necessary to maintain densities. The sex of 83% of prawns was determined at 16 weeks of age and the remainder sexed at 30 weeks of age. Juvenile and adolescent prawns were fed a commercially available pellet *ad libitum* four times each day and water quality was maintained as described by Benzie et al. (1997).

Growth rates and family survival

Individual body weights were measured in weeks 7, 10, and 16 post hatching. In addition, weights in week 30 were taken in G1 and G2, and weights in weeks 40 and 54 were taken in G1 and G3 (Table 1). Growth rates were calculated between 16–30, 16–40 and 16–54 weeks. Family survival records were calculated for family groups between weeks 0–4, 4–10, 10–16 and 16–35. The 16–35 week survival analysis was derived from 16–30 week survival data in generations 1 and 2, and 3, there were 25, 118 and 35, 0–4 week survival records; 40, 132 and 49, 4–10 week survival records; 42, 121 and 50, 10–16 week survival records; and 33, 146 and 149, 16–35 week survival records respectively, averaging approximately 4 replicates per family.

Table 1. Observed weight in grams (± SD) of untagged and tagged prawns in generations 1,2 and 3

Age (weeks)	4	7	10	16	30	40	54	30	40	54
	Untagged				Tagged					
	Pooled sex				Males			Females		
G1	0.008 (0.005) 402*	0.34 (0.34) 692*	1.2 (0.8) 687*	6.5 (2.6) 1015*	25 (5) 390*	41 (7) 296 *	60 (7) 94*	28 (7) 304*	54 (13) 214*	83 (18) 93*
G2	0.004 (0.002) 2309*	0.12 (0.07) 2516*	0.7 (0.3) 2487*	2.6 (0.9) 2953*	18 (3) 1045*	0*	0*	20 (4) 1041*	0*	0*
G3	0.005 (0.003) 594*	0.08 (0.08) 968*	0.4 (0.3) 965*	2.7 (1.1) 1180*	0*	29 (5) 318*	42 (6) 227*	0*	36 (7) 302*	56 (9) 222*

*.Sample size

Statistical and genetic analysis

The normality of weight and growth data were assessed with log transformations applied where necessary. Family survival records were calculated from percentage family survival within each LRT (0–4 weeks), mesh cage (4–10 weeks and 10–16 weeks), or growout tank (16–30 pooled with 16–40 weeks) and transformed using empirical logits (Townsend and Skalski, 1997) enabling 0%survival and 100%survival records to be included with the addition and subtraction of a constant α=0.5 (for full details see Kenway et al. 2006).

7.2.2 Results

Family survival

Family survival from spawning to 4 weeks was highly variable and it was not possible to estimate heritabilities as variance component solutions converged out of bounds. Heritabilities in periods 4–10, 10–16 and 16–35 weeks were significantly different from zero (Table 3). Back transformation of empirical logits gave percentage family survivals of 68%, 83% and 71% for the periods 4–10 weeks, 10–16 weeks and 16–35 weeks respectively. Genetic correlations between family survival periods were not significantly different from zero

Weight at age

Weights at 10 and 16 weeks were significantly positively skewed and were transformed using logs. The heritability for log week-10 weight was low at 0.13± 0.30. Genetic correlation of week 10 weight with week 30, 40 and 54 weight could not be obtained because solutions did not converge and are not reported. Both males and females had a similar phenotypic mean and standard deviation for log 16-week weight (1.07± 0.44 g), but displayed sexual dimorphism at week 30 (19.7±3.9 g males, 21.5±5.2 g females), week 40 (34.9± 6.2 g males, 43.5±10.3 g females) and week 54 (47.1± 7.4 g males, 64±13 g females). The genetic correlations between body weights of males and females were above 0.97 except at week 54 weight with a genetic correlation of 0.84±0.10. When variances of male and female body weight were standardised prior to analysis, heritability of weight at age ranged from 0.45 to 0.56 (Table 3). In general, the genetic correlations between weights at 16, 30, 40 and 54 weeks were highest at similar weights with week 30 and week 40 having a genetic correlation close to unity (Table 2).

Table 2. Family survival, weight at age and growth rate heritabilities (bold diagonal) and phenotypic correlations (above diagonal)

	Logit survival			Weight log (g)	Weight (g)			Growth rate (mg/day)		
week(s)	4-10	10–16	16–35	16	30	40	54	16–30	16–40	16–54
Logit survival Week(s) 4–10	**0.51**									
Week(s) 10–16	−0.25	**0.36**								
Week(s) 16–35	−0.10	0.22	**0.72**							
Log(week 16 weight)	0.4	−0.06	−0.05	**0.56**	0.52	0.43	0.43			
Weight Week 30	0.59	−0.18	0.12	0.56	**0.55**	0.81	0.7			
Week 40	−0.33	−0.15	0.24	0.57	0.97	**0.45**	0.86			
Week 54	−0.18	0.05	−0.07	0.67	0.73	0.78	**0.53**			
Growth rate Week(s) 16–30	0	−0.02	0.46					**0.5**	0.78	0.65
Week(s) 16–40	0.13	0.03	0.18					0.92	**0.48**	0.871
Week(s) 16–54	0.15	0.41	0.18					0.63	0.86	**0.55**

Growth rate

Sexual dimorphism was significant for growth rate with means and standard deviations at weeks 16–30 (166±35 mg/day males, 186±50 mg/day females), weeks 16–40 (179±33 mg/day males, 234±56 mg/day females), weeks 16–54 (169±26 mg/day males, 234± 43 mg/day females). Sex differences within each generation are provided in Table 1.

Family survival, weight at age and growth rate

Genetic correlations between family survival and weight at age were negative in some instances (Table 2). The correlations were generally low with large standard errors (see Kenway et al. 2006). Genetic correlations between family survival and growth rate were all positive except for

logit 10–16 week family survival and 16–30 week growth rate which was close to zero and not significantly negative (Table 2). The positive correlations were all less than 0.41 and not significantly different from zero.

7.2.3 Discussion

The growth rates of the domesticated EC *P. monodon* stocks reared for three generations in tanks at AIMS were significantly lower than those of the more recently domesticated *P. monodon* stocks reared in tanks and raceways (see Chapter 1). Females reared in the AIMS tanks reached a mean weight of 83 g in 54 weeks and males a mean weight of 60 g. In comparison to this first generation domesticated female *P. monodon* grown in tanks at CMR reached a mean weight of 100 g in only 44 weeks and males reached 85 g. (Coman et al. 2006). As discussed Chapter 1, improvements in growth rate of tank reared *P. monodon* over the past decade are, in part, due to incremental improvements to the rearing environment, diet, health and husbandry techniques for captive breeding of this species.

The heritability for *P. monodon* juvenile weight at age of 0.13±0.30 reported in this study was similar to that reported for juvenile *P. monodon* by Benzie et al. (1997). A large maternal genetic or environmental component was observed by Benzie et al. (1997) but data in the present study were not sufficient to determine maternal components. The estimates of heritability for weight from 16 weeks up to 54 weeks ranged from 0.36 to 0.73 and were much higher than the earlier reported juvenile heritabilities for weight in *P. monodon*.

The estimates of heritability for weight at age in this study, were lower than those of 0.78 to 0.83 for weight at 22 to 24 g reported by Argue et al. (2002) for *P. vannamei*, but generally higher than those reported in other prawn studies including those or *P. vannamei*, 0.47±0.25 for weight at 50 g (Arcos et al. 2004); 0.20±0.17 for weight at 17 weeks and 0.34±0.17 or weight at 29 weeks (Perez-Rostro and Ibarra, 2003); 0.24+0.05 and 0.17+ 0.04 for weight at 23 weeks (Gitterle et al. 2005); 0.42± 0.05 for weight at 14 weeks (Carr et al.1997); those for *P. stylirostris*, 0.11 for weight of approximately 30 g (Goyard et al. 2002) and those for *P. japonicus* 0.25 at 6 months (Hetzel et al. 2000). The heritability of growth rate, as opposed to weight at age, has been analysed in fewer species. In *P. vannamei,* heritabilities recorded were 0.63±0.20 for 17–23 week growth and 0.54±0.20 for 23–29 week growth (Perez- Rostro and Ibarra, 2003). These were similar to the present study, which found heritabilities averaging 0.56 over the 16–30 week period. Heritability for growth rate from approximately 8 weeks through to approximately 27 weeks, ranged from 0.77 to 0.80 in *P. vannamei* (Argue et al. 2002).

In a practical situation, selection for growth would be carried out by selecting the heaviest males and females within each sex. Weight at a given age would be preferred over growth rate as a selection trait, as it is easier to measure and as shown in this study, has a similar heritability. Direct selection pressure for weight is best applied at market weight, although this may not correspond to weight at sexual maturity. If larger broodstock yield more nauplii per spawn (Cavalli et al. 1997), a two stage selection program may be preferred with the heaviest females selected at both market weight (30 weeks) and at mating (54 weeks) since the genetic correlations between these two weights are less than unity (0.67±0.08).

There are already a number of examples of successful selection for weight in penaeid species. For example, one generation of selection produced a 4.4% growth increase in *P. vannamei* (Fjalestad et al. 1997), a 21% growth increase in *P. vannamei* (Argue et al. 2002) and an average 11% growth increase in *P. japonicus* (Hetzel et al. 2000). Using genetic parameters from this study, selecting the heaviest 40% at 30 weeks should increase 30 week weight by 10%. Survival in artificial culture conditions is one trait that may not be intentionally selected, but will inevitably change from inadvertent selection during a domestication program (Doyle,

1983). With low survival rates, high selection intensity is generated for survival, and hence the selection response should increase with lower survival rates. This assumes the same environmental factors are causing mortalities in different generations, a result which is supported by a significantly positive family survival heritability over more than one generation.

In a commercial selection program the genetic correlations of survival and growth would be monitored to ensure selection effort is directed to improving profitability. The heritability of family survival of *P. monodon* in the present study ranged from 0.36 to 0.72, which is in line with 0.42 estimated for *P. vannamei* from full sibs (Carr et al. 1997), but higher than that predicted by Gillerle et al. (2005) which in two lines predicted heritability for survival of 0.04±0.02 and 0.10±0.02. *De Donato* et al. (2001) did not find any significant effect for general survival between families in *P. vannamei*, whereas resistance in the same species to Taura Syndrome Virus gave a realised heritability of 0.28± 0.14 (Argue et al. 2002).

The limited numbers of sires and dams in the base population mean that random sampling of the general population could result in under or over estimates of heritability for population inference. Heritability and particularly genetic correlations must also be interpreted carefully which may have produced inconsistent estimates due to limited data and which may have also been biased by common environmental effects or unaccounted maternal genetic effects. The logit transformed family survival records, together with other traits were assumed to be normally distributed quantitative traits yielding valid estimates of heritability. It should also be noted that the data used in the present paper derive from animals that were grown in tanks throughout their life cycle, and therefore strictly speaking the calculated heritabilities reflect those for weight, growth rate and family survival in tanks. Work in other penaeid species has demonstrated a good correlation between measurements based on tank reared animals and those reared in ponds (Moss et al. 2001; Gillerle et al. 2005).

Overall, the results presented here indicate that there is plenty of genetic variation for effective selection programs to improve both growth and survival in *P. monodon*. Assuming that the biological risks of maintaining families in a long term program can be managed (Browdy, 1998), the medium to high heritabilities for both growth and family survival are a good foundation for establishing cost effective improvement via selective breeding of *P. monodon*.

7.3 Genetic parameters for reproductive traits

7.3.1 Methods

Production of family lines

Sampling of broodstock and production of family lines are described in detail by Kenway et al. (2006). Reproductive, weight and pedigree data were derived from three generations (G1, G2 and G3). The G1 consisted of nine full-sib families; the G2 comprised seven paternal half-sib, three maternal half-sib and 19 full sib families; and the G3 comprised two paternal half-sib families and 11 full-sib families. Reproductive data was determined from 13, 65, and 14 spawning events in generations G1, G2 and G3 respectively.

Mating protocols, egg and nauplii sampling

Mating and feeding protocols were described by Kenway et al. (2006). Females with fully mature ovaries were transferred to individual 120L spawning tanks. The following morning each batch of eggs was harvested, washed and placed into a separate container with 4L of seawater. After thorough mixing, five 2ml sub-samples were fixed in 70% ethanol and later counted on a “Bogerov” tray under a stereo dissecting microscope to estimate egg number. The remaining eggs were transferred to an upweller tray for hatching. Nauplii were harvested,

processed and sampled in a similar way to egg batches to estimate nauplii number and hatch rate. Under the protocol, nauplii were retained as separate family batches. Ablation and spawning dates were recorded with the difference between these dates was recorded as the number of days to spawning.

Statistical and genetic analysis
Heritability ($\underline{h}^2$) of reproductive traits, egg number, nauplii number and hatch rate were derived from the genetic contribution of the female prawns that spawned. Heritability estimates and the genetic correlations of the reproductive traits with growth rate, and weight at age, were derived using an animal model.

Variance component estimates were determined from multivariate analysis with unstructured variances using ASReml release 1.1 (Gilmour et al. 2002) with model:

$$\mathbf{y} = \mathbf{Xb} + \boldsymbol{Za} + \mathbf{e}$$

where **y** is a vector of observations of each trait; **b** is a vector of fixed effects; **a** is a vector of random additive animal genetic effects; **X** and **Z** are incidence matrices that relate observations to the respective effects and **e** is the random residual error component.

Reproductive traits, i.e. days to spawn, egg number, nauplii number and hatch rate were corrected for environmental effects using generation as a fixed effect in the model. Other fixed effects such as spawnings (first or subsequent) and the number of spermatophores received by each female were initially considered but excluded from the model due to the limited information available. The appropriate data transformation was determined by assessment of plotted residuals against the corresponding fitted values. Days to spawn, egg number and nauplii number were square root transformed and hatch rate transformed using the arcsine of the square root of the proportion hatched.

Sexual dimorphism in the variances and means between males and females was evident for growth rate and weight at age (Kenway et al. 2006). When the sexes were pooled for analysis, the variances and means between males and females for growth rate and weight at age, within each period, were standardised prior to determining their genetic correlations with reproductive traits. The weight and growth rate of animals were corrected for grow-out tank as a fixed effect (Kenway et al. 2006).

7.3.2 Results

Phenotypic means and variation
Reproductive traits were highly variable between generations with all traits having a large coefficient of variation (Table 1), with the highest being 68% for nauplii production and 71% for hatch rate. Generation 3 produced twice the number of eggs compared to earlier generations. The poorer hatch rate of G3 reduced the number of nauplii to comparable nauplii numbers in G2.

Table 3. Means and coefficient of variation of transformed reproductive traits, days to spawn, egg number, nauplii number and hatch rate and means of untransformed reproductive data over three generations.

Transformed data	√ (days to spawn)	√ (egg number)	√ (nauplii number)	Hatch#
Mean (backtransfomed)	4.1 (16)	411 (169,000)	196 (38,000)	0.56 (0.28)
Coefficient variation (%)	34	35	68	71
Untransformed means	days to spawn	egg number	nauplii number	hatch rate (%)
Generation 1	9	159815	16006	9
Generation 2	22	164400	61269	41
Generation 3	12	343815	69000	24
Pooled	19	189376	56050	34

arcsin √ (proportion hatched)

Heritabilities and correlations for reproductive traits

For the reproductive traits recorded, heritability estimates were low to high with the highest heritability of 0.47 for days to spawn (Table 2). There was a strong positive phenotypic correlation between hatch rate and nauplii number which was also manifested in a significant genetic correlation. The analysis indicates that egg number has a negative genetic correlation with hatch rate, but like most other genetic correlations it was associated with large standard errors (Table 2).

Table 4. Heritability estimates (h2) (diagonal), genetic correlations (below diagonal) and phenotypic correlations (above diagonal), of transformed reproductive data (± standard error), for days to spawn, egg number, nauplii number and hatch rate.

	√ (days to spawn)	√ (egg number)	√ (nauplii number)	Hatch#
√(days to spawn)	**0.47±0.15**	0.10 ± 0.13	0.13 ± 0.12	0.09 ± 0.13
√(egg number)	0.08±0.36	**0.41 ± 0.18**	0.23 ± 0.12	-0.16 ± 0.13
√(nauplii number)	0.80±0.32	-0.12 ± 0.61	**0.27 ± 0.16**	0.85 ± 0.04
Hatch#	0.55±0.48	-0.75 ± 0.42	0.76 ± 0.23	**0.18 ± 0.16**

arcsin √ (proportion hatched)

Table 5. Heritability estimates (h2) of weight at age and growth rate, and genetic correlations of transformed reproductive data (± standard error) for days to spawn, egg number, nauplii number and hatch rate.

Age (weeks)	h2	√ (days to spawn)	√ (egg number)	√ (nauplii number)	Hatch#
	Weight at age				
30	0.55	-0.06 + 0.46	0.22 + 0.33	0.02 + 0.41	-0.02 + 0.54
40	0.45	-0.18 + 0.47	0.38 + 0.29	0.09 + 0.40	-0.01 + 0.49
54	0.53	-0.11 + 0.75	0.93 + 0.19	0.37 + 0.40	-0.07 + 0.71
	Growth rate				
16-30	0.50	0.09 + 0.42	0.35 + 0.30	-0.27 + 0.45	-0.46 + 0.38
16-40	0.48	-0.21 + 0.38	0.54 + 0.25	0.24 + 0.42	-0.05 + 0.51
16-54	0.55	0.14 + 0.41	0.63 + 0.29	0.38 + 0.40	-0.48 + 0.53

arcsin √ (proportion hatched)

Genetic correlations between reproductive and growth traits

Genetic correlations of reproductive traits and those of growth rate and weight at age were determined with male and female weight data pooled (Table 3). Genetic correlations between

both growth traits (weight at age and growth rate), and egg number were positive (Table 3) and significantly different from zero at week 54. The genetic correlations between growth traits and other reproductive traits, days to spawn, nauplii number and hatch rate were all less than 0.5 in magnitude and not significantly different from zero.

7.3.3 Discussion

There were too few data to partition the contribution of the male (sperm donor) versus the female spawner (egg producer) to hatch rate and nauplii number. These traits were assumed to be entirely influenced by the female spawner. However, there is some evidence that reduced hatch rate in penaeid species may be attributable to the male as much as to the female (Perez-Velazquez et al. 2001; Perez-Velazquez et al. 2003; Ceballos-Vázquez et al. 2003; Hall et al. 2003). If the latter is the case, it is likely that some of the genetic variance attributed to sperm viability was statistically repartitioned to the female spawner resulting in an overestimate of heritability. It is expected that selection of the female spawner, for hatch rate and nauplii number, would account for indirect selection pressure of sperm viability with the "overestimated" heritability estimates still yielding a similar predicted response. Ideally, the appropriate model would include a separate genetic component for the female egg producer and the male spermatophore donor, this will be possible when substantially more data has been collected.

These estimates of heritability obtained for *P. monodon* egg number in this study (0.41+0.18) are in the same order of heritability estimates for other aquaculture species including coho salmon (Gall and Neira, 2004) and rainbow trout (Su et al. 1997) but significantly higher than estimates obtained for *P. vannamei* when egg diameter was used as a covariate (Arcos et al. 2004).

In this study, significant genetic correlations were found between egg number and broodstock weight at 54 weeks and between egg number and 16-54 week growth rate. This is consistent with results reported for *P. vannamei* showing a significant genetic correlation between egg number and total weight (Arcos et al. 2004). As suggested by Palacios and Racotta (2003) selection for large spawners could result in added reproductive output based on higher fecundity.

In contrast to the positive correlation observed between *P. monodon* egg number and broodstock weight there was negative genetic correlation between hatch rate and weight at age or growth rate. However, the genetic correlation of nauplii number with weight at age or growth rate was less than 0.38 and not significantly different from zero, indicating that there is no evidence to suggest that selection for growth will adversely reduce nauplii production. This finding is in agreement with Palacios and Racotta (2003) who reported that selection for nauplii production was not so directly related to spawner weight.

The high variability and medium heritability for *P. monodon* nauplii production observed in this study indicates significant potential to improve nauplii production through selection. The response to selection could be improved through the use of correlated traits to increase the variance of estimated breeding values (EBVs) for nauplii production. Hatch rate may be useful in improving EBVs for nauplii production, as it had a high genetic correlation of 0.75+0.23 with nauplii number. The high genetic correlation of nauplii number and days to spawn of 0.80+0.32 also make it a possible candidate for inclusion as a correlated trait. In this case the negative economic impact of increasing days to spawn would be taken into account through the use of an economic selection index to achieve maximum economic gains from selection. Genetic correlations of nauplii number with weight at age may also be included in the same selection index to maximise economic response to selection for the whole production system.

The heritability estimates for reproductive parameters, particularly those for days to spawn and egg number indicate that improvements in reproductive performance of *P. monodon* will be achieved by selectively breeding for reproductive traits. The results also indicted that selection for growth will not adversely impact on reproductive performance.

BENEFITS AND ADOPTION

The results of this project have provided the Australian prawn farming industry with the knowledge and technology to progress from the use of wild *Penaeus monodon* broodstock to commercial production of domesticated broodstock of known health status. This is a major advance beyond the total reliance of the industry on unpredictable supplies of wild broodstock. Industry now has the capability to begin controlling the quantity, quality and timing of supplies of postlarvae for stocking farm ponds. Over the past two decades variability in the availability of wild broodstock has resulted in significant losses to farmers. For example, in 1999-2000 a chronic shortage of *P. monodon* broodstock meant that many ponds were either unstocked, stocked late, or were stocked with alternative species. This resulted in an estimated $5M in lost production for the season. Delays in stocking ponds are particularly critical to farmers at higher latitudes because of the restricted grow-out seasons (Jackson & Wang 1998; Preston et al. 1999).

Broodstock rearing systems

Comparison of different broodstock rearing environments demonstrated the benefits of using controlled environment tanks and raceways to produce mature broodstock within twelve months. This is in synchrony with the annual production cycle of most Australian *P. monodon* farms. Previous studies of the effect of temperature on the growth rate of *P. monodon* indicate an optimum water temperature of around 30°C (Jackson & Wang 1998). This is consistent with the results of this study in which the controlled environment tanks and raceways were maintained between 28°C and 30°C. The broodstock reared in the unheated ponds at Bribe Island Research Centre (BIARC) had slower growth rates during the winter, thus requiring more time to reach maturity than those grown in temperature controlled environments. However, ponds, particularly those in warmer regions, offer the potential to rear larger numbers of broodstock at lower capital costs.

The growth rates of captive reared *P. monodon* reared in controlled environment tanks in this study were a significant improvement over previous trials, with a 200% increase in the average wet weight of 11 month old females compared to previous trials in 1997 (Coman et al. 2005) and in 2000 (Kenway et al. 2006). This achievement highlights the importance of progressive improvements to the environment, diet, health and husbandry techniques for captive breeding of *P. monodon*.

Broodstock health

This project produced new knowledge about the origin and nature of viruses in wild founder stock populations of *P. monodon* and subsequent changes in the level of infections in successive generations of the domesticated stocks. Viral screening of EC stocks revealed progressive reduction in levels of infections of endemic Gill Associated Virus (GAV) and Mourilyan Virus (MoV) over 3 generations of captive rearing in broodstock ponds at BIARC. Wild GoC stocks were free from GAV but MoV was endemic at low levels. GoC stocks acquired GAV infection during the first generation of domestication, which persisted, along with MoV, in successive generations. These infections did not impair the growth, survival or reproductive output of broodstock or the grow-out performance of their progeny in commercial ponds.

The precise location of GAV and MoV virus particles in *P. monodon* gonads, eggs, larvae and postlarvae was examined using histology and fluorescent *in situ* hybridisation (FISH). Determining the location of these viruses is important in understanding their modes of transmission and the potential effectiveness of egg washing, with or without virucidal compounds. Egg washing can only be effective if the source of the infection is external to the gametes, for example in the spermatophore, seminal fluid or ovarian fluids. No evidence of viral infection within eggs was evident either prior to or immediately after spawning. The results indicate that horizontal transmission is the main route of GAV infection in early life history

stages. However, the results also indicated that MoV could be vertically transmitted via male reproductive tissues and that virus particles are released with seminal fluids during spawning.

The effects of washing newly spawned eggs with different virucidal indicated that Betadine (0.1 ppm) has the best potential to eliminate or significantly reduce levels of GAV without adversely affecting hatch rates. This finding is consistent with the recommendations of the OIE International Aquatic Health Code for washing the eggs of Penaeid prawns (see web page: http://www.oie.int/eng/normes/fmanual/A_00014.htm, Methods for Disinfection of Crustacean Farms). The protocol relies on the use of formalin (100 ppm) for 1 min followed Betadine (0.1 ppm) for 1 min and is based on results from studies by Brock and Bullis (2001) and Chen et al. (1992).

Genetic improvement

Improvements in the growth, survival and reproductive output of *P. monodon* broodstock were achieved with each generation in each of the rearing environments. By the second generation reproductive output of domesticated broodstock had reached the level considered to be the minimum required to be commercially viable by industry (mean egg production of >200,000 per spawning and mean nauplii production of >100,000 per spawning). Genetic analysis of domesticated EC stocks reared in tanks indicated high heritabilities for growth and for survival (Kenway et al. 2006) and low to moderate heritabilities for some reproduction traits (Macbeth et al. 2007). These results indicate that future investment in selective breeding of *P. monodon* will result in concurrent improvements in growth, survival and reproductive performance.

Adoption of the results

*T*he project produced twenty four third generation families, derived from EC broodstock, as potential founder stocks for future selective breeding and ten families derived from GoC broodstock for on-going commercial-scale domestication and selective breeding. So far only the core industry participants, Gold Coast Marine Aquaculture and Seafarm Pty Ltd have directly contributed to on farm components of the research and/or directly adopted the results of the project. Seafarm contributed their sixth generation (G6) domesticated stocks to the EC broodstock. Subsequent to the completion of the project, representatives of all the families derived from the EC stocks have been provided to Seafarm for them, and potentially other farms, to commence on-farm trials with these stocks.

The first trials of domesticated stocks in commercial ponds at GCMA in 2005 demonstrated that growth and survival of the progeny of second generation GoC broodstock was equivalent or superior to that of progeny of wild broodstock. Subsequent to the completion of the project, the second commercial trials in 2006, with third generation GoC progeny, resulted in an average yield of 11.8 tonnes ha^{-1}. The third commercial trials in 2007, with fourth generation GoC progeny, further increased the average yield to 12.8 tonnes ha^{-1}.

Over the next few years we anticipate progressive adoption of the results of the project across the whole industry as the progeny of domesticated stocks become increasingly available to more and more farms. The benefits from domestication alone include; eliminating total reliance on wild stocks; all-year round availability of postlarvae and the production of disease free or high health seedstock. All of these will lead to greater profits for the prawn farmers. This in turn will make the Australian prawn farming industry more competitive. Beyond successful, industry-wide, domestication we anticipate subsequent cumulative and permanent genetic gains in commercially important traits and associated economic gains to the industry.

FURTHER DEVELOPMENT

Although commercial scale production of domesticated broodstock has now been achieved, constraints to the production of these broodstock are still restricting the number of ponds that Australian prawn farmers can stock with domesticated, selectively bred stocks. The two key constraints are; the low number of farms that have the infrastructure to rear broodstock and low hatch rates in most domesticated broodstock (as reported in Chapter 1).

An increase in the number of farms investing in broodstock rearing facilities is already starting to ease the first constraint. However, a follow-up survey of all current domesticated broodstock producers identified low hatch rates as the major factor limiting the supplies of domesticated postlarvae to industry. A number of factors appear to be contributing to the low hatch rates including the rearing environment, broodstock nutrition, mating behaviour and success, variation in viral infections and genetic variation in reproductive performance.

The results of the current study have clearly demonstrated that the rearing environment affects broodstock performance, with more consistent results obtained from controlled environment tanks or raceways, than ambient condition ponds. The results have also demonstrated the importance of maintaining duplicate stocks at different locations. The relatively small numbers of duplicate sibling stocks, held in controlled environment tanks at CSIRO, were critical in conserving domesticated lines of both GoC and EC stocks when the sibling pond-reared stocks were lost due to low fecundity and/or low survival.

Previous studies have demonstrated that variation in broodstock diet has a significant impact on gamete quality and hatch rates. In particular, increasing the level of unsaturated fatty acids (HUFAs) in maturation diets appear to have beneficial effects on egg fertilization and hatching (Cahu et al. 1994; Xu et al. 1994; Coman et al. 2007). The results of the current study revealed that there were significant numbers of non-hatching and/or unfertilised spawnings. Follow-up studies have demonstrated that significant proportions of the tank-domesticated females had simply not mated and thus had no implanted spermatophores at the time of spawning. The current method of determining mating success by visual observation of *P. monodon* females is difficult and unreliable. A rapid and reliable assay for mating success would significantly enhance the efficiency of seedstock production. It would also permit a more rigorous analysis of the full impacts of variation in mating success in commercial nauplii production from domesticated broodstock.

Problems with the condition of the male reproductive tract and spermatophore degeneration have been reported in domesticated stocks of several penaeid species (Talbot et al. 1989; Alfaro et al. 1993; Alfaro and Lozano 1993). The results of viral screening in the current study showed that male reproductive tissues are a site of MoV infection. In follow on studies, third generation East Coast male *P. monodon* reared in the both nursery and grow-out ponds at BIARC developed a high prevalence and high-level MoV infections. These infections coincided with very poor spermatophore development, severely limiting the production of families from this source of males. Further studies are needed to determine the effects of variation in the level of MoV infections and spermatophore development. Of particular interest are the factors that result in the progression of infections with either GAV or MoV to disease in tissues or organs including testes and ovaries.

Further research in needed to address the problem of low hatch rates by examining and optimizing the individual and combined influences of rearing environment, broodstock nutrition, mating behaviour, variation in viral infections and genetic variation in reproductive performance on seedstock production of domesticated *P. monodon* stocks.

PLANNED OUTCOMES

Outcomes that apply to the whole of the Australian prawn farming industry:
The key outcome anticipated from this project was to provide the Australian prawn farming industry with the knowledge and technology to commence the progression of the industry beyond their dependency on wild broodstock to the controlled production of domesticated broodstock of known health status. As outlined above, and detailed in this final report, this outcome was successfully achieved.

It was also anticipated that successful domestication would reduce the risks of disease outbreaks and thus improve farm production efficiency, marketability, and hence profitability. The results of the project have demonstrated the critical need to determine the nature and level of viral infections in founder stocks used in domestication. The results revealed significant changes to the level of viral infections in successive generations of domesticated stocks. Initially there was a progressive reduction in levels of infections of GAV and MoV in broodstock reared in ponds at BIARC. However, the males of the third generation pond reared stocks were infected with high levels of MoV and their reproductive capacity was critically impaired. The development and implementation of the practice of maintaining duplicate sibling East Coast and Gulf of Carpentaria stocks in biosecure, controlled environment conditions, was critical in preventing major stock losses. Whilst the risks of disease outbreaks can never be entirely eliminated, well designed domestication practices can clearly reduce the level of risk.

Success in identifying or producing Specific Pathogen Free (SPF) broodstock was anticipated to provide the opportunity for Australian producers to respond to the strong market demand for SPF broodstock to supply the US$4 billion *P. monodon* industry in S.E. Asia. Although the domesticated stocks produced in this project are free from exotic viruses, including White Spot Syndrome Virus (WSSV), SPF status for GAV and MoV is yet to be achieved.

Finally, it was anticipated that successful domestication will provide the means to commence selective breeding for commercially desirable traits, such as improved growth rates. As detailed in Chapter 8, the results of genetic analysis do indicate that future investment in selective breeding of *P. monodon* will result in concurrent improvements in growth, survival and reproductive performance.

Specific outcomes for industry core partners:
It was anticipated that access to new knowledge, technology and to on-farm domesticated broodstock during the course of the project would be of direct benefit to the core industry partners. This proved to be the case, predominantly at GCMA where all the on-farm trials were done. During the course of the project GCMA progressively developed the infrastructure, technology and rearing protocols for the successful production of domesticated *P. monodon* broodstock in controlled environment raceways on-site. The use of controlled environment raceways permits commercially viable numbers of broodstock to be produced all year round and in synchrony with pond stocking cycles. This is a major advance for the Australian industry with further development and expansion of this technology likely to have a major impact on increasing the number of farms producing domesticated broodstock on demand on-site.

The first trials of domesticated stocks in commercial ponds at GCMA in 2005 demonstrated that growth and survival of the progeny of second generation GoC broodstock was equivalent or superior to that of progeny of wild broodstock. Subsequent to the completion of the project, the second commercial trials in 2006, with third generation GoC progeny, resulted in an average yield of 11.8 tonnes ha^{-1}. The third commercial trials in 2007, with fourth generation GoC progeny, further increased the average yield to 12.8 tonnes ha^{-1}, the highest yields ever recorded for the production of farmed *P. monodon* in Australia.

CONCLUSION

Domestication of *P. monodon* has been the highest research priority for the Australian prawn farming industry for the past several years. In response to this priority the Australian Prawn Farmer's Association (APFA) initiated a major research initiative to understand and overcome the barriers to domesticating *P. monodon*. The initiative integrated research skills from the Australian Institute of Marine Science, CSIRO Marine and Atmospheric Research and Livestock Industries and the Queensland Department of Primary Industries (Bribie Island Research Centre). As core industry partners Gold Coast Marine Aquaculture and Seafarm Pty Ltd provided infrastructure, farm stocks and commercial expertise. The project was co-funded by industry, the research providers and the Fisheries Research and Development Corporation.

The industry led multi-partner, multidisciplinary approach adopted in this project has achieved the success that eluded the previous decade of un-coordinated research by individual research institutions and farms. The results of the project have provided the knowledge and technology to permit the industry to begin producing domesticated stocks of *P. monodon* on a commercial scale. The results of the project have also clearly demonstrated the benefits of commercial scale on-farm domestication of *P. monodon*, with the progeny of domesticated stocks producing the highest ponds yields ever recorded in Australia. The advances achieved in this project are a major step towards the whole industry acquiring the ability to control the quantity, quality and timing of supplies of postlarvae for stocking farm ponds. However, constraints to broodstock production and performance are still restricting the number of ponds that Australian prawn farmers can stock with the progeny of domesticated broodstock.

The two key constraints to industry wide access to domesticated broodstock are; the low number of farms that have the infrastructure to rear broodstock and the low hatch rates in most domesticated broodstock. An increase in the number of farms investing in broodstock rearing facilities is already starting to ease the first constraint. However, a recent survey of all current domesticated broodstock producers identified low hatch rates as a major factor limiting the supply of domesticated postlarvae for the industry. There is a critical need to address the problem of low hatch rates by examining and optimizing the individual and combined influences of nutrition, genetics, health, mating behaviour and environment on seedstock production of domesticated *P. monodon* stocks.

Over the next few years we anticipate progressive adoption of the results of the project across the whole industry as improvements to hatch rates are achieved and the progeny of domesticated stocks become increasingly available to more and more farms. The benefits from domestication alone include; eliminating total reliance on wild stocks; all-year round availability of postlarvae and the production of disease free or high health seedstock, all of these will lead to greater profits for the prawn farmers. This in turn will make the Australian prawn farming industry more competitive. In addition to the progression towards industry-wide, domestication we anticipate the development of selective breeding programs that will results in cumulative and permanent genetic gains in commercially important traits with associated economic benefits to the industry.

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APPENDIX 1: INTELLECTUAL PROPERTY

At the start of the project the background intellectual of the all the participants was documented. This document is held on file by the applicant (Australian Prawn Farmer's Association)

INTELLECTUAL PROPERTY AND VALUABLE INFORMATION ARISING FROM THE PROJECT

The principle objective of this project was to provide knowledge and technology on the domestication of P. monodon to the Australian prawn farming industry via the Australian Prawn farmer's Association (APFA).

The key areas of new knowledge and technology are:

- *Broodstock rearing systems*. Quantitative comparison of the growth, survival and reproductive output of *P. monodon* broodstock from the East Coast (EC) and from the Gulf of Carpentaria (GoC) reared through three successive generations in tanks, raceways or specialised ponds.
- *Molecular virology*. Details on the origin and nature of viruses in wild founder stock populations of *P. monodon* and subsequent changes in the level of infections in successive generations of the domesticated stocks. The location of GAV and MoV virus particles in *P. monodon* gonads, eggs, larvae and postlarvae
- *Virucidal treatments*. Details of the effects of washing newly spawned eggs with different virucidal treatments and their effectiveness in eliminating or significantly reducing levels of GAV without adversely affecting hatch rates
- *Genetic analysis*. Quantitative information on the heritabilities for growth, survival and reproduction traits of domesticated EC stocks reared in tanks
- *Domesticated stocks*. Third generation families, derived from EC broodstock, as potential founder stocks for future selective breeding and fourth generation families derived from GoC broodstock for on-going stocking of farm ponds and selective breeding.

The ownership of the progeny of the domesticated broodstock produced in this project is subject to the terms and conditions described in a Deed of Agreement between the project participants (FRDC**,** APFA, CSIRO, AIMS, QDPI&F, GCMA & SEAFARM).