RNA interference (RNAi) as an antiviral mechanism against *Penaeus merguiensis* densovirus (*Pmerg*DNV)

Thesis submitted by

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ABSTRACT

The Australian hepatopancreatic parvovirus (HPV) isolate is currently known to have a high prevalence in wild and cultured penaeid species throughout Queensland, Australia. Despite penaeid prawns being the main crustacean cultured in Queensland, there has been no investigation into the production losses resulting from HPV. The aim of this project was to characterise the virus from Australian *Penaeus merguiensis*, estimate production losses on the culture of *P. merguiensis* due to HPV disease and determine if RNA interference (RNAi) can mediate a sequence specific anti-viral effect against the Australian HPV isolate.

The Australian HPV isolate from *P. merguiensis* is the fourth strain of penaeid prawn HPV identified. Following the convention of the International Committee for the Taxonomy of Viruses, this virus has been named *Penaeus merguiensis* densovirus (*Pmerg*DNV). Complete genome sequence (6299 bp) analysis revealed a nucleotide similarity (86%) closer to the complete genome sequence of the Korean HPV isolate from *P. chinensis* than to the complete genome sequence of the Thai HPV isolate from *P. monodon* (83%) and the Indian HPV isolate from *P. semisulcatus* (83%). Hence, HPV strains may be following the phylogenetic relationship of the penaeid prawn hosts rather than their geography.

A TaqMan based real-time PCR assay was developed for the detection of *Pmerg*DNV. The TaqMan assay was developed within the capsid protein region of the genome and was optimised to detect as little as 10 copies of the targeted sequence per reaction. The assay was specific for *Pmerg*DNV as it did not detect related crustacean and canine parvoviruses from Australia. This assay has the potential to be used for diagnostic purposes and in robotic applications, particularly for the detection and quantitation of low-grade infections, permitting exclusion of potential carriers of the virus from culture facilities and subsequently preventing disease outbreaks.

A PCR analysis of 10-day old postlarvae of *P. merguiensis* from three northern Queensland farms from 190 ponds over two years revealed that the odds were approximately 2:1 that ponds with moderate to heavy loads of *Pmerg*DNV will have below mean survival. Of particular interest is that production will increase by at least 14% across the farms if survival is improved to match the current mean loss by removing *Pmerg*DNV.

Since natural infections of *Pmerg*DNV in *P. merguiensis* can interfere with results of infection studies, infection experiments were conducted on the house cricket (*Acheta domesticus*) and mealworm beetle larvae (*Tenebrio molitor*) to find an alternate bioassay species. *Acheta domesticus* and *T. molitor* were challenged with approximately 1 x 10^6 virions of *Pmerg*DNV by inoculation. *Pmerg*DNV was detected in 20% of *T. molitor* and 86.6% of *A. domesticus* challenged with *Pmerg*DNV. During a subsequent time course experiment, there was a slight increase in *Pmerg*DNV titres (10^{4-5} virions), reaching a maximum peak at day 5 (10^6 copies). A threshold of *Pmerg*DNV DNA level equal to or greater than 10^3 virions was necessary for mortality in *A. domesticus*. As the inoculum increased from 10^3 DNA copies to 10^4, 10^5, 10^6, mortality increased from 20% to 60%, 80% and 100% respectively.

Since an alternate bioassay species (*A. domesticus*) had been established, investigations continued to determine if RNAi can be used to provide protection against *Pmerg*DNV in *A. domesticus*. Adult *A. domesticus* were injected with 5 µg of stealth RNAi or control stealth RNAi, targeting the capsid protein and challenged with *Pmerg*DNV twenty four hours post-injection. Crickets injected with RNAi targeting *Pmerg*DNV had the lowest mortality rate (11.5%) compared to crickets injected with control dsRNAi (33%) and *Pmerg*DNV alone (25%). The introduction of dsRNAi corresponding to the capsid protein of *Pmerg*DNV, was effective in reducing viral replication in *A. domesticus*. Crickets challenged with specific dsRNA significantly reduced *Pmerg*DNV production by one log (3.58 x 10^2) compared to crickets challenged with *Pmerg*DNV alone (3.42 x 10^3). Control dsRNA also resulted in a one log reduction of *Pmerg*DNV (3.95 x 10^2), but did not produce an inhibitory effect quite as strong as the targeted dsRNAi for the capsid protein of *Pmerg*DNV.

The RNAi assay targeting the NS2 gene was repeated jointly with a post-doctoral scientist from India as part of the Australia-India strategic research fund. The greatest cumulative percentage mortality (70%) was recorded in the target RNAi + *Pmerg*DNV treatment, followed by in the control RNAi + *Pmerg*DNV (50%) and *Pmerg*DNV only treatment (46.5%) and the target RNAi only treatment (43%). Similarly, the introduction of dsRNA corresponding to the NS2 protein of *Pmerg*DNV was effective in reducing viral replication in *A. domesticus*. However, a 10-fold reduction in *Pmerg*DNV titres was only recorded in the target RNAi + *Pmerg*DNV treatment. Crickets challenged with specific dsRNA significantly reduced *Pmerg*DNV production by one log (8.1×10^3) compared to crickets challenged with *Pmerg*DNV alone (9.85×10^4). Control dsRNA also resulted in a reduction of *Pmerg*DNV (2.2×10^4), but did not produce an inhibitory effect as the targeted stealth interfering RNAs.

As mud crabs *Scylla serrata* cohabit the environment around prawn farms and have been used as a maturation diet for prawn broodstock, they were examined in conjunction with other students for *Pmerg*DNV. Approximately 74% of haemolymph from adults and 100% of inclusion body positive batches of larvae were positive for *Pmerg*DNV by quantitative real-time polymerase chain reaction (PCR), with *Pmerg*DNV titres ranging from 6×10^2 to 1.5×10^5. Sequencing of 2,475 base pairs of viral genome confirmed the virus shared 99% similarity to *Pmerg*DNV. This is the first record of an isolate of hepatopancreatic virus to be found outside penaeid prawns and further complicates the epidemiology of *Pmerg*DNV since control of the virus must involve excluding both wild host species.

This project establishes an *A. domesticus* model for genetic studies of the virus-host interactions and demonstrates the RNAi pathway is a potent antiviral mechanism against *Pmerg*DNV. In this context, administration of *Pmerg*DNV-specific dsRNAi may provide an efficient counter measure against *Pmerg*DNV in prawns and therefore holds considerable promise as a preventative of viral diseases in aquaculture.

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LIST OF ABBREVIATIONS

^{o}C	Degrees Celcius
A_{260}	Absorbance at 260 nanometres
A_{280}	Absorbance at 280 nanometres
*Aae*DNV	*Aedes aegypti* densovirus
*Aal*DNV	*Aedes albopictus* densovirus
AAV	Adeno-associated virus
ABARE	Australian Bureau of Agricultural and Resource Economics
Ad	Adenovirus
ANOVA	Analysis of variance
bp	Base pair
BLAST	Basic Local Alignment Search Tool
*Bm*DNV	*Bombyx mori* densovirus
cap	Capsid
DNA	Deoxyribonucleic acid
dsBRD	Double-stranded RNA binding domain
dsDNA	Double stranded DNA
*Ds*DNV	*Diatraea saccharalis* densovirus
dsRNA	Double-stranded RNA
EGFP	Enhanced green fluorescent protein
g	Gravity
GAV	Gill associated virus
*Gm*DNV	*Galleria mellonella* densovirus
H & E	Haematoxylin and eosin
HIV	Human immunodeficiency virus
HPV	Hepatopancreatic parvovirus
HPVchin	Strain of hepatopancratic parvovirus from *Penaeus chinensis* of Korea
HPV-mac	Strain of hepatopancreatic parvovirus from *Macrobrachium rosenbergii*
HPVsemi	Strain of hepatopancreatic parvovirus from *Penaeus semisulcatus* of India
HSV	Herpes simplex virus

IHHNV	Hawaiian strain of infectious hypodermal and haematopoietic necrosis virus
INIB	Intranuclear inclusion body
ITR	Inverted terminal repeat
*Jc*DNV	*Junonia coenia* densovirus
kb	Kilobase
LPS	Lipopolysaccharide
LSD	Least significant differences
LTS	Long terminal repeats
M	Molar (unit of concentration)
MBV	Monodon baculovirus
MCMS	Mid crop mortality syndrom
mg	Millilitre
mm	Millimetre
mM	Millimoles
mRNA	Messenger RNA
miRNA	MicroRNA
ng	Nanogram
nm	Nanometre
nM	Nanomoles
nt	Nucleotide
ORF	Open reading frame
PCR	Polymerase chain reaction
PCR-ELISA	Polymerase chain reaction-enzyme linked immunosorbent assay
PEG	Polyethylene glycol
PEI	Polyethylenimine
*Pf*DNV	*Periplaneta fuliginosa* densovirus
PL	Postlarvae
*Pmerg*DNV	*Penaeus merguiensis* densovirus
*Pm*DNV	*Penaeus monodon* densovirus (hepatopancreatic parvovirus from *Penaeus monodon* of Thailand)
*Pst*DNV	*Penaeus stylirostris* densovirus
PTGS	Post-transcriptional gene silencing

RdRP	RNA-dependent RNA polymerase
RDS	Runt-deformity syndrome
rep	Replicase
RISC	RNA inducing silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
siRNA	Short-interfering RNA
shRNA	Short-hairpin RNA
SMV	Spawner-isolated mortality virus
SPF	Specific pathogen free
SPSS	Statistical Package for the Social Sciences
ssDNA	Single-stranded DNA
ssRNA	Single-stranded RNA
tRNA	Terminal RNA
μm	Micrometre
μl	Microlitre
WSSV	White spot syndrome virus
YHD	Yellow head disease

CHAPTER 1

General introduction

Aquaculture is the fastest growing food sector in the world. It has a world average annual growth rate of 8.8 percent per year since 1970, compared with only 2.8 percent for terrestrial farmed meat production and 1.2 percent for capture fisheries over the same period (FAO, 2007). Its contribution to global supplies of fish, crustaceans, molluscs and other aquatic animals continues to grow, increasing in total production by weight from 3.9 percent in 1970 to 27.1 percent in 2000 and 32.4 percent in 2004 (FAO, 2007). In terms of trade, prawns continue to be the most valuable commodity accounting for 16.5 percent of the total value of internationally traded fishery products in 2004, followed by groundfish (10.2 percent – i.e. Alaska pollock, cod, haddock and kake), tuna (8.7 percent) and salmon (8.5 percent) (FAO, 2007).

The value of the Queensland component of Australian prawn aquaculture was estimated to be approximately 93 percent ($46.5 million) during 2005-06. As prawn farming expands and intensifies, requirements for improved diets and feeding strategies become increasingly important and diseases become a greater threat. Hence, the management of disease has become of major importance to the long term success of this industry and is now considered a primary constraint to the culture of penaeid species, affecting the quantity of harvest and quality and regularity of production, impeding both economic and social development. Thus, the aquaculture industry has had its share of diseases and problems caused by bacteria, fungi, parasites and viruses. While therapeutic methods such as antibiotics and probiotics can be utilized to treat bacterial, fungal and parasitic infections, there are no current methods to treat disease of viral aetiology in crustaceans since they lack an adaptive immune response. The most important diseases, in terms of economic impact, of cultured marine prawns are of viral aetiology.

The current lack of continuous prawn cell lines suitable for the cultivation and isolation of viral pathogens of prawns is a serious impediment to effective disease control for prawn farming. Progress has been reported in culturing cells from the freshwater crayfish, *Orconectes limosus* using neuronal tissue (Neumann *et al.*, 2000) and *Pacifastacus leniusculus* using primary haematopoietic stem cells to study interactions between the host and its viral pathogen (Soderhall *et al.*, 2005; Jiravanichpaisal *et al.*, 2006). In marine crustacea, short-term primary cell cultures have been used to study host-virus interactions (Tapay *et al.*, 1997; Itami *et al.*, 1999; Tirasophon *et al.*, 2005), but no long-term or permanent cell lines have been established from marine prawns. Thus, information regarding antiviral immunity in prawns has been derived from studies concerning what prawns require to enhance resistance to viral infection. Immuno-stimulants that have been proposed to play a role in prawns include lipopolysaccharide (LPS) from bacteria (Takahashi *et al.*, 2000), glucans derived from yeast (Huang and Song, 1999; Chang *et al.*, 2003), inactivated virus (Bright Singh *et al.*, 2005), virus-encoded proteins (Witteveldt *et al.*, 2004a; Witteveldt *et al.*, 2004b), and double stranded RNA (dsRNA) (Robalino *et al.*, 2004; Robalino *et al.*, 2005; Tirasophon *et al.*, 2005; Westenberg *et al.*, 2005; Yodmuang *et al.*, 2006; Kim *et al.*, 2007; Tirasophon *et al.*, 2007; Xu *et al.*, 2007). The latter is of particular interest since they are likely to be the targets in natural infections, triggering a series of gene silencing mechanisms known as RNA interference (RNAi).

RNA interference (RNAi) is a form of post-transcriptional gene silencing in most eukaryotic organisms for RNA-guided regulation of gene expression. Hence, the RNAi pathway is part of a cell's innate immunity and acts as an intracellular mechanism to eliminate unwanted or invading genetic material. It was first recognised in the 1990s in plants (Napoli *et al.*, 1990) and the nematode worm *Caenorhabditis elegans* (Fire *et al.*, 1998). Nowadays, RNA-guided RNA interference antiviral immunity is recognised to occur in almost all eukaryotic organisms including protozoans (Bastin *et al.*, 2001; Malhotra *et al.*, 2002; Cottrell and Doering, 2003), fungi (Raoni and Arndt, 2003), algae (Wu-Scharf *et al.*, 2000), nematodes (Fire *et al.*, 1998; Caplen *et al.*, 2001; Schott *et al.*, 2005), plants (Fagard and Vaucheret, 2000), insects (Misquitta and Paterson, 1999;

Hughes and Kaufman, 2000), fish (Nasevicuis and Ekker, 2000; Dang *et al.*, 2008) and mammals (Wianny and Zernicka-Goetz, 2000; Caplen *et al.*, 2001).

Hepatopancreatic parvovirus belongs to the subfamily *Densoviridae* of the family *Parvoviridae*, one of the smallest and structurally simplest of the DNA animal viruses (Berns, 1990b; Berns *et al.*, 2000). Although mortalities during the larval stages have been associated with HPV disease (Spann *et al.*, 1997), infection is primarily associated with reduced growth rates of juvenile prawns (Flegel *et al.*, 1999). Hence, farmers are unable to sell infected stock at full market value, and this can result in substantial economic losses. However, there is currently no quantitative information regarding the impact of this disease in Australian penaeid aquaculture, despite the disease being prevalent on Queensland penaeid farms culturing *P. merguiensis*.

The aims of this project were to characterise the HPV isolate from Australian *P. merguiensis*, determine the impact of HPV disease on Queensland *P. merguiensis* farms and to determine if it RNAi was a method that could control the disease.

The work during this project has been published in scientific journals. However, throughout this thesis, the relevant chapter sections will be referenced. Due to conflicting molecular evidence within the literature surrounding the division of of *Penaeus* into six subgenera (Dall, 2007), all species will be referred to as *Penaeus* throughout this project.

CHAPTER 2

Review of literature

2.1. Introduction

The worldwide growing demand for seafood products has resulted in an expansion of the aquaculture industry. The Class Crustacea is a large and diverse group comprising as many as 45 000 species (Castro and Huber, 2000) and, while most exist as part of the food chain, there are some species (crabs, crayfish, lobsters and penaeid prawns) that have become commercially important for fishing and aquaculture. However, infectious diseases of viral origin are considered the most diverse and numerous of the microbial agents causing infectious diseases in crustaceans (Brock and Lightner, 1990) and have increasingly hampered the success of crustacean aquaculture.

Viruses are comprised of a nucleic acid encased within a protein capsid and lack the biosynthetic machinery required for replication, so they are heavily dependent on the cellular machinery of their host for replication (Black, 1996). Among crustacean viruses, members from the family *Parvoviridae* have been responsible for serious economic losses within crustacean aquaculture worldwide. Hepatopancreatic parvovirus (HPV) has been associated with substantial production losses in the penaeid prawn farming industry around the world due to stunting of infected prawns and/or overt mortality (Lightner and Redman, 1985; Lightner *et al.*, 1992; Flegel *et al.*, 1999).

Prevention and control of HPV disease is a priority for the durability of the penaeid prawn industry. However, invertebrates have an innate immune response system, comprising of cellular and humoral mechanisms that contribute to the defence reaction by limiting microbial invasion or for the clearance and killing of invading microbes from tissues and blood circulation (Bachere, 2000). Since they lack an adaptive peptide-based immune response, viral diseases cannot be controlled through the use of vaccines. However, in these organisms, RNA interference (RNAi) may provide antiviral immunity, exerting a specific, potent

and rapid response which is in contrast to their broad spectrum innate immunity and could therefore aid in the control of viral disease in these organisms.

This review will summarise our current understanding of HPV and RNAi as a novel nucleic acid-based antiviral immunity against HPV disease. The literature on RNAi is substantial, and this review is far from exhaustive. Hence, this review will focus on the mechanisms and the different strategies for eliciting RNAi.

2.2. Hepatopancreatic parvovirus

Hepatopancreatic parvovirus is a member of the family *Parvoviridae* (Subfamily: *Densovirinae*), a family of viruses that are amongst the smallest (18-26 nm in diameter) and structurally simplest of the DNA animal viruses (Berns, 1990a). They are icosahedral, non-enveloped, intracellular viruses 18-26 nm in diameter and contain a linear, single stranded genome approximately 4-6 kb in size (Siegl *et al.*, 1985; Berns *et al.*, 2000).

2.2.1. History of discovery

Hepatopancreatic parvovirus was first visualised in wild *Penaeus merguiensis* and *P. indicus* in Singapore (Chong and Loh, 1984). Less than a year later, it was reported in *P. semisulcatus* from Kuwait and in *P. monodon* and *P. chinensis* postlarvae from the Philippines (Lightner and Redman, 1985).

The first Australian record of HPV came from samples of *P. esculentus* from Moreton Bay and the Gulf of Carpentaria in 1985 (Paynter *et al.*, 1985). In 1988, it was reported in *P. merguiensis* (Owens and Hall-Mendelin, 1988), in 1989 by Roubal and colleagues (Roubal *et al.*, 1989) and it has since been recorded in *P. monodon* and *P. japonicus* (Lightner, 1996; Spann *et al.*, 1997).

2.2.2. Geographic range

Hepatopancreatic parvovirus is widely distributed in captive, wild and hatchery-reared prawns throughout the world including Australia, Asia, Africa, Korea, North and South America, Malaysia, China, Taiwan, Philippines, Indonesia, Singapore, Kenya, Israel and Kuwait (Lightner, 1996). Much of what seems to be a cosmopolitan distribution is due to the movement of infected prawns for culture. For example, HPV was introduced to areas throughout North and South America, Mexico and along the coast of El Salvador with the movement of infected *P. vannamei* (Lightner, 1996). It was introduced into Israel and Hawaii through the importation of infected *P. penicillatus* from Kenya and *P. chinensis*, respectively (Brock and Lightner, 1990). However, it was successfully eradicated from Hawaii through the destruction of the infected stock (Brock and Lightner, 1990).

2.2.3. Host range and strains of hepatopancreatic parvovirus

Natural infections have been observed in ten species of penaeid prawns including commercially valuable *P. monodon*, *P. merguiensis* and *P. chinensis* (Table 2.1) (Lightner, 1996). A HPV-like agent has been recorded in postlarvae of the freshwater prawn *Macrobrachium rosenbergii* (Anderson *et al.*, 1990), and is genetically different (as shown by *in situ* hybridisation studies) to the HPV strains in penaeid prawns (Lightner *et al.*, 1994). Therefore, it is not surprising that several strains of HPV have emerged. To date, four penaeid prawn strains of HPV (HPVchin, *Pm*DNV, HPVsemi and *Pmerg*DNV) have been described. The first strain (HPVchin) was reported in 1995 from *P. chinensis* in Korea (Bonami *et al.*, 1995), followed by HPV from *P. monodon* in Thailand (*Pm*DNV) four years later (Sukhumsirichart *et al.*, 1999). In 2005, HPVsemi was reported in wild stocks of *P. semisulcatus* from India (Manjanaik *et al.*, 2005) and the fourth penaeid prawn strain was described from Australian *P. merguiensis* (La Fauce, 2005).

Table 2.1: Recorded natural occurrences of hepatopancreatic parvovirus in penaeid prawns (Lightner, 1996)

Penaeus chinensis	*Penaeus esculentus*
Penaeus indicus	*Penaeus japonicus*
Penaeus merguiensis	*Penaeus monodon*
Penaeus penicillatus	*Penaeus semisulcatus*
Penaeus stylirostris	*Penaeus vannamei*

2.2.4. Transmission

The first report on the successful horizontal transmission of HPV was in 2003 and involved orally challenging *P. monodon* postlarvae with HPV (Catap *et al.*, 2003). Additionally, HPV is thought to be vertically transmitted, after progeny of *P. chinensis* held in quarantine in Hawaii developed the disease (Lightner, 1996). However, this has not been proven experimentally and since HPV is not known to occur in the reproductive tissues of infected prawns, it may be that the eggs and larvae were infected with the virus via infected water (HPV being released via the faeces of infected individuals) and not directly from the broodstock.

2.2.5. Clinical signs of disease

Hepatopancreatic parvovirus typically affects the mid-juvenile stages of penaeid prawns. Prawns affected by HPV usually show non-specific gross signs of disease including anorexia, reduced preening activity (resulting in an increase in fouling on the carapace by epicommensals and higher susceptibility of the prawn to secondary infections by bacteria and fungi) and in particular, reduced growth rates (referred to as runt-deformity syndrome) (Lightner and Redman, 1985; Paynter *et al.*, 1985; Brock and Lightner, 1990; Lavilla-Pitogo, 1996; Lightner, 1996). Mortalities often follow the onset of clinical signs of disease in chronic infections. However, HPV is seldom observed alone in epizootics and usually occurs in multiple agent epizootics with high mortality rates (Spann *et al.*, 1997). Hence, mortalities due to HPV are difficult to document.

The severity of HPV-infection was suggested by Lightner (1996) to be enhanced by subjecting infected individuals to the stress of overcrowding. However, other factors must be considered when subjecting animals to overcrowding stress. For example, environmental factors such as low dissolved oxygen concentrations (due to the increased numbers of prawns) or dual infections may have contributed to enhanced expression of disease. Furthermore, increased level of HPV may have been due to the surviving animals feeding on sick and dying individuals. The role of various factors including dual infections and the susceptibility of different life stages of the host species to HPV necessitates further investigation.

2.2.6. Histopathology

Hepatopancreatic parvovirus infection is primarily restricted to the hepatopancreas of infected penaeid prawns, except in Australian *P. monodon* where the single case of HPV infection was observed in the midgut caecae (Leigh Owens pers. comm.). The hepatopancreas comprises of long, finger-like, blind-ending tubules lined by an epithelium layer consisting of four cell types: E-(embryonic); F-(fibrillar); R-(resorptive) and B-(secretory) cells (Gibson, 1979). However, HPV only affects the rapidly dividing E- and F- cells, located at the distal end of the tubules (Lightner *et al.*, 1993).

Infected cells are hypertrophic and contain a single (rarely more than one), prominent basophilic (with haemotoxylin & eosin (H & E) stain) intranuclear inclusion body (Figure 2.1), containing densely packed HPV virions that range in size from 22-24 nm in diameter (Lightner and Redman, 1985; Bonami *et al.*, 1995; Sukhumsirichart *et al.*, 1999; Manivannan *et al.*, 2002; Catap *et al.*, 2003). Inclusions early in development are small, centrally located within the nucleus and closely associated with the nucleolus. During development, inclusions enlarge, and occupy most of the karyoplasm of hypertrophied nuclei (Lightner and Redman, 1985; Catap *et al.*, 2003; Rukpratanporn *et al.*, 2005) (except in the freshwater prawn *Macrobrachium rosenbergii* where the cell nuclei do not possess a laterally displaced nor prominent nucleolus) (Lightner *et al.*, 1994).

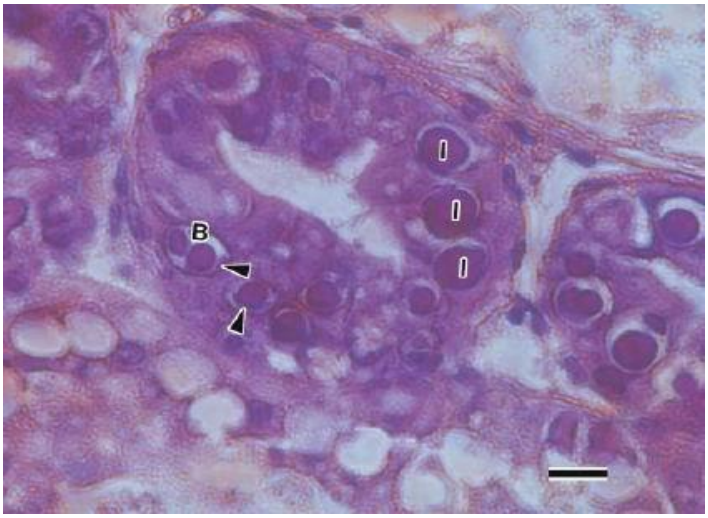


Figure 2.1: Tubules of hepatopancratic parvovirus-infected hepatopancreatic tissue of *Penaeus monodon* with hypertrophied nuclei and inclusion bodies (I). The cell nucleolus may be compressed by the developing inclusion body (arrowheads) and some hepatopancreatocytes can contain two inclusion bodies (B). H & E stain; scale bar = 10 µm (Catap *et al.*, 2003)

2.2.7. Economic losses due to hepatopancreatic parvovirus

Hepatopancreatic parvovirus is commonly observed in association with other pathogens including *Penaeus stylirostris* densovirus (*Pst*DNV), white spot syndrome virus (WSSV) and monodon baculovirus (MBV) (Lightner and Redman, 1985; Umesha *et al.*, 2003; Chayaburakul *et al.*, 2004). For this reason, the effects of HPV are often masked by the effects of the other pathogens. For example, HPV infection alone was only recorded in 2% of 95 *P. monodon* postlarvae whereas close to one third of postlarvae screened had multiple infections involving HPV (Table 2.2) (Umesha *et al.*, 2003). It would therefore be difficult to confirm that HPV was the sole cause of mortalities when it occurs in association with other pathogens. Furthermore, HPV was implicated for causing mortalities of up to 80% in pond-reared postlarval *P. monodon*, despite MBV being present at a high prevalence in moribund individuals (Flegel and Fegan, 1995). Hence, quantitative information regarding economic losses due to HPV is difficult to document.

Table 2.2: Prevalence of multiple viral infections in *Penaeus monodon* postlarvae from India (Umesha *et al.*, 2003)

	Number of samples
HPV, MBV, WSSV positive	26
HPV and MBV positive	1
HPV and WSSV positive	3
MBV and WSSV positive	21
HPV positive	2
MBV positive	8
WSSV positive	26
HPV, MBV and WSSV negative	8
Total	95

(HPV: Hepatopancreatic parvovirus; MBV: Monodon baculovirus; WSSV: White Spot Syndrome Virus)

2.2.7.1. Mortalities

Economic losses from HPV infection may be due to the direct mortality of infected stock. For example, mortalities ranging from 50-100% were recorded within 4-8 weeks of disease onset in juvenile *P. merguiensis* in the 1980's (Lightner and Redman, 1985). It has also been suggested the percentage of HPV infected larvae prior to stocking closely corresponds to the expected minimum mortality for that pond (Sukhumsirichart *et al.*, 1999).

2.2.7.2. Runt-deformity syndrome

Infected prawns have a reduced growth rate (referred to as runt-deformity syndrome (RDS)) which can result in substantial production losses for farmers because they continue to spend money on the maintenance of ponds and food for prawns that are not growing. A negative correlation has been recorded between length of prawns and severity of HPV infection (Flegel *et al.*, 1999). Despite the correlation, it cannot be assumed the relationship between HPV infection and RDS is a causative one. Nonetheless, stunting has become a major source of economic losses, particularly in Thailand for farmed *P. monodon* (Table 2.3).

Table 2.3: Production and value of *Penaeus monodon* in Thailand from 2000 to 2002. Note the reduction in production and hence economic value over the years due to increasing problems with runt-deformity syndrome (Chayaburakul *et al.*, 2004)

Year	Number of tonnes (t) of *Penaeus monodon* produced	Value (US$ million)
2000	249 633 t	$2697
2001	255 568 t	$2467
2002	212 019 t	$1846

2.2.8. Summary of hepatopancreatic parvovirus

Hepatopancreatic parvovirus has a worldwide geographic distribution and caused disease in at least ten penaeid species, including commercially produced species such as *P. chinensis*, *P. merguiensis* and *P. monodon*. Hepatopancreatic parvovirus infection results in a reduced growth rate of infected prawns (consequently reducing their market value) and in chronic infections, the onset of clinical signs of disease are often followed by mortality. Hence, this virus has been responsible for causing substantial economic losses within the penaeid prawn industry worldwide. Hepatopancreatic parvovirus seems to be an emerging disease so novel control strategies against this virus are highly desirable.

2.3. Protecting Crustacea against viral diseases

In contrast to vertebrates, invertebrates lack a true adaptive immune response system (but rather rely on various innate immune responses) and although the invertebrate immune system has been well studied in the context of antibacterial and antifungal responses (Destoumieux-Garzon *et al.*, 1997; Destoumieux-Garzon *et al.*, 2000; Destoumieux-Garzon *et al.*, 2001; Acharya *et al.*, 2004; Cheng *et al.*, 2005), information is limited regarding the invertebrate immune response directed against viruses.

The innate immune system is activated by pathogens or environmental antigens and is mediated by the interaction between receptors or pattern recognition proteins and pathogens (Medzhitov and Janeway, 1997). The innate defence system includes a series of humoral and cellular immune mechanisms that contribute to the pathogen defence reaction by limiting microbial invasion or by

helping the clearance/killing of the invading microbes from the blood and tissues (Soderhall and Cerenius, 1992). The prophenoloxidase system (Soderhall and Cerenius, 1992; Aspan *et al.*, 1995) and antimicrobial peptides (Destoumieux-Garzon *et al.*, 1997; Destoumieux-Garzon *et al.*, 2000; Destoumieux-Garzon *et al.*, 2001) have been linked with prawn innate immunity.

Almost all studies concerning innate immunity of prawns have concerned bacteria, fungi or parasites rather than viruses. Previous studies showed the existence of non-protein antiviral substance in crustaceans, but so far little is known about the possible innate antiviral factor generated by the interaction between host cell and virus, and neither antiviral gene nor antiviral protein has been characterised from crustaceans (Luo *et al.*, 2003; Zhang *et al.*, 2004). A better understanding of the immune response of crustacea will help in the design of more efficient strategies for disease control. However, since they possess an innate immune response, vaccines cannot be used as a method to control or prevent viral infections. One method that does show promise as an antiviral mechanism in invertebrates is RNA interference (RNAi).

2.4. RNA interference

RNA interference (RNAi) is an evolutionary conserved, natural gene silencing phenomenon in which double stranded RNA (dsRNA) silences gene expression, by either inducing sequence-specific degradation of complementary messenger RNA (mRNA) or by inhibiting translation (Mittal, 2004; Hammond, 2005). This homology-dependent gene silencing was first discovered in transgenic plants (Matzke *et al.*, 1989; Napoli *et al.*, 1990; Van der krol *et al.*, 1990) where pioneering work by plant virologists established plants responded to viral infections by inducing RNAi, resulting in the specific recognition and destruction of the invading viral RNAs and homologous host RNAs (Baulcombe, 2004; Qu and Morris, 2005). The phenomenon of RNAi has subsequently been described in a wide range of eukaryotic organisms including arthropods and mammals (Fire *et al.*, 1998; Elbashir *et al.*, 2001c; Li *et al.*, 2002a; Ge *et al.*, 2003; McCaffrey *et al.*, 2003). To date, RNAi has been used as a strategy to investigate gene function (Alper *et al.*, 2008; Berns *et al.*, 2008; Zhu *et al.*, 2008) (for example, it has been used to analysis the function of close to 17 000 of the 19 000 (approximate

figure) genes in *C. elegans* (Kamath *et al.*, 2003; Tucker and Han, 2008) and as an antiviral mechanism to combat viral infections in plants (Tenllado *et al.*, 2004; Zao *et al.*, 2006), invertebrates (Travanty *et al.*, 2004; Zambon *et al.*, 2006; Dang *et al.*, 2008) and vertebrates (in particular, influenza, cancer and human immunodeficiency virus (HIV) (Gu *et al.*, 2006; Gu *et al.*, 2008; McSwiggen and Seth, 2008; Singh, 2008; Wang *et al.*, 2008)).

The use of RNAi as an antiviral mechanism is particularly important for invertebrates such as crustaceans that lack an adaptive immune response (have innate immunity) and therefore, cannot be vaccinated as a preventative measure against viral diseases. Viruses contribute to substantial economic losses within penaeid prawn aquaculture so strategies to prevent or combat viral infections are highly desirable.

2.5. Discovery and current understandings RNA interference

The first indication of the existence of homology-dependent gene silencing emerged when researchers attempting to deepen the colour of petunia flowers by expressing higher levels of the enzyme (chalcone synthase) responsible for the synthesis of pigment, unexpectedly resulted in reduced or lack of floral pigmentation and a reduction in gene expression (Matzke *et al.*, 1989; Napoli *et al.*, 1990; Van der krol *et al.*, 1990). This occurrence was designated the term co-suppression, indicating that transgenes themselves were inactive and that the DNA sequences were somehow affecting the expression of the endogenous loci (Hamilton and Baulcombe, 1999). The silencing of homologous genes in the genome for plants may also be referred to post-transcriptional gene silencing (PTGS) or quelling (Rao and Sockanathan, 2005).

The term RNAi emerged following the 1998 demonstration of the development of sequence-specific gene silencing by injection of dsRNA into *Caenorhabditis elegans* (Fire *et al.*, 1998). Fire and colleagues demonstrated a mixture of antisense and sense RNA (dsRNA) was able to direct the degradation of mRNA and was at least tenfold more potent as a silencing trigger than were sense or antisense RNAs alone. This 'gene silencing' was achieved by simply feeding *C. elegans* the bacterium *Escherichia coli* (the preferred diet of *C. elegans*) containing plasmids with the dsRNA-encoding DNA sequences.

Following the work by Fire and colleagues, there was increased interest in the use of RNAi in both plants and animals. To date, RNAi related events have been recognised in almost all eukaryotic animals including algae (Wu-Scharf *et al.*, 2000), yeast (Raoni and Arndt, 2003), protozoans (LaCount *et al.*, 2000; Bastin *et al.*, 2001; Malhotra *et al.*, 2002; Cottrell and Doering, 2003), plants (Fagard and Vaucheret, 2000), insects (Aldeman *et al.*, 2002; Beye *et al.*, 2002; Caplen *et al.*, 2002; Li *et al.*, 2002a; Kavi *et al.*, 2005), fish (Nasevicuis and Ekker, 2000; Dang *et al.*, 2008) and in mammals (Wianny and Zernicka-Goetz, 2000; Elbashir *et al.*, 2001a; Ge *et al.*, 2003; McCaffrey *et al.*, 2003).

2.6. RNA interference pathway

The mechanism for RNAi remained unexplained for some time. An early crucial observation in determining the mechanism behind RNAi was the production of short RNAs (20-25 nucleotides long) matching the gene being silenced by plants that were silencing genes in a RNAi related process (PTGS) (Hamilton and Baulcombe, 1999). Similarly, it was revealed by reconstituting the biochemical pathways of RNAi *in vitro* using fruitfly extracts that long dsRNAs were diced up into short RNAs (Elbashir *et al.*, 2001b). Furthermore, the short RNAs had a specific structure involving two 21-nucleotide strands of RNA in a staggered duplex, with 19 nucleotides of dsRNA and two unpaired nucleotides at the ends (Elbashir *et al.*, 2001b).

Current understandings of the mechanisms underlying RNAi are derived from genetic studies involving the introduction of dsRNA in *C. elegans* (Fire *et al.*, 1998; Tabara *et al.*, 1999; Ketting *et al.*, 2001; Parrish and Fire, 2001) and *Drosophila* (Kennerdell and Carthew, 1998; Elbashir *et al.*, 2001b). To date, the process of RNAi can be divided into two stages: the initiator and the effector stage. More commonly, the mechanisms underlying RNAi are referred to as a 'dice and slice mechanism' (summarised in Figure 2.2) (Hannon and Rossi, 2004; Novina and Sharp, 2004; Shanker *et al.*, 2005; Lee and Shinko, 2006).

2.6.1. Initiator step

The initiator stage is limited to the cytoplasm of the cell (Hutvagner and Zamore, 2002; Zeng and Cullen, 2002) and involves generating short interfering RNAs (siRNA) from long dsRNA, or microRNAs (miRNA) from endogenous hairpin RNA precursors, that appear in the cell from their primary transcripts by an RNase III-like protein, known as Dicer (Hannon, 2002; Doench *et al.*, 2003; Baulcombe, 2005). Dicer is a large protein (approximately 220 kDa) containing a dsRNA binding domain (dsBRD), two catalytic RNAse III domains, a helicase domain and a piwi-argonaute-zwille (PAZ) interaction domain which binds small RNAs (Bernstein *et al.*, 2003; Ma *et al.*, 2004). Dicer unwinds and cleaves long dsRNA duplexes into siRNAs 21-23 nucleotide DNA fragments with symmetric 2-3 nucleotide 3' overhangs and 5'-phosphate and 3'-hydroxyl groups (Dykxhoorn *et al.*, 2003). Any siRNAs that lack a 5' phosphate are rapidly phosphorylated by an endogenous kinase (Nykanen *et al.*, 2001; Schwarz *et al.*, 2002).

The siRNAs are incorporated and bound into a multiprotein RNA-inducing silencing complex (RISC). Functional RISC contains four different subunits including endonuclease, exonuclease, helicase and homology searching domains (Lee and Shinko, 2006). The duplex siRNAs are unwound by an RNA helicase (Nykanen *et al.*, 2001), leaving the antisense strand (referred to as the guide strand) to guide RISC to its homologous target mRNA for endonucleolytic cleavage (Dykxhoorn *et al.*, 2003). Small interfering RNAs that do not require Dicer processing are able to directly enter the RISC, but induce silencing less efficiently than siRNA precursors induced by Dicer (Kim *et al.*, 2004a).

2.6.2. Effector step

The effector step is essentially the last process of the RNAi pathway. It is achieved by the multiprotein complex RISC and involves the endonucleolytic destruction of the targeted mRNA. The target mRNA is cleaved at a single site in the centre of the duplex region between the guide siRNA and the target mRNA, 10 nucleotides (nt) from the 5' end of the siRNA (Elbashir *et al.*, 2001b). This is achieved through the proteins of the Argonaute family (e.g. *rde-4*, *rde-1* and *drh-1/2* in *C. elegans*) (Tabara *et al.*, 2003) or Argonaute2 in *D. melanogaster* (Williams and Rubin, 2002) which possess nuclease activity responsible for mRNA target cleavage. Cleavage is endonucleolytic, and occurs only in the region homologous to the siRNA (Zamore *et al.*, 2000; Elbashir *et al.*, 2001c). The cleaved RNA is rapidly degraded and consequently, the protein for which it encodes is not produced.

Alternatively, translation may be suppressed without mRNA cleavage, but is more typical for miRNA-mediated gene silencing (Hammond, 2005). Compared with siRNAs that can be both artificial or endogenous, miRNAs are always encoded by the genome itself as hairpin structures (Hamilton and Baulcombe, 1999; Aravin *et al.*, 2001; Reinhart and Bartel, 2002; Ambrose *et al.*, 2003). However, both molecules are involved in the same process that leads to mRNA degradation or the inhibition of protein synthesis. As a general rule, siRNAs cause mRNA destruction, whereas miRNAs can cause both mRNA destruction and the inhibition of protein synthesis. If there are variations or mismatches in nucleotide base pairs in the siRNA/miRNA duplex (more common for miRNAs), the mRNA is not cleaved and gene silencing results from translational inhibition (Hammond, 2005).

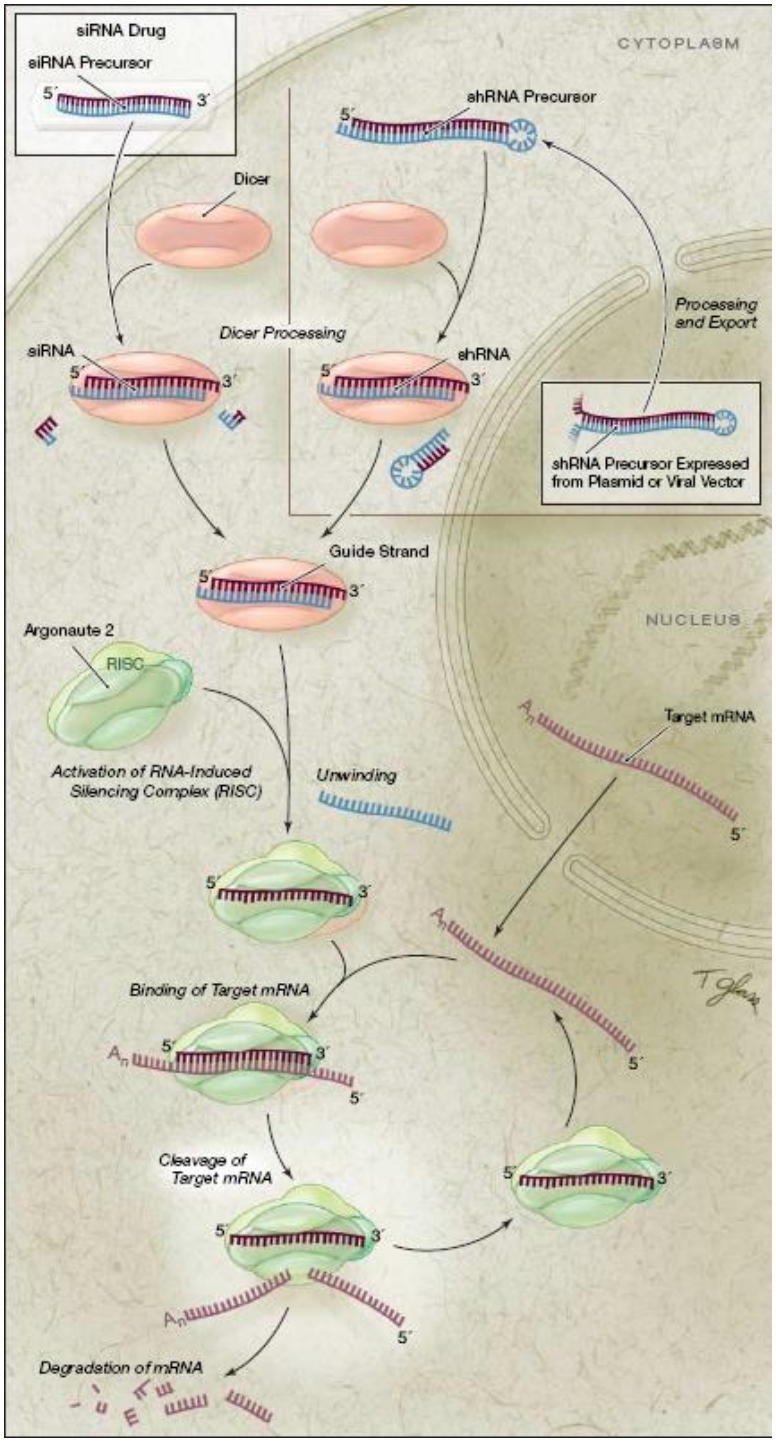


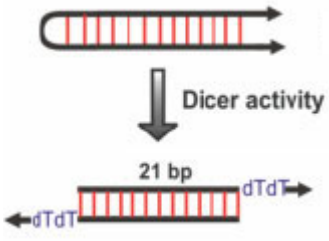
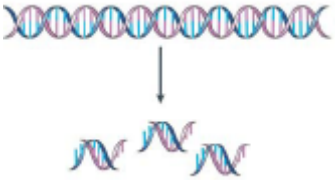
Figure 2.2: Mechanism of RNA interference. The processing of small interfering RNA precursors by Dicer leads to the formation of siRNAs that are incorporated with cellular proteins to form an RNA-induced silencing complex. The duplex siRNAs are unwound and the antisense strand guides RISC to the target mRNA for endonucleolytic cleavage (Shanker *et al.*, 2005)

2.7. Triggers of RNA interference

RNA interference is a conserved eukaryotic gene regulatory system that suppresses gene expression through RNA-mediated sequence-specific interactions. It is initiated by the presence of dsRNA and can be achieved in the laboratory through a number of difference strategies. To date, six types of double stranded RNA have been identified that trigger RNAi (Table 2.4). Although all six types of dsRNA have proven to be effective to various degrees (Yang *et al.*, 2000; Zamore *et al.*, 2000; Elbashir *et al.*, 2001a; Elbashir *et al.*, 2002; Paddison *et al.*, 2002a; Ambrose *et al.*, 2003), siRNAs tend to produce the greatest silencing (often with the least toxicity) (Rao *et al.*, 2004) and have therefore been the common approach for inducing RNAi based gene silencing. They are incorporated into the RNAi pathway at a later stage and are therefore less likely to interfere with gene regulation by endogenous microRNAs (Grimm *et al.*, 2006; John *et al.*, 2007). Furthermore, siRNAs can be manufactured by processes that are amenable to large-scale production and can be modified to contain drug-like properties, making them particularly attractive as therapeutics. Hence, they are the class of RNAi therapeutics that is the most advanced in preclinical and clinical studies (de Fougerolles, 2008).

Three strategies involving chemical synthesis, *in vitro* enzymatic synthesis, and DNA plasmid vector exist for generating siRNAs for gene silencing. Each method is associated with specific advantages and disadvantages (Table 2.5).

Table 2.4: Description of the different types of double stranded RNA that triggers RNA interference*

Short interfering RNAs (siRNAs)	A class of double stranded RNAs of 21-22 nucleotides in length, generated from dsRNA. siRNAs promote gene silencing through the cleavage of mRNAs with exact complementary sequences.	
MicroRNAs (miRNAs)	A class of 19-25 nucleotide, single stranded RNAs that are encoded in the genomes of most multicellular organisms studied. Gene silencing occurs at the stage of protein synthesis.	
Short hairpin RNAs (shRNAs)	A class of single stranded RNA molecules containing inverted repeats that allow them to form intramolecular duplexes. shRNAs are processed by Dicer and serve as a template for sequence-specific silencing.	
Long double stranded RNA (dsRNA)	A class of double stranded RNAs that are introduced into cells and processed by Dicer into shorter fragments in 21-23 nucleotide intervals (siRNAs) that silence gene expression.	
Short interfering RNA (siRNA)-based hairpin RNA	A class of siRNAs produced from the cleavage of duplex hairpin RNA by Dicer	
MicroRNA (miRNA)-based hairpin RNA	Imperfect duplex hairpin RNA cleaved by Dicer into miRNAs and direct gene silencing	

*(Zamore *et al.*, 2000; Elbashir *et al.*, 2001b; Caplen *et al.*, 2002; Paddison *et al.*, 2002a; Paddison *et al.*, 2002b; Ambrose *et al.*, 2003; Novina and Sharp, 2004; Rao and Sockanathan, 2005)

Table 2.5: Advantages and disadvantages of short interfering RNA synthesis strategies (Duxbury and Whang, 2004)

	Advantages	**Disadvantages**
Chemical and *in vitro* enzymatic synthesis	• Rapid synthesis • High purity using chemical synthesis • RNase III: produces siRNA mixtures, reducing the need to test the efficacy of multiple siRNAs separately	• Purity and specificity using enzymatic synthesis is variable • Chemical synthesis expensive for multiple siRNAs • Transient RNA
DNA plasmid vector	• Stable RNAi achievable using selection marker • More economical for multiple sequences	• Construction of a DNA plasmid vector is labour intensive • Transfection-dependent

2.7.1. Chemically synthesised short interfering RNAs

The use of siRNAs that are synthesised, purified and annealed by chemical processes (Elbashir *et al.*, 2001b) are becoming increasingly popular. Chemically synthesised siRNAs are directly introduced into the cytoplasm bypassing the 'Dicer' step. Perfect duplex hairpin RNA is cleaved by Dicer into siRNAs and imperfect duplex hairpin RNA is cleaved by Dicer into miRNAs that are responsible for directing gene silencing. MicroRNAs are a class of short noncoding RNAs that have an incomplete sequence homology to their targets (Dillon *et al.*, 2005). MicroRNAs primarily function as siRNAs by preventing the translation of mRNA into protein rather than destroying the mRNA transcript (Shanker *et al.*, 2005). However, miRNAs cannot be designed by computational methods, unlike siRNAs.

Chemically manufactured siRNAs can be synthesised at higher amounts and have been proven to be the most efficient (despite the higher cost and increased synthesis time) triggers of sequence-specific mRNA degradation when they contain 2 nt 3' overhangs (Elbashir *et al.*, 2001c; Kim *et al.*, 2004b). However, this was originally noted in *D. melanogaster* and what may work best for one species, may not necessarily work optimally for other species. Nevertheless, it should be used as a starting point when chemically synthesising siRNAs. Dicing kinetics have been recorded with the use of siRNAs longer than 27 bp (Amarzguioui *et al.*, 2005). Even so, chemically generated siRNAs have since been employed as the most efficient means of inducing RNAi.

2.7.2. *In vitro* enzymatic synthesis of short interfering RNAs

Enzymatically generated siRNAs (*in vitro* siRNA synthesis) involves T7 phage RNA polymerase mediated *in vitro* transcription and is the quickest and most cost effective method for siRNA synthesis. The polymerase produces individual sense and antisense siRNA strands that form siRNAs when annealed (Donze and Picard, 2002; Yang *et al.*, 2002; Sohail *et al.*, 2003).

Transcription is generated from short double-stranded oligo cassettes containing the promoter sequence upstream of the siRNA template sequence that is to be transcribed (Donze and Picard, 2002; Sohail *et al.*, 2003). Transcription begins and terminates at specific initiation and termination sequences, determined by the promoter (Elbashir *et al.*, 2001c). Small inverted repeats are produced, separated by three to nine nucleotides, termed short hairpin RNAs (shRNAs), that are subsequently converted into siRNAs by Dicer (Elbashir *et al.*, 2001c; Paddison *et al.*, 2002b; Sui *et al.*, 2002). The siRNA strands are synthesised in separate reactions and often contain a GGG leader sequence. Consequently, the siRNA needs to be processed by T7 ribonuclease to remove the single stranded 5' GGG overhang. Furthermore, siRNAs must not be transcribed with a UU 3' overhang, otherwise T7 processing may be incomplete due to the formation of two G:U wobble base pairs, resulting in non-specific inhibition of gene expression (Kim *et al.*, 2004b).

2.7.3. Plasmid vector

Polymerase III promoter-based DNA plasmids can be used to produce siRNAs for gene silencing (Sui *et al.*, 2002). Short hairpin RNAs are produced which are subsequently processed by Dicer into siRNAs. The use of plasmid generated siRNAs enables stable RNAi expression for up to 2 months post-transfection (Brummelkamp *et al.*, 2002).

2.8. Delivery strategies

Effective delivery to the appropriate cells or tissue remains a major hurdle for successful RNAi. Numerous *in vivo* delivery strategies exist, ranging from the simple local delivery of 'naked' siRNA duplexes to the more complicated methods involving the systemic delivery of siRNA complexes as conjugates, liposome/lipoplexes, as complexes with peptides, polymers or antibodies or vector mediated. Vectors used in DNA based technology can be broadly categorised as those derived from viruses (Kay *et al.*, 2001) and those that are not (for example, plasmids) (Liu and Huang, 2002; Schmidt-Wolf and Schmidt-Wolf, 2003). Each approach has its advantages and disadvantages which must be considered when choosing an appropriate delivery strategy. This section provides a review of the various delivery approaches used with siRNA. However, a comprehensive listing of successful *in vivo* efficacy studies can be found in numerous review publications (de Fougerolles *et al.*, 2005; Aigner, 2006; Bumcrot *et al.*, 2006).

2.8.1. Virus vector mediated

The use of viral vectors or plasmids to express RNAi effector molecules results in the stable expression of RNAi which may otherwise have varied in transfection efficiency (Kay *et al.*, 2001; Liu and Huang, 2002). Gene transfer using viral vectors is referred to as transduction. Transduction is defined as the introduction of functional genetic information of the host genome (either plasmid or chromosomal) to a recipient cell through the use of a recombinant viral vector (Russell *et al.*, 1995; Snyder, 1999; Kay *et al.*, 2001). The life cycle of a virus requires them to efficiently transfer their own genetic material to the cells they infect. The introduction of the viral genome into the host cell leads to an early phase of gene expression characterised by the appearance of viral regulatory

products. This is followed by the expression of structural genes and the assembly of new viral particles. In the case of gene therapy vectors, elements of the viral genome that contribute to replication, virulence and disease are deleted to reduce pathogenicity and immunogenicity and replaced by a therapeutic gene cassette carrying the genes of interest (Figure 2.3). The residual viral genome and the gene of interest (referred to as a transgene) are integrated into the vector construct which contains the transgene and *cis*-acting sequences that are essential for encapsidation of the vector genome and viral transduction of the target cell. The vector and packaging constructs are subsequently expressed in the packaging cells, which produce the recombinant viral particles (Figure 2.4) (Kay *et al.*, 2001; Pfeifer and Verma, 2001).

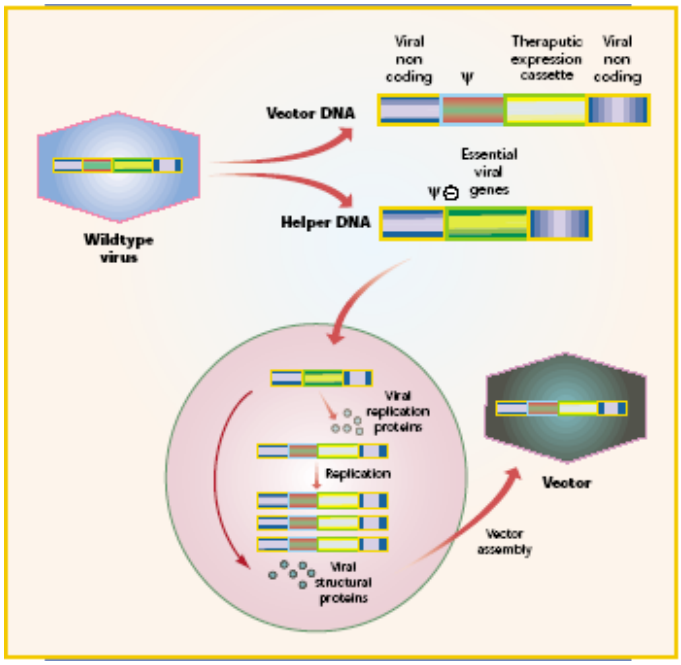


Figure 2.3: Basic principle for the assembly of a virus vector (Kay *et al.*, 2001)

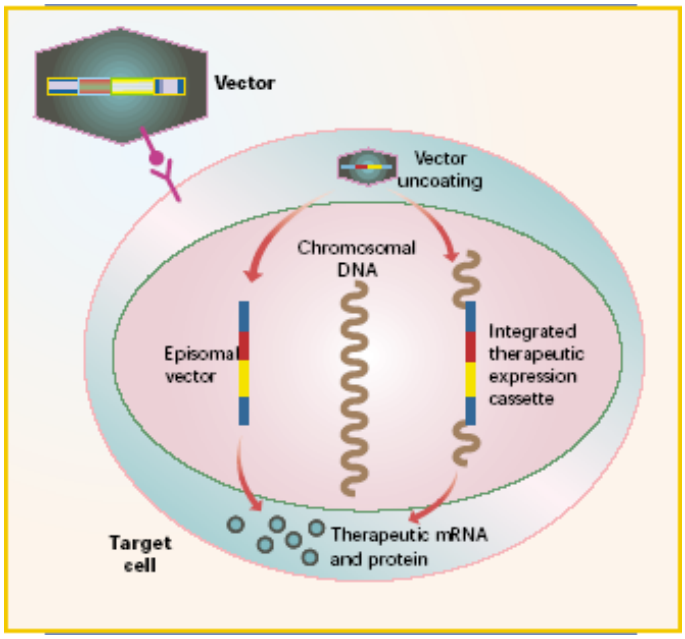


Figure 2.4: Transduction of the target cell. The vector particle containing the gene of interest binds to and enters the cell, in which the genome enters the cell nucleus, resulting in the expression of the gene (Kay *et al.*, 2001)

For gene therapy to be successful, an appropriate amount of a gene must be delivered into the target tissue without substantial toxicity. Viral vectors can be divided into three categories: (a) integrating vectors, (b) non-integrating vectors and (c) hybrid vectors. Each viral vector is characterised by an inherent set of properties that affect its suitability for specific gene therapy applications. For example, gene therapies designed to interfere with a viral infectious process may require gene transfer into a large number of cells. For the delivery of therapeutic nucleic acids, viruses provide an efficient means for the delivery of therapeutic nucleic acids (even in cells resistant to transfection with dsRNA and plasmid) due to the inherent ability of viruses to transport genetic material into cells (Verma and Somia, 1997). However, viral delivery systems have a limited loading capacity since it is difficult to produce the genetic material for large scale applications, construction of a viral vector is labour intensive and their use poses several safety risks (Chapter 2.9.4) such as their oncogenic potential and their inflammatory and immunogenic effects which prevent them from repeated administration (Lehrman, 1999; Liu and Muruve, 2003; Sun *et al.*, 2003).

This section will briefly describe the properties of viral vectors commonly used for gene therapy and their advantages and limitations in inducing RNAi (Table 2.6). There are difficulties associated with determining the vector system most applicable for research applications because each vector has its own specific properties which determine its suitability for research applications. It is therefore up to the discretion of the researcher as to what vector system would be suitable for their specific research application. A description of each vector system and its modification for application to gene transfer is presented here.

2.8.1.1. Integrating viral vectors

Integrating viral vectors are capable of providing long-life expression of the transgene and include adeno-associated virus (AAV), lentivirus and retroviral derived vectors. This is in contrast to gene delivery using adenovirus, herpes simplex virus (HSV) and baculovirus viral vectors where the viral genome remains episomal.

Table 2.6: Properties of the prominent types of virus vectors used for RNA interference*

Type of viral vector	Virus	Salient properties
Integrating	Adeno-associated virus (AAV)	• Transduces non-dividing and dividing cells • Infects a variety of cell types • Limited packaging capacity • Difficult to produce • Recombinant virus may not integrate • Long-term gene expression reported • Remains infectious after heating and freeze-drying
	Retrovirus-Lentivirus	• Transduces dividing and non-dividing cells • Difficult to produce • Moderate packaging capacity
	Retrovirus-*Oncoretrovirus*	• Only transduces dividing cells • Efficient integration into the chromatin of target cells • Moderate packaging capacity • Straightforward production • Moderately high titers possible
Non-integrating vectors	Adenovirus (Ad)	• Transduces dividing and non-dividing cells • Tranduces a variety of cell types • Straightforward production • Packaging capacity moderate to high, depending on construct • High titers of the transgene are expressed • Efficient for short-term expression of the transgene • Virions highly antigenic • Early generation vectors express viral proteins in transduced cells • Later generation vectors do not express viral proteins in transduced cells
	Herpes simplex virus	• Transduces non-dividing cells • Very large packaging capacity • Difficult to produce • High titers achieved • Early generations are cytotoxic • Efficient in a variety of cell types • Difficulties associated with long-term expression • Multiple sites for foreign DNA insertion
	Baculovirus	• Large packaging capacity • Large scale production of protein • Stable and prolonged silencing can be achieved • Cell lysis-associated proteolysis may become a problem • Restricted host range (invertebrates) • Non pathogenic to vertebrates • Multiple sites for foreign DNA insertion

*(Miller, 1988; Maeda, 1989; Lewis *et al.*, 1992; Verma and Somia, 1997; Lee *et al.*, 2000; Kay *et al.*, 2001; Mitani and Kubo, 2002; Evans *et al.*, 2006; Lin *et al.*, 2006)

2.8.1.1.1. Adeno-associated virus

Adeno-associated virus (AAV) is a member of the parvovirus family and contains a 4.7 kilobase (kb) single stranded DNA (ssDNA) genome. The viral genome consists of two genes (capsid and replicase genes) situated along the genome between two inverted terminal repeats (ITRs) that define the beginning and end of the virus and contain the packaging sequence (Verma and Somia, 1997; Kay *et al.*, 2001). Each gene produces multiple polypeptides. The capsid (*cap*) gene encodes the viral capsid protein and the replicase (*rep*) gene product is involved in viral replication and integration. Thus, to generate a recombinant AAV vector, *rep* and *cap* genes can be deleted to leave only the virus's terminal repeats involved in viral replication and integration (Rolling and Samulski, 1995). A helper virus (commonly adenovirus and herpes simplex virus) provides AAV with the additional genes it requires for replication. The virus can infect a variety of dividing and non-dividing cell types and remains infectious after heating or freeze-drying (Verma and Somia, 1997; Kay *et al.*, 2001; Evans *et al.*, 2006).

However, in contrast to wild-type AAV, recombinant virus carrying a therapeutic gene in place of the normal viral *rep* and *cap* genes, loses its ability to specifically integrate and in certain cell types, the recombinant AAV is maintained episomally (rather than being integrated into the host genome) where it only expresses the therapeutic gene after second-strand synthesis (Ferrari *et al.*, 1996). Despite this, efficient, long-term, stable gene expression has been achieved using recombinant AAV (Koeberl *et al.*, 1997).

2.8.1.1.2. Retroviruses

Retroviruses are lipid-enveloped particles comprising of linear, positive sense, single stranded RNA (ssRNA) genomes of 7 to 11 kb. Retroviruses are a group of viruses whose genome is retro-transcribed into linear dsDNA and integrated into the cell chromatin following entry into the target cell (Kay *et al.*, 2001). The genome consists of three genes (*gag*, *pol* and *env*), which are required in *trans* for viral replication and packaging. These three genes are situated along the genome between two long terminal repeats (LTRs) that define the beginning and end of the viral genome (Verma and Somia, 1997). The LTRs contain a

promoter, polyadenylation, and integration sequences, a packaging site termed *psi* (which allows the viral RNA to be distinguished from other RNAs in the cell) and a terminal RNA (tRNA) binding site, as well as several additional sequences involved in reverse transcription and are also involved in controlling the expression of the viral genes. The viral genes encoding the three proteins can be removed and replaced with heterologous genes and transcriptional regulatory sequences which are subsequently transcribed under the control of the LTRs or enhancer-promotor elements engineered with the transgene (Verma and Somia, 1997; Kay *et al.*, 2001; Evans *et al.*, 2006).

Recombinant retrovirus infects target cells through a specific interaction between the target cells surface receptor and the envelope protein of the virus. The virus is internalised, where it is uncoated and the RNA is reverse-transcribed into proviral dsDNA by means of the virally encoded *pol* gene, and stably integrated into the host genome after being transported to the cell nucleus. The ability of retroviruses to insert their genome into the host DNA allows for stable genetic modification for the life of the host cell (Naldini *et al.*, 1996a; Naldini *et al.*, 1996b).

Two members of the retrovirus family are commonly used for gene transfer: lentiviruses (eg. human immunodeficiency virus) and *Oncoretrovirus* (eg. Moloney murine leukemia virus) (Evans *et al.*, 2006). The salient properties of lentiviruse and *Oncoretrovirus* vectors are summarised in Table 2.7. Briefly, lentiviruses are capable of infecting both dividing and non-dividing cells but are unfortunately difficult to produce (Lewis *et al.*, 1992). Conversely, *Oncoretrovirus* vectors only transduces dividing cells but is relatively simple to produce (Verma and Somia, 1997; Kay *et al.*, 2001).

Table 2.7: Potential determinants of efficient short interfering RNA-directed gene silencing (Dykxhoorn *et al.*, 2003)

Short interfering RNA (siRNA)	Messenger RNA (mRNA)
Incorporation into the RNA-inducing silencing complex (RISC) and stability in RISC	The position of the siRNA-binding target region
Basepairing with mRNA	Secondary and tertiary structures in mRNA
Cleavage of mRNA	Binding of mRNA-associated proteins
Turnover of mRNA after cleavage	Basepairing with siRNA
	The rate of mRNA translation
	The number of polysomes that are associated with translating mRNA
	The abundance and half-life of mRNA
	The subcellular location of mRNA

2.8.1.2. Non-integrating viral vectors

Non-integrating viral vectors include those based on adenoviruses (Ad), herpes simplex viruses and baculovirus-derived vectors in which the viral DNA is maintained in an episome in the infected cell.

2.8.1.2.1. Adenovirus

Adenoviruses are a family of DNA viruses that contain a linear, 30-40 kb double-stranded DNA (dsDNA) genome encoding over 50 polypeptides (Graham and Prevec, 1995; Kay *et al.*, 2001; Evans *et al.*, 2006). Adenoviruses can infect both dividing and non-dividing cells efficiently in culture and *in vivo*. Since they do not integrate into the host genome, they are replicated as extrachromosomal elements in the nucleus of the host cell. They are capable of infecting a wide variety of cells through a specific interaction between the viral fiber protein and at least one cell surface receptor and are further enhanced through a specific interaction of the fiber with an integrin 'co-receptor'. Thus, the host range is altered by modifying the fiber protein so it can bind to other components of the cell surface more efficiently (Wickham *et al.*, 1995; Wickham *et al.*, 1996). They are extremely useful if expression of the transgene is required for short periods because the incoming adenoviral proteins that package DNA can be transported to the cytoplasm where they are processed and presented on the cell surface, tagging the cell as infected for destruction. Adenoviruses can hold up to 8 kb of foreign DNA (Verma and Somia, 1997; Kay *et al.*, 2001; Evans *et al.*, 2006).

A recombinant adenovirus for gene transfer is generated by removing the E1 gene important for viral gene expression and replaced with the therapeutic gene under the regulation of a heterologous promoter. However, E1-deleted viruses can be propagated only in a cell line that provides the E1 gene products in *trans*, such as 293 cells and express low levels of viral antigens following infection, resulting in a low level of DNA replication (particularly at a high multiplicity of infection) and often, the loss of therapeutic gene expression after seven to fourteen days (Graham and Prevec, 1995; Yang and Wilson, 1995; Yang *et al.*, 1996). Alternate recombinant adenoviruses and their complementary cells lines for production have been constructed by deleting the E2 and/or E4 genes. However, these viruses show reduced antigenicity *in vivo* and a reduction in the duration of gene expression, possible due to a loss of viral DNA replication or transregulation of gene expression (Krougliak and Graham, 1995; Wang and Finer, 1996).

2.8.1.2.2. Herpes simplex virus

Herpes simplex virus (HSV) is an enveloped virus with a large genome composed of 152 kb of linear dsDNA containing 84 viral genes, approximately half of which are nonessential for replication in cell culture. These features provide for multiple sites of foreign DNA insertion, capable of harbouring up to 75 kb or more of foreign DNA (Burton *et al.*, 2002). Herpes simplex viruses can be used for efficient gene transduction in a variety of cell types. However, there are difficulties associated with long-term transgene expression in certain tissues. This is thought to be since the mechanism of herpes simplex virus attachment is complex, involving multiple viral envelope glycoproteins (Kay *et al.*, 2001). Using at least two of the viral envelope glycoproteins (gB and gD), the virus binds to cells through an interaction with heparin sulfate moieties on the cell surface. The virus enters the host cell by fusion, and there the linear DNA circularizes. Gene expression is initiated by the viral protein VP16, which is carried into cells as part of the viral tegument, stimulating expression of a set of proteins that activate a series of genes required for DNA synthesis and viral packaging. The newly synthesised viral particles are released from the infected cells by lysis. Since many of the HSV proteins are nonessential for viral

replication, they can be removed and replaced with the target therapeutic genes (Howard *et al.*, 1998; Lilley *et al.*, 2001).

2.8.1.2.3. Baculovirus

Baculoviruses provide a powerful tool for foreign gene expression at high levels in insect cells. Viruses within the Family *Baculoviridae* possess a closed dsDNA genome ranging from 80-220 kb in length, encoding for over 100 genes (Miller, 1988; Maeda, 1989). Very little is known about the function of many baculovirus genes. However, two genes (p10 and polyhedrin) have been mapped and sequenced (Hooft van Iddekinge *et al.*, 1983; Kuzio *et al.*, 1984; Leisy *et al.*, 1986). The polyhedrin gene has an important role in the transmission of the virus in nature, but is nonessential for viral replication. Hence, the polyhedrin gene can be exchanged with other genes to create a productive recombinant virus (Roy, 2004). Furthermore, it is a gene with a strong promoter, and it can be utilised as a marker, allowing easy detection by light microscopy (Maeda, 1989).
Recombinant viruses for transfection are obtained by the recombination in host cells transfected with the wild-type DNA and a recombinant transfer plasmid vector containing the foreign gene and the 3' and 5' flanking region of the polyhedrin gene, including the promoter (Miller, 1988; Maeda, 1989). During the final phase of gene expression (20 to 72 hours post infection), polyhedrin becomes the predominant protein of the cell, expressing the gene of interest (Miller, 1988).

Baculoviral vectors are able to encapsidate large amounts of foreign DNA (Smith *et al.*, 1983) and result in stable and prolonged silencing of targeted genes (Lin *et al.*, 2006). However, in cell lysis-associated proteolysis (if the protein of interest is a secreted protein), proteinases from lysed cells can compromise protein production (Lee *et al.*, 2000). Furthermore, the restricted host range (invertebrates) obviously makes baculovirus expression inconvenient for some applications.

2.8.2. Non-viral vectors

In light of the limitations of viral vectors such as the size of genes, random integration in the host genome, insertional mutagenesis and immunogenicity of the viral vector, there have been substantial considerations for the utilisation of non-viral vectors as a promising alternative for gene therapy since they avoid some of the potential hazards associated with the use of viral vectors. Gene transfer through the use of non-viral vectors is referred to as transfection. Transfection is the uptake by a cell of a fragment of naked DNA and the incorporation of this DNA into the host cell chromosome in an inheritable form (Schmidt-Wolf and Schmidt-Wolf, 2003).

Non-viral carriers have several advantages over the use of viral vectors: (1) they are generally safer *in vivo* and are cheaper to produce, (2) they do not elicit a specific immune response (less immunogenetic than viral vectors) and can therefore be administered repeatedly, (3) they are easy to prepare and (4) they are more flexible with regard to the size of the DNA fragment being transferred (Schmidt-Wolf and Schmidt-Wolf, 2003).

Several DNA-vector-mediated mechanisms have been developed to use for RNAi to avoid some of the potential hazards associated with the use of viral vectors. There are three main non-viral vector systems that involve the treatment of cells by chemical (cationic liposomes and polymer complexes) or physical means (direct injection of naked plasmid DNA) (Kuemmerle *et al.*, 2000; Liu and Huang, 2002; Herweijer and Wolff, 2003; Schmidt-Wolf and Schmidt-Wolf, 2003). Technically, the use of chemical and physical methods of gene transfer is relatively simple and it does not provoke specific immune responses of the host. However, they are generally less efficient in delivering DNA and in initiating gene expression compared to the use of viral vectors (Liu and Huang, 2002; Schmidt-Wolf and Schmidt-Wolf, 2003).

2.8.2.1. Liposomes and lipoplexes

There are two types of liposomes, anionic and cationic, with the cationic liposomes more frequently used for gene therapy. Liposomes are artificial carrier molecules composed of lipids that are used to encapsulate DNA and permit foreign DNA to be introduced into cells. Liposomes form large complexes (either positive (cationic) or negatively (anionic) charged) that facilitate DNA fusion with the host cell. The majority of cationic liposomes used for transfection consist of a positively charged lipid mixed with a neutral helper lipid, facilitating the formation of stable lipid bilayers and interaction with the targeted cell membrane. The positively charged DNA complex is taken up from the extracellular compartment by endocytosis and transferred into the nucleus of the target cell (Lechardeur *et al.*, 2005). Conversely, lipoplexes are spontaneously formed from the interaction of cationic lipids and negatively charged nucleic acids. However, they are structurally more heterogeneous and unstable compared to liposomes, which has major disadvantages for reproducibility, manufacturing, and drug administration (de Fougerolles, 2008).

In contrast to viral vectors, liposomes and lipoplexes are non-pathogenic, can be used for multiple treatments, and are relatively cheap and easy to produce. Furthermore, there is no limitation on the length of DNA that can be incorporated into cationic liposomes for gene delivery. However, the efficiency of transfection using liposomes is often less than that achieved using viral vectors. Hence, liposomes can be conjugated to defective viral particles, viral protein, or virally derived peptides that are able to disrupt the lysosome and/or increase DNA transport to the nucleus (Gao and Huang, 1995; Robbins and Ghivizzani, 1998; Judge *et al.*, 2005; Lee *et al.*, 2005).

2.8.2.2. Polymer complexes

Polymer complexes offer an alternative to liposomes and are believed to protect DNA from degradation by condensing DNA molecules (Thomas and Klibanov, 2003; Lee *et al.*, 2005). Polymer complexes can be naturally or synthetically derived. Natural polymer complexes that have been employed as vectors for gene delivery include proteins such as histones (Fischer *et al.*, 2001) and aminopolysaccharides such as chitosan (Borchard, 2001). Synthetic polymer complexes include numerous peptides and polyamines (Pouton *et al.*, 1998).

Dynamic polyconjugates and cyclodextrin-based nanoparticles have yielded robust results involving siRNA *in vivo*. Dynamic polyconjugates are a multicomponent polymer system involving a membrane-active polymer to which siRNAs are covalently coupled via a disulfide bond where a polyethylene glycol (PEG) lipid and N-acetylgalactosamine groups are linked via pH-sensitive bonds (Rosema *et al.*, 2007). The polymer complex disassembles in the low-pH environment of the endosome, exposing its positive charge, resulting in endosomal escape and cytoplasmic release of the siRNA from the polymer (Rosema *et al.*, 2007). Cyclodextrin-based nanoparticles contain polycation nanoparticles and like dynamic polyconjugates, involve targeted delivery and endosomal escape mechanisms (Heidel *et al.*, 2007). Atelocollagen and chitosan are two additional polymer-type approaches that have also been reported to effectively deliver siRNA *in vivo* (Takei *et al.*, 2004; Howard *et al.*, 2006; Pille *et al.*, 2006).

2.8.2.3. Peptide and protein complexes

Short interfering RNAs may also be integrated with positively charged peptides or proteins. Briefly, cationic peptides and proteins are used to form complexes with the negatively charged phosphate backbone of the siRNA duplex. These complexes may incorporate a targeting element, such as a receptor-specific peptide or antibody (Song *et al.*, 2005; Kumar *et al.*, 2007). Peptide or protein complexes can also be non-targeted such as polyethylenimine (PEI) polymers and cell-penetrating peptides (Boussif *et al.*, 1995; Aigner, 2006).

Polyethylenimine polymers are synthetic linear or branched structures with high cationic charge densities and protonable amino groups that interact with the targeted cell surface through electrostatic interaction after incorporation with the siRNA. They are taken up by cells through endocytosis whereby endosomal escape is hypothesised to occur, resulting in the osmotic release of the polyplexes into the cytoplasm (Boussif *et al.*, 1995; Aigner, 2006). Despite successful *in vitro* delivery of siRNA, there has yet to be successful published reports of *in vivo* silencing utilising non-targeting peptide or protein complexes.

2.8.2.4. 'Naked' short interfering RNA

The direct injection of naked plasmid DNA is the simplest non-viral gene transfer system and refers to the delivery of siRNA (unmodified or modified) in an excipient such as saline. Hence, the ease of formulation and administration of the delivery of naked siRNA to tissues makes this an attractive therapeutic approach. However, the expression level after the direct injection of naked plasmid DNA is severely limited due to rapid degradation of the DNA by nucleases (Kuemmerle *et al.*, 2000; Herweijer and Wolff, 2003; Mehier-Humbert and Guy, 2005). Furthermore, plasmid based strategies require transcription and in the case of hairpin DNA, Dicer processing (compared with siRNA transfection that do not require Dicer processing) so the initiation of siRNA-transfected silencing is not immediate. The utility of plasmids is also limited in cell lines that are unable to be propagated for long periods (such as primary cells) and are difficult to transfect. The advantage with the use of plasmid DNA compared with the use of siRNAs is that plasmid DNA can be readily generated (Dykxhoorn *et al.*, 2003).

2.8.3. Hybrid vectors

Hybrid vectors are designed to combine the advantages of more than one vector into a single reagent to achieve greater infection efficiency. For example, adenovirus/retroviral hybrid vectors combine the integrating potential of retroviral vectors (leading to persistent, long term gene expression) with the ability to infect dividing and non-dividing cells) and the high output of Ad vectors (Murphy *et al.*, 2002). Similarly, prolonged transgene expression has been achieved through the use of an Ad/AAV hybrid vector compared with the

single use of Ad vectors (Goncalves *et al.*, 2001). Adenovirus and AAV hybrids (Goncalves *et al.*, 2001; Goncalves *et al.*, 2002), Ad and lentivirus hybrids (Kubo and Mitani, 2003), Ad and retrovirus hybrids (Yoshida *et al.*, 1997; Murphy *et al.*, 2002) and AAV and baculovirus hybrids (Palombo *et al.*, 1998) have successfully been established and have greater infection efficiencies compared to the use of either of the single virus vectors.

An alternative approach to the use of hybrid vectors involves the use of vectors that combine the merits of both viral and non-viral systems. For example, virus-cationic-liposome DNA complexes are capable of achieving a level of gene transfer greater than 2000-fold above the achieved baseline using a cationic liposome (transferrin-polylysine) alone (Curiel *et al.*, 1991). Furthermore, the inclusion of the adenovirus in a hybrid vector with transferrin-polylysine permits gene transfer in a number of cell lines that would otherwise have been refractory to gene transfer using transferrin-polylysine conjugates (Curiel *et al.*, 1991).

While these hybrid vectors have shown promise, at least *in vitro* systems, their efficiency for *in vivo* applications should be a focus for further investigation because there may be additional factors operating *in vivo* that reduce the efficiency of the hybrid vector. Furthermore, hybrid vectors have primarily been investigated in mammalian systems so the use of hybrid vectors in plant and alternative animal systems, warrants investigation before it can be assumed they work just as efficiently as they do in mammalian systems. However, the evidence from research to date suggests there is potential for the use of hybrid vectors to be used in preclinical and clinical applications for gene therapy.

2.8.4. Summary of vectors for gene delivery

The delivery of siRNAs to target cells or tissues remains the primary challenge regarding RNAi therapeutics. A delivery vector, of either viral or non-viral origin, must be used to carry the foreign gene into a cell. No single gene-delivery vector currently contains all the desirable characteristics, and each has its advantages and limitations. Viral vectors take advantage of the facile integration of the gene of interest into the host cell and high probability of its long-term expression but are plagued by safety concerns including toxicity and mutagenesis. Non-viral vectors, although less efficient in delivering DNA and in initiating and maintaining foreign gene expression and lacking specificity, are non-pathogenic and amenable to large scale production. Hence, non-viral gene transfer will probably become more important as better delivery methods become available. Whatever the method, all approaches have been demonstrated to provide selective and potent target gene suppression and offer great promise for the development of RNAi therapeutics.

Ideally, a gene transfer vector would have the properties from viral and non-viral vectors including high infection efficiency, convenience and reproducibility of production, ability to target the desired cell type and non-pathogenic to the host. All the desirable properties do exist individually in disparate vectors so perhaps the use of a combination of several vector systems will achieve the desired result of the vector being non-pathogenic, efficient and a specific gene delivery method for RNAi. However, the selection of the delivery approach will also be influenced by the nature of the application, the route of administration to be used, and the cell types and tissues to be targeted.

2.9. Limitations on the use of RNA interference

Although RNA interference is an extremely powerful tool as a gene silencing and antiviral mechanism, RNAi has several limitations.

2.9.1. 'Off-target' effects

Although the effects of RNA interference are generally thought to be sequence specific, there is still debate within the literature as to whether or not some of the effects are 'off-target'. 'Off-target' effects include silencing of the wrong genes or the degradation of closely related but non-identical mRNAs (Bartell, 2004; Downward, 2004; Hannon and Rossi, 2004). Conversely, the use of some siRNAs can enhance expression instead of having a silencing effect. There may also be cross reactivity of the siRNAs with targets of limited similarity (Jackson *et al.*, 2003; Kawasaki *et al.*, 2003; Saxena *et al.*, 2003). Hence, 'off-target' gene silencing is clearly unwarranted since the cellular consequence of altered gene activity is unknown and largely unpredictable.

Genome-wide monitoring gene activity by microarray technology has demonstrated that 'off-target' silencing of genes can result from siRNA treated cells. Initial analyses demonstrated that a match of as few as eleven nucleotides can result in 'off-target' knockdown (Jackson *et al.*, 2003). More recently, a six to seven nucleotide match to the siRNA has been identified in experimentally verified 'off-targets' (Lim *et al.*, 2005; Lin *et al.*, 2005; Burmingham *et al.*, 2006; Jackson *et al.*, 2006). Thus, an understanding of the specificity of siRNA-mediated gene silencing is essential for the appropriate design and interpretation of RNAi experiments and RNAi-based therapeutic strategies. However, as the intensive research into the specificity of siRNAs and miRNAs continues, it is likely that sequences can be targeted with minimal side effects.

2.9.2. RNA interference suppressor proteins

There is the additional concern of post-transcriptional gene silencing (PTGS)/RNAi in that suppressor proteins have been found in several plant and animal viruses. Viruses encoding proteins that are suppressors of RNA silencing was first reported from plant viruses in 1998 (Anandalakshmi *et al.*, 1998; Beclin *et al.*, 1998; Brigneti *et al.*, 1998). A number of plant (Voinnet *et al.*, 1999; Silhavy *et al.*, 2002; Qu and Morris, 2005) and insect (Li *et al.*, 2002a) viruses have since been reported to express different RNAi suppressor proteins. For example, the tomato bushy stunt virus p19 protein suppresses PTGS in plants by binding siRNAs generated after virus infection (Silhavy *et al.*, 2002) and the

cucumber mosaic virus 2b protein inhibits the spread of PTGS throughout the host plant (Reavy *et al.*, 2004). The insect virus, flock house virus B2 protein suppresses RNAi activity in both plants and *Drosophila* S2 cells (Li *et al.*, 2002b) and the B2 protein of betanodaviruses of fish have demonstrated RNAi activity (Ou *et al.*, 2007), suggesting an evolutionary conserved RNAi pathway (or at least part of) having a natural antiviral role. Hence, viral suppressor proteins may be useful tools to investigate the mechanism of RNAi.

The molecular mechanism of RNAi inhibition is still relatively unknown. Research concerning RNAi suppressor proteins has primarily concerned plants and to date, three types of viral suppressors have been identified using a variety of silencing suppression assays. Two types of viral suppressors are capable of a complete or partial reversal of pre-existing RNA silencing, whereas the third does not reverse RNA silencing but prevents RNAi signalling (Li and Ding, 2001). Although the suppression of RNAi could be essential for efficient viral infection, many of the viral suppressors identified have previously been shown to be required for virulence determination (Li and Ding, 2001). Hence, the suppression of RNA silencing by plant and animal viruses probably represents a viral adaptation to a novel host antiviral defence mechanism. Since the RNA silencing pathway seems to be conserved in a range of eukaryotic organisms and as viruses of plant and animal hosts encode homologous proteins, the same assays for silencing suppression established in plants should be applied to look for animal viral suppressors (preferably in animal viruses that have proteins which share an evolutionary origin with known plant viral suppressors for initial investigations).

2.9.3. The use of synthesised short interfering RNAs

The siRNA sequence is not, surprisingly, a crucial aspect in determining the efficiency of the siRNA-directed mRNA cleavage which results in gene silencing. However, there are a number of other factors including the binding of the RNA-binding proteins and the secondary structure of the mRNA target that are likely to affect the efficiency of gene silencing involving siRNAs (Dykxhoorn *et al.*, 2003). Not every siRNA sequence is efficient in mediating gene silencing and it has been suggested that most researchers achieve a 33%

success rate (Downward, 2004). Furthermore, siRNAs that are not perfectly homologous to their target sequence can repress their translation (although only recorded in plant and mammalian systems and not in invertebrates) (Doench *et al.*, 2003; Saxena *et al.*, 2003). However, further research is required to determine if there is any interplay between the characteristics of the mRNA target sequence and the effectiveness of siRNA-directed gene silencing.

Another drawback with the use of siRNAs is cost, particularly since different siRNAs targeting the same gene can have a range of efficacies. Consequently, several siRNA sequences may need to be assessed for knockdown efficiency before finding the one that works well enough to use reliably. However, it has been suggested that whichever strand of the RNA duplex is most easily unwound from 5' to 3' will be preferentially assembled into the RISC complex (Schwarz *et al.*, 2003). This has led to the conclusion that siRNAs designed to be unstable at the 5' end of the antisense strand are the most effective, and have the least likelihood of sense strand-directed silencing (Khvorova *et al.*, 2003).

Multiple sequences are often required to be screened because different siRNA sequences have different efficacies. Hence, it has been suggested that by pooling enzymatically generated siRNAs, there is a greater chance of achieving the required result with increased efficiency, eliminating the need to identify a single effective siRNA. Although this may be a quicker and more cost effective process, there are increased concerns of non-specific translational inhibition due to any residual unprocessed long dsRNAs and increased off-target effects (Holen *et al.*, 2002; Jackson *et al.*, 2003; Saxena *et al.*, 2003; Jackson and Linsley, 2004; Scacheri *et al.*, 2004; Amarzguioui *et al.*, 2005). If several siRNAs were pooled, one would have to assume there would be some competition between the highly efficient and less efficient siRNAs resulting in fewer efficacies compared to the use of one optimal siRNA sequence. By utilising only one optimal siRNA, any phenotypic changes can be observed and linked to that siRNA. This can be verified through the application of a second siRNA targeting the same gene. This certainly has its advantage over pooling siRNAs where the specific sequence for the phenotypic changes is unknown, requiring additional confirmation through

the use of target-specific and mismatched siRNAs to verify the sequence specificity of the observed phenotype.

The development of siRNA gene based therapies can also be difficult, particularly for RNA viruses which can have a high degree of sequence diversity between difference genotypes. In addition, rapid evolution through mutations and recombination can also make the development of siRNA gene based therapies difficult (Kronke *et al.*, 2004). Furthermore, large sections of highly unstructured translated regions of viral RNA genomes are resistant to PTGS (Kronke *et al.*, 2004). Hence, gene targeting must avoid these areas of the RNA genome for effective silencing.

The use of siRNAs has two other potential problems. The first is that the downregulation of gene expression mediated by siRNA frequently only lasts for 3-5 days in cell culture or 3-5 cell divisions post-transfection, due to dilution rather than degradation of siRNAs (Holen *et al.*, 2002; Duxbury and Whang, 2004), which may not provide a sufficient amount of time to determine if functional depletion has occurred. Secondly, there is variability in transfection efficiency, particularly when working with difficult-to-transfect cell lines. Despite the potential problems associated with inducing RNAi with siRNAs, they have been applied and continue to be successfully applied in plant, invertebrate and vertebrate systems (Caplen *et al.*, 2001; Elbashir *et al.*, 2001a; Miki *et al.*, 2005; Sos-Hegedus *et al.*, 2005; Westenberg *et al.*, 2005).

2.9.4. Concerns with the use of viral vectors

In general, viruses are very efficient vectors for gene transfer but there are significant limitations and concerns associated with their use. There are obvious safety issues that arise when working with viral vectors such as carcinogenesis and viral integration into the host genome which has the potential for insertional mutagenesis, resulting in inappropriate activation or inactivation of endogenous genes. Furthermore, repeated administration of a viral vector can induce an immune response which abolishes the transgene expression (Liu and Huang, 2002). However, the question still remains as to the long term effect of the integrated transgene and of the virus in the host individual and should be a focus

for further research, particularly if viral vectors are to be used for therapeutic purposes.

Toxic immunological reactions are another major drawback of viral vectors. RNA interference is an endogenous pathway with its own cellular machinery so there are concerns that utilising this machinery for therapeutic purposes could disrupt its intrinsic functions (Rao and Sockanathan, 2005). Furthermore, viral vectors have been implicated in the death of at least one patient. In 1999, a teenager died following the administration of recombinant adenoviral vector received during gene therapy treatment. The recombinant adenoviral vector invaded not just the intended target organ (the liver), but also other organs triggering an activation of innate immunity that led to respiratory system distress and fatal multiple organ failure (Marshall, 1999; Verma, 2000).

2.10. Biological applications for RNA interference

2.10.1. Pathogen resistance and maintenance of normal gene expression

The primary focus for the use of RNAi has been for pathogen resistance and there is no doubt that RNAi has a potential role in pathogen (viral) resistance in plants, invertebrates and vertebrates. The appearance of dsRNA within a cell (for example, as a result of viral infection) triggers a RNA interference response. The cellular enzyme Dicer cleaves the dsRNA into siRNAs. The siRNAs bind to the cellular enzyme RISC which degrades the mRNA, silencing the expression of the viral gene (Figure 2.5).

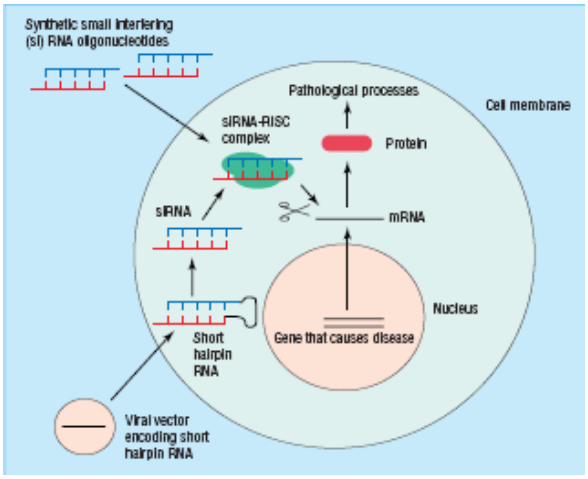


Figure 2.5: Targeting disease by RNA interference. An RNA interference response is triggered by the appearance of double stranded DNA within a cell (Downward, 2004)

In mammalian systems, RNA interference has been suggested to be involved in maintaining order in the genome (by suppressing the movement of mobile genetic elements such as transposons and repetitive sequences) and maintaining normal cellular gene expression (Elbashir *et al.*, 2001a). However, since it has only been suggested for mammalian systems and the mechanism of RNAi seems to be evolutionary, it may be likely that it has the same role in plants and invertebrates. Additional research is required to determine the possible role RNAi has in maintaining order in the genome and normal gene expression in plant and animal systems.

2.10.2. Transgenic RNA interference

The application of RNAi technology has progressed towards the development of transgenic animals (the passing and amplification of the RNAi signal from cell to cell), often done by the infection of embryonic stem cells (Alder *et al.*, 2003; Dykxhoorn *et al.*, 2003). The first indication that RNAi silencing could be amplified came from the observation that RNAi had the ability to spread throughout *C. elegans* after the ingestion of bacterially expressed dsRNAs (Fire *et al.*, 1998; Sijen *et al.*, 2001; Timmons *et al.*, 2001). However, transitive RNAi can result in the silencing of a number of unintended genes within the genome. In this process, siRNAs bind to complementary transcripts after being denatured. New dsRNAs (secondary siRNAs) are created via a 5’ to 3’ extension reaction

performed by an RNA-dependent RNA polymerase (RdRP) (Dillon, 2003). Consequently, there may be non-specific gene silencing if the secondary siRNAs are complementary to other gene sequences.

Much of the work concerning transitive RNAi has been with plants (Van Houdt *et al.*, 2003; Vanitharani *et al.*, 2003; Petersen and Albrechtsen, 2005). However, the phenomenon of transitive RNAi has also been reported in *C. elegans* (where the transitive RNAi phenomenon was first observed) (Alder *et al.*, 2003), in mice and rats (Hasuwa *et al.*, 2002; Lois *et al.*, 2002). For example, mice injected with a polymerase III expression vector as embryos were crossed to produce F1 progeny that showed virtually complete silencing of enhanced green fluorescent protein (EGFP) in the brain, liver, kidney and spleen suggesting the silencing effect was widespread throughout the body (Hasuwa *et al.*, 2002). Similarly, transgenic mice and rats have been produced after infecting embryos with a lentivirus vector containing the gene of interest (Lois *et al.*, 2002). These finding suggest transgenic RNAi could function as an alternative method for gene silencing even in species where cell lines are not established.

The work concerning transitive RNAi in plants suggests the targets of RNA silencing are involved in the expansion of the pool of functional siRNAs. There is also some indication that RNA silencing can expand across target RNAs and to regions downstream of the primary genome target (Braunstein *et al.*, 2002; Han and Grierson, 2002; Vaistij *et al.*, 2002). Since transitive RNAi has been reported in plant, invertebrate and vertebrate systems, it is likely that this phenomenon is more widespread than what has been reported but the lack of studies in this area has hampered our knowledge on how widespread the phenomenon of transitive RNAi is. Furthermore, experiments concerning transitive RNAi have involved a limited number of siRNAs and cell types. It is therefore necessary to extend these findings to other cell types and test different siRNAs (since difference siRNAs have different efficiencies), particularly if the siRNAs are to be used as therapeutic agents. Nonetheless, the results to date indicate RNAi-mediated gene silencing is heritable and stable and that RNAi functions in all cell and tissue types tested from embryos to adult animals.

2.11. Future research concerning RNA interference based technologies

Although the effects of RNAi seem to be beneficial, the available literature lacks the answers to some potential problems that may be associated with RNAi. For example, it is still unknown whether cells undergoing RNAi are as healthy as cells not undergoing RNAi. There have been few studies concerning the overall health of the cells undergoing RNAi. However, available literature suggests cells not undergoing RNAi are healthier than their affected counterparts. There is evidence that siRNAs and members of the RNAi machinery have a role in chromosome architecture in several organisms including the fission yeast *Schizosaccharomyces pombe* (the enzyme Dicer is involved in chromatin silencing and heterochromatin assembly) (Hall *et al.*, 2002; Volpe *et al.*, 2002). Secondly, there is evidence that the RNAi machinery can be saturated (Kamath *et al.*, 2000). Consequently, the question remains whether a cell devoting much of its RNA machinery to the process of mRNA degradation could become more susceptible to virus infection or even defective in chromosome function. Therefore, siRNA directed against a particular gene may silence that gene but may also affect normal cell division.

The injection of dsRNA into the nematode *C. elegans* triggers its spread to several tissues but it is unknown how the dsRNAs exit the cell in which they are produced, how they are systemically disseminated, or how they are taken up by distant target cells. It has been proposed for plants and lower eukaryotes such as *C. elegans* and embryos of *Drosophila*, that siRNAs possess the capability to function as primers that are extended on the targeted RNA by an RNA-dependent RNA polymerase (RdRP) to amplify the dsRNA trigger (Lipardi *et al.*, 2001; Sijen *et al.*, 2001). Although a similar phenomenon has been detected in *Anopheles gambiae* and in *Drosophila* (work following the initial observations in *Drosophila* embryos) (Hoa *et al.*, 2003; Roignant *et al.*, 2003), siRNAs that function as primers to amplify the dsRNA trigger have not proven to play a significant role in the RNAi pathway. It is also unknown if there is a threshold level of dsRNA needed to trigger an RNAi response. Additional research is therefore required into the spread of RNAi silencing.

2.12. Summary of RNA interference

The field of RNAi has progressed at an amazing rate since its discovery in *C. elegans* and has become an effective method for the analysis of gene function and the sequence-specific downregulation of gene expression at both the transcriptional and post-transcriptional levels in plants and animals (invertebrates and vertebrates).

Several delivery methods for dsRNA are currently available (viral or non-viral vectors) and have been demonstrated to provide successful gene suppression. However, their ultimate success depends on many factors including (i) transfection efficacy (ii) efficacy at low concentrations, (iii) toxicity and absence of non-specific effects, (iv) applicability in various treatment regimens and in various diseases as well as, (v) the ability of the transfer vector to overcome numerous biological barriers to reach their target tissue/organ.
There are a number of limitations and concerns associated with the use of RNAi technology, which must be overcome before RNAi can be harnessed as a therapeutic modality. These include effectively difficulty of the siRNAs to the target cells/organ for *in vivo* use, the potential for 'off-target' effects and that several plant and animal viruses contain proteins with RNAi suppressor activity. However, the RNAi pathway is nucleotide specific so by targeting conserved sequences, it is possible to target individual or groups of similar genes. RNA interference can also be applied to many cell types and because the genomic sequences of many organisms are available, it is possible to harness the technology of RNAi to investigate the function of all genes in an organism's genome.

More importantly, RNAi can be utilised to target viral genes which is promising for the development of preventative measures to control viral outbreaks for which neither vaccines nor treatments are available. However, much of the work concerning RNAi has involved establishing the RNAi antiviral mechanism in cells lines and until the same experiments are performed in a plant/animal model, much of the work concerning RNAi should be viewed as preliminary since it is unknown if the plants or animals will respond to virus infection in a similar matter.

The field of RNAi has progressed immensely and has revolutionised cell biology in many systems since it was first discovered in *C. elegans* by Fire and colleagues in 1998. There is no doubt RNAi can be used as a tool for understanding biological functions of genes and as a therapeutic. Despite some limitations, the use of RNAi technology to target either viral or cellular genes is promising for the development of treatment of diseases for which neither vaccines nor efficient therapies are currently available.

2.13. Conclusion

The cultivation of penaeid prawns is a economically important activity worldwide. However, the worldwide growing demand for seafood products has resulted in an intensification of farming practices and the industry is now suffering serious problems linked to infectious diseases, particularly those of viral origin, for which there are currently no treatment methods available.

Hepatopancreatic parvovirus disease affects a number of economically important species and is found in captive, wild and hatchery-reared prawns. Its current geographic distribution maybe due to the transportation of infected prawns for culture and ranges from East Africa to Korea including Australia and the Pacific, and the Atlantic coasts of the Americas (Brock and Lightner, 1990; Lightner, 1996). Economic losses can result from HPV disease directly by mortality or indirectly through reduced growth rates, resulting in remarkably lower market values compared to prawns that have grown to a premium size (Lightner and Redman, 1985; Flegel *et al.*, 1999). In this context, control of HPV disease is very important to ensure long-term sustainability of prawn aquaculture.

Crustaceans, like other invertebrates, do not have a true adaptive immune response (Hoffman *et al.*, 1999) and therefore lack true antibodies. For this reason, they cannot be vaccinated against viral disease. The use of RNAi as a viral defence mechanism has the potential to have widespread use for the control of not only vertebrate but also invertebrate viruses by inducing sequence specific degradation of the homologous endogenous transcripts, resulting in the reduction, or loss, of gene activity without there being detectable effects on the

expression of genes unrelated in sequence (Wagner and Sun, 1998; Robalino *et al.*, 2004). The therapeutic potential for RNAi is enormous, with application for a wide spectrum of diseases from plants, vertebrates and invertebrates (Fire *et al.*, 1998; Elbashir *et al.*, 2001c; Li *et al.*, 2002a; Ge *et al.*, 2003; McCaffrey *et al.*, 2003). Recent studies have demonstrated there is the potential to use RNAi for providing protection of *P. monodon* and *P. vannamei* to yellow head virus and white spot syndrome virus (Robalino *et al.*, 2004; Robalino *et al.*, 2005; Tirasophon *et al.*, 2005; Westenberg *et al.*, 2005; Yodmuang *et al.*, 2006; Kim *et al.*, 2007; Tirasophon *et al.*, 2007; Xu *et al.*, 2007). Consequently, RNAi could prove to be a novel alternate method for the control of HPV and other prawn viruses for which neither vaccines nor efficient therapies are currently available.

CHAPTER 3

General Materials and Methods

3.1. Strain of hepatopancreatic parvovirus

Samples of *Penaeus merguiensis* (20) stored frozen at James Cook University, Queensland were used as the source of hepatopancreatic parvovirus. Infected specimens were originally obtained from three commercial prawn farms in northern Queensland as early as June 2003. Infected specimens originated from the same HPV outbreak and had previously been classified as being HPV-positive by an experimental PCR.

3.2. Viral purification

The general method for purifying hepatopancreatic parvovirus from the hepatopancreai of *P. merguiensis* was modified from the procedure previously described by Bonami *et al*. (1995) and Rukpratanporn *et al*. (2005). Hepatopancreai were removed from the gnathothoracies of *P. merguiensis* and homogenised in TN buffer (0.02 M Tris-HCl, 0.4 M NaCl, pH 7.4) (Appendix A), at a ratio of 1:5, using a Waring Commercial Laboratory Blender. The homogenate was centrifuged at 7,000 *g* and then 13,000 *g* respectively, for 15 minutes each at 4°C using Suprafuge 22 12.50 rotor. Liquid supernatant was vacuum filtered through Whatman GF/B, Whatman GF/F and Millipore Nitrocellulose 0.45 µm membrane filter (Millipore, Sydney, Australia), respectively using Whatman Sterile Aseptic System and Holder (47 mm).

The filtered supernatant was subsequently centrifuged at 4°C for one hour at 142,459 *g* using Beckman Coulter Optima L-90K Ultracentrifuge (Beckman Coulter, USA) 70 Ti rotor. The pellet was gently resuspended in 500 µl of TN buffer, layered onto the top of a 20-40% sucrose gradient and re-centrifuged at 113,652 *g* in a SW 40 Ti rotor for 3 hours at 4°C. The viral pellet was resuspended in 200 µl of TN buffer and stored at -80°C until required.

3.3. Spectrophotometry

The amount of DNA in a preparation was quantified by spectrophotometry using an Eppendorf Biophotometer (Eppendorf, Germany), with a 10 mm optical path length. Fifty microlitres of the DNA sample was dispensed into a sterile Eppendorf disposable UVette (Eppendorf, Germany) and the absorbance readings at wavelengths of 260nm (A_{260}) and 280nm (A_{280}) were recorded against nuclease-free water (blank). Absorbance readings at 260nm permit the calculation of the amount of nucleic acid in the sample while the ratio (A_{260}/A_{280}) provides an estimate of the purity of the DNA sample. A ratio between 1.8 and 2.0 is a sign of pure samples.

Chapter 4

Molecular characterisation of hepatopancreatic parvovirus from Australian *Penaeus merguiensis*

4.1. Introduction

The increasing demand for seafood around the world has led to a considerable expansion in aquaculture. Penaeid prawns are one of the most valuable commercially produced species but the increase in production has been associated with an increase in disease problems, particularly those with infectious aetiologies (Lightner and Redman, 1992). Among these, viral pathogens have contributed to substantial economic losses within penaeid culture (Fraser and Owens, 1996; Flegel, 1997).

Hepatopancreatic parvovirus (HPV) is currently considered as a member of the *family Parvoviridae* (Bonami *et al.*, 1995). Its host range encompasses both wild and cultured penaeid species worldwide (Paynter *et al.*, 1985; Lightner, 1996; Spann *et al.*, 1997) and the freshwater prawn *Macrobrachium rosenbergii* (Anderson *et al.*, 1990). Mortalities during the larval stages of penaeid prawns have been associated with HPV (Spann *et al.*, 1997). Furthermore, HPV-infection is associated with reduced growth rates of juvenile prawns (Flegel *et al.*, 1999). However, there are no specific gross signs for HPV so diagnosis may be difficult, particularly in the presence of other pathogens that may mask the effect (Manivannan *et al.*, 2002; Chayaburakul *et al.*, 2004).

To date, three strains of HPV have been reported suggesting HPV isolated from different prawn species and/or different geographic regions are genetically different. Diagnosis of HPV has primarily depended on histology which cannot detect low grade infections and differentiate between strains. Conversely, sensitive methods of detection such as polymerase chain reaction (PCR) and gene probes are strain specific (Lightner *et al.*, 1994; Phromjai *et al.*, 2001) as small nucleotide changes lead to negative test results. Consequently, the known strains of HPV may only represent a small proportion of the existing strains, as

some may have escaped detection by the current diagnostic methods. Hence, there is the need to characterise HPV in different geographical areas and from different species to effectively diagnose the virus in potential carriers.

In Australian penaeid species, HPV has been reported in *Penaeus esculentus, P. japonicus, P. merguiensis* and *P. monodon* (Paynter *et al.*, 1985; Lightner, 1996; Spann *et al.*, 1997) but it is unknown whether these are similar strains to each other or to those already described overseas. This paper describes the first sequence data obtained for HPV from any *P. merguiensis* worldwide and from any Australian host and its relationship to other known arthropod parvoviruses.

4.2. Materials and Methods

4.2.1. Viral purification

Hepatopancreatic parvovirus virions were purified from the hepatopancreai of *Penaeus merguiensis* stored frozen at James Cook University, Queensland (Chapter 3.2).

4.2.2. Nucleic acid extraction

Total DNA was extracted from the viral suspension using the High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the manufacturer's instructions.

4.2.3. Polymerase chain reaction amplification

Ten sets of primers were used to amplify segments of the HPV genome (Table 4.1). The PCR program and primer set (1120F and 1120R) described from Pantoja and Lightner (2001) were expected to yield a 592 bp amplicon from the HPV template. The PCR program and primer set (7490F and 7852R) described from Phromjai *et al*. (2001) were expected to yield a 350 bp amplicon from the HPV DNA template.

Additional primers were subsequently designed from the genome of *Pm*DNV (DQ002873), the HPVchin genome (AY008257) and from new sequences obtained from amplifications of the genome of the Australian HPV isolate. All primers were designed using Oligo 6.60 Software (Molecular Biology Insights, USA) and synthesised by Sigma-Genosys, Australia. The PCR reaction mixture contained 1 µl (20-50 ng) of HPV template, 1 x *Taq* buffer (750 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween20), 2.5 mM $MgCl_2$, 0.75 U *Taq* polymerase (MBI Fermentas), 200 µM each dNTP and 50 pmol of each primer. The PCR reaction volume was adjusted with sterile distilled water to a final volume of 25 µl. Amplification was performed in an Eppendorf Mastercycler Gradient Thermocycler (Eppendorf, Germany) with a PCR profile consisting of an initial 94^{o}C for 7 minutes, 35 cycles at 94^{o}C for 45 seconds denaturation, 55^{o}C annealing for 45 seconds and polymerisation at 72^{o}C for 1 minute and an additional 5 minutes at 72^{o}C following the last cycle. Amplified products were visualised on 1% agarose gel containing ethidium bromide at a concentration of 0.5 µg ml^{-1}.

Furthermore, primers HPV4657F, HPV5836F and HPV5765 (Table 4.1) were designed at the ends of the genome to primer walk the ends of the genome incorporating the inverted terminal repeats. The reaction mixture contained 1 µl (50-100 ng) of DNA template, 1 x *Taq* buffer (100 mM Tris-HCl (pH 8.8 at 25^{o}C),500 mM KCl, 0.8% Nonidet P40), 5.0 mM $MgCl_2$, 5 U *Taq* polymerase (MBI Fermentas), 10 mM dNTPs and 0.6 µM of each primer. The PCR reaction volume was adjusted with sterile distilled water to a final volume of 50 µl. Amplification was performed in an Eppendorf Mastercycler Gradient Thermocycler (Eppendorf, Germany) with a PCR profile consisting of 10 cycles at 95^{o}C for 30 seconds denaturation, 40^{o}C for 1 minute annealing and polymerization at 72^{o}C for 4 minutes and 30 seconds, followed by 30 cycles at 95^{o}C for 30 seconds denaturation, annealing at 60^{o}C for 1 minute and polymerization at 72^{o}C for 4 minutes and 30 seconds and an additional 5 minutes at 72^{o}C following the last cycle. Amplified products were visualised on 1% agarose gel containing ethidium bromide at a concentration of 0.5 µg ml^{-1}.

To obtain the sequence for the inverted terminal repeat on the 3' end of the genome, a 40-mer primer (HPV148R) was designed to provide a single primer read of the terminal repeat. Amplification was performed using KOD Hot Start DNA Polymerase (Merck Biosciences, Victoria, Australia). The reaction mixture consisted of 1µl (50-100 ng) of DNA template, 1 x 10 buffer for KOD Hot Start DNA polymerase, 1.5 mM $MgCl_2$, 200 µM each dNTP, 0.3 µM of primer, 0.02 U/µl KOD Hot Start DNA polymerase and sterile distilled water to a final volume of 50 µl. Amplification was performed in an Eppendorf Mastercycler Gradient Thermocycler (Eppendorf, Germany) with a PCR profile consisting of 95^{o}C for 2 minutes polymerase activation, 35 cycles of 95^{o}C for 20 seconds denaturation, 60^{o}C for 1 minute annealing and 70^{o}C for 20 seconds extension. Following the last cycle, sampes were polymerized for an additional 5 minutes at 70^{o}C. Amplified products were visualised on 1% agarose gel containing ethidium bromide at a concentration of 0.5 µg ml^{-1}.

Table 4.1: The sequences of the primers used to amplify the genome of the Australian hepatopancreatic parvovirus isolate from *Penaeus merguiensis*. The initial primer sequences and sequences on the final consensus sequence are provided. Primer bases that differed in the final consensus sequence are underlined, the letter D indicates a base pair deletion in the position of the original primer sequence, italicized letters indicate an additional base pair not in the original primer sequence, dashes indicate the consensus sequence was completely different to the primer and primer sequences that did not differ are represented as N/A. All primers were designed on the negative sense strand of the genome

Primer name	**Nucleotide sequence (5'-3')**	**Consensus Nucleotide sequence (5'-3')**
HPV434F	TATCCGGAAACTCATTATCTA	N/A
HPV434R	TCCATCACAAACATTTACCTT	-
HPV452F	TCCCTCTTGTCCTTGCCCTTC	-
HPV452R	CATCATCCAAAATTGCCTTAG	CATCATCCAAAACTGTCTTAA
1120F	GGTGATGTGGAGGAGAGA	AGTGACATGGAGGAGAGA
1120R	GTAACTATCGCCGCCAAC	TCCTTCCCCTCCTCCAAC
HPV504F	GTGCCGATCTACGTGAAGCTG	GAGCCGATCAACGTGAAGCTG
HPV504R	CTGAAAATCCTGATGCGTATG	N/A
HPV1500F	TGAAGAGAGAAGGTATGG	N/A
HPV1500R	TCTATAGCCCTCCTGTCATCA	N/A
HPV780F	GAATGGCATGTCTCGCTTTGA	CAGTTGATTGADCGCTTTGA
HPV445F	AGCAGCTTTCGTGGTGGCAGAGA	-
HPV445R	CGCCACCAGTAAAGTAATTGA	N/A
HPV1135R	ATTTCTTTCCTCTTGCCCTAA	N/A
HPV250F	AGCATTAGGGCAAGAGGAAAG	N/A
HPV250R	TGTCTGAAAATCCTGATGCGT	N/A
7490F	TGGAGGTGAGACAGCAGG	-
7852R	AAGAGCGAGGACAGTTGG	-
HPV600F	TGTCTAAGCGCCAGTAACCAA	N/A
HPV600R	TATACAGTTACTTGGCATGAC	N/A
HPV4657F	TTTGAGGGAGCACAGGAAGGTT ATCTGATGGACGACGACA	N/A
HPV5836F	GTGGAACTCATGGAAGTAAGTA TGCAGATAGAGGACCAAT	GTGGAACACATGGAAGCAAGTA TT*T*CAGATAGAGGACDAAG
HPV5900F	CACGCGTGCGTGGTTTACCCTT	CACACGTGCGTGGTTTACCCTT
HPV148R	TCTCCACTCATCTCCATAACTGATA AATACACTGCGGCGG	N/A

4.2.4. Cloning and sequencing

DNA fragments were purified from agarose gels using Wizard®SV Gel and PCR Clean-Up System (Promega, New South Wales, Australia) and cloned into *Escherichia coli* JM 109 cells, using pGEM-T® Easy Vector System (Promega, New South Wales, Australia), according to the manufacture's instructions. At least three white colonies were selected for isolation of the recombinant plasmids according to the protocol of the Eppendorf Fast Plasmid™ Mini Prep Kit (Eppendorf, Germany). Recombinant plasmids were digested with *Spe* I, followed by electrophoresis on 1% agarose gel to screen for DNA inserts. Recombinant plasmids containing DNA inserts were sequenced using the Amersham Chemistry DYEnamic ET terminator sequencing kit (Amersham Biosciences) and M13 universal primers. Three forward and three reverse reactions were performed for each clone. Samples were either analysed at the Advanced Analytical Centre at James Cook University, Townsville with a MegaBACE Sequence Analyser (Amersham Biosciences) or by Macrogen Inc (Korea). Sequencher™ software (Gene Codes Corporation) was used to analyse and align overlapping sequences for each clone.

4.2.5. Nucleotide sequence analysis

Sequence information derived from HPV in this study was compared with sequence information from other strains of HPV using BLAST. Putative open reading frames (ORFs) in the nucleotide sequence were determined by computer analysis using NCBI ORF finder.

4.2.6. Phlylogenetic comparisons

Alignment and phylogenetic analysis of sequences was performed using ClustalX Multiple Sequence Alignment Program version 1.8 (National Centre for Biotechnology Information) and GeneDoc Multiple Sequence Alignment Editor and Shading Utility Version 2.6.002 (Pittsburgh Supercomputing Centre) using sequence information from known arthropod parvoviruses (Table 4.2). Alignments were visualised using NJplot (http://pbil.univ-lyon1.fr/software/njplot.html) (Perriere and Guoy, 1996). Bootstrap values for the phylogenetic analysis were calculated from 1000 replications.

Table 4.2: Source and origin of arthropod sequences used for the phylogenetic comparison with the sequence of the Australian hepatopancreatic parvovirus isolate

Abbreviation	Full name	Host species	GenBank accession number and/or source
HPVchinensis (HPVchin)	Hepatopancreatic parvovirus	*Penaeus chinensis*	AY008257
HPVmonodon (*Pm*DNV)	Hepatopancreatic parvovirus	*Penaeus monodon*	DQ002873
HPVsemisulcatus (HPVsemi)	Hepatopancreatic parvovirus	*Penaeus semisulcatus*	Manjanaik *et al*., 2005
IHHNV (Australian strain)	Infectious hypodermal and haematopoietic necrosis virus	*Penaeus monodon*	AY590120, K. Krabsetsve unpubl. data, pers. comm.
IHHNV (*Pst*DNV)	Infectious hypodermal and haematopoietic necrosis virus	*Penaeus stylirostris*	AF218266
SMV	Spawner isolated mortality virus	*Penaeus monodon*	AF499102
*Aae*DNV	*Aedes aegypti* densovirus	*Aedes aegypti*	M37899
*Aal*DNV	*Aedes albopictus* densovirus	*Aedes albopictus*	X74945
*Bm*DNV	*Bombyx mori* densovirus	*Bombyx mori*	AB042597
*Ds*DNV	*Diatraea saccharalis* densovirus	*Diatraea saccharalis*	NC001899
*Gm*DNV	*Galleria mellonella* densovirus	*Galleria mellonella*	L32896
*Jc*DNV	*Junonia coenia* densovirus	*Junonia coenia*	S47266
*Pf*DNV	*Periplaneta fuliginosa* densovirus	*Periplaneta fuliginosa*	NC000936

4.3. Results

4.3.1. Polymerase chain reaction amplification

Using PCR, the complete genome (6299 bp) of HPV from Australian *P. merguiensis* was successfully sequenced (Genbank accession number: DQ458781).

Primers from the literature, 7490F-7852R (Phromjai *et al.*, 2001) and 1120F-1120R (Pantoja and Lightner, 2001), specific for the HPVchin genome failed to give the expected amplicon sizes of 350 bp and 592 bp, respectively but rather produced amplicons of 240 bp and approximately 1 kb, respectively.

4.3.2. Nucleotide sequence analysis

The base composition of the sequence obtained was 34.85% A, 15.75% C, 25.67% G and 23.73% T. The total G + C content was 41.42% and A + T content was 58.58%.

The Australian HPV isolate from *P. merguiensis* shared the highest nucleotide similarity of 86% with the complete genome sequence of the Korean HPV isolate from *P. chinensis* and 83% similarity with the complete genome sequence of the Thai HPV isolate from *P. monodon* using BLAST searches for the sequence at the Genbank NCBI database. A nucleotide similarity of 83% was shared with the partial 391 bp fragment of DNA from HPV DNA from Indian *P. semisulcatus*.

4.3.3. Potential open reading frames

Potential open reading frames in the HPV sequence obtained during this study were determined by the NCBI ORF finder. The HPV genome contained three major ORFs in which there was a slight overlap between the first and second ORF (Figure 4.1), but in different reading frames, namely 2 and 3. All small ORFs did not contain significant sequence homology using BLAST.

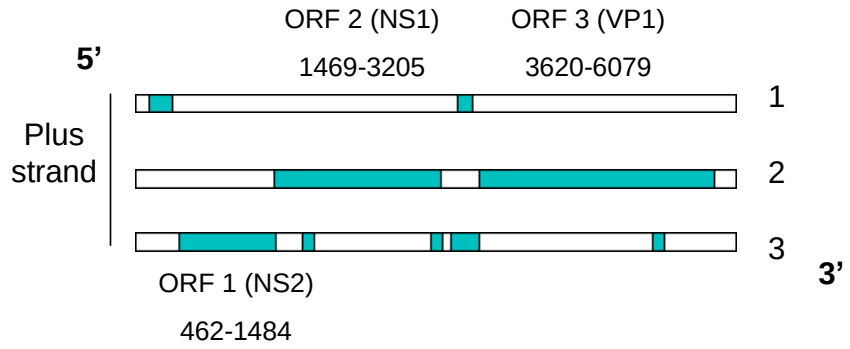


Figure 4.1: Organisation of open reading frames on the plus strand of the hepatopancreatic parvovirus genome relative to how other hepatopancreatic parvovirus entries to Genbank have been submitted

4.3.3.1. Open reading frame one

The first ORF (ORF 1) started at nucleotide 462 in reading frame 3+ and terminated with a TAA codon at position 1484. It contained 340 amino acids, corresponding to a molecular weight of 39.84 kDa. This ORF shared an amino acid similarity of 86% with HPV from *P. monodon* of Thailand (*Pm*DNV). There was no significant sequence homology between ORF1 of *Pmerg*DNV and other prawn, insect or indeed any other parvoviruses. This protein is speculated to encode a nonstructural protein (NS2).

4.3.3.2. Open reading frame two

The second ORF (ORF 2) was the second largest of the ORFs identified for hepatopancreatic parvovirus from Australian *P. merguiensis*. It started at nucleotide 1469 in reading frame 2+ and terminated with a TAA codon at position 3205. It contained 578 amino acids, corresponding to a molecular weight of approximately 68.3 kDa. Using the entries in the protein database via BLAST, this ORF shared 93% amino acid similarity the non-structural protein 1 of *Pm*DNV (DQ002873) and 26% similarity with the non-structural protein 1 of *Pst*DNV (AF218266). There were no additional significant matches with other parvoviruses. This sequence also contains sequence homology to the replication initiator motifs involved in parvovirus rolling hairpin mechanism (Bergoin and Tijssen, 2000). These motifs were located between amino acids 86 and 415 and shared similar conserved regions with IHHNV and other parvoviruses (Shike *et al.*, 2000) (Figure 4.2a). The amino acid sequence also contains sequence

homology to parvoviruses NTP-binding and helicase domains of the NS1 polypeptide. These domains were located between amino acids 390 and 488 and shared similar conserved regions with IHHNV and other parvoviruses (Shike *et al.*, 2000) (Figure 4.2b). Hence, this protein is speculated to encode a nonstructural protein (NS1).

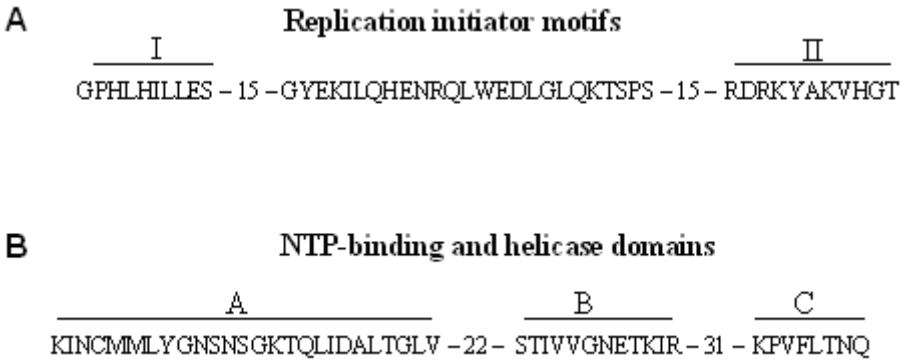


Figure 4.2: Region of non-structural protein one with A) Replication initiator motif I and II and B) NTP-binding (A and B) and helicase domains (C). Bold letters indicate conserved amino acid sequences with other parvoviruses

4.3.3.3. Open reading frame three

The third ORF (ORF 3) started at nucleotide 3620 in reading frame 2+ and terminated with a TAA codon at position 6079. It contained 819 amino acids, corresponding to a molecular weight of 91.622 kDa. This ORF shares 73% amino acid similarity with the structural protein of *Pm*DNV and capsid protein of HPV from *P. chinensis* of Korea (HPVchin), using entries in the protein database via BLAST. There were no additional significant matches with other parvoviruses. Although the function of this protein is yet unknown, it is speculated that this ORF encodes the structural protein (VP1). Alignment of the four partially sequenced HPV strains shows highly conserved regions within the viral protein region across all four HPV strains (Figure 4.3).

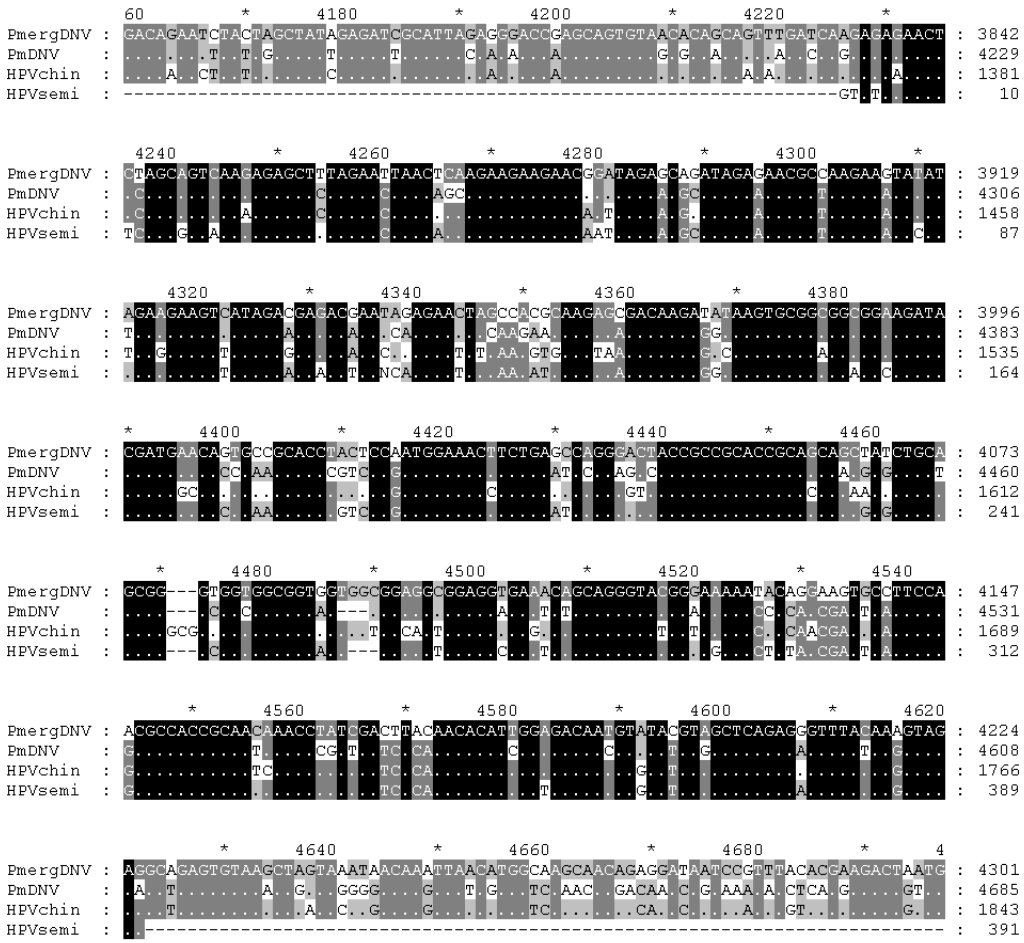


Figure 4.3: Ancestral alignments of all four hepatopancreatic parvovirus strains shows highly conserved nucleotide sequences in the viral protein (VP1) region of the genome. The numbers above aligned sequences represent the position along the sequence of the Australian isolate. Numbers at the end represent the nucleotide position of each strain. The Australian isolate of hepatopancreatic parvovirus is abbreviated as *Pmerq*DNV

4.3.4. Phylogenetic comparison

A phylogenetic tree (Figure 4.4) based on the alignment of genomic DNA of prawn and insect parvoviruses gave two main clades. Clade 1 included the insect parvoviruses *Bombyx mori* densovirus (*Bm*DNV), *Periplaneta fuliginosa* densovirus (*Pf*DNV), *Junonia coenia* densovirus (*Jc*DNV), *Galleria mellonella* densovirus (*Gm*DNV) *Diatraea saccharalis* densovirus (*Ds*DNV) and the prawn parvovirus spawner isolated mortality virus (SMV). Clade 2 included all strains of HPV, infectious hypodermal and haematopoietic necrosis virus (IHHNV) and the insect parvovirus *Aedes aegypti* densovirus (*Aae*DNV) and *Aedes albopictus* densovirus (*Aal*DNV). The Australian HPV isolate (*Pmerg*DNV) is phylogenetically more closely related to the South Korean strain (HPVchin) than to the Indian (HPVsemi) or Thai (*Pm*DNV) isolates, respectively.

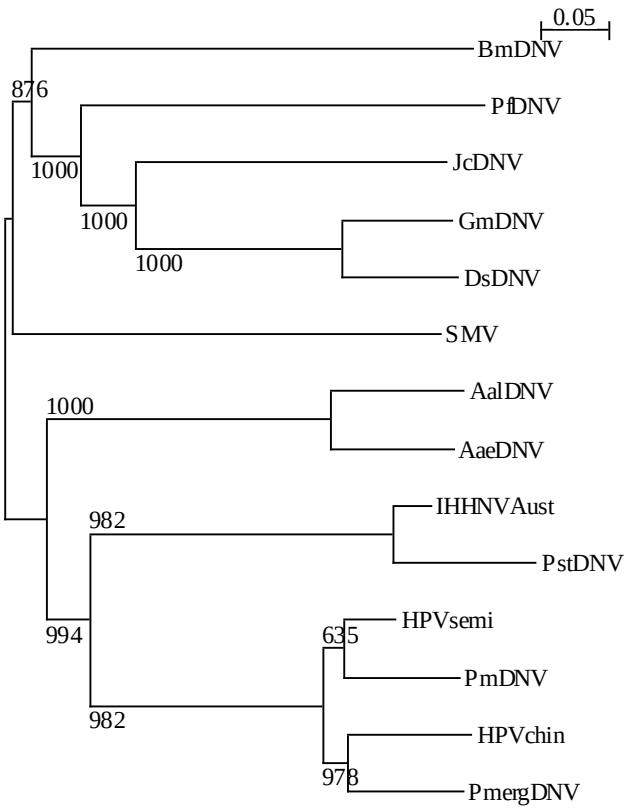


Figure 4.4: Phylogenetic tree generated from aligned shrimp and insect parvovirus sequences. Aligned sequences consisted of both entire genomes and fragments of sequenced genomes. Bootstrap values are indicated as a number on each branch and were calculated from 1000 replications. A list of full names and the source of each sequence is presented in Table 4.2. The Australian isolate of hepatopancreatic parvovirus is abbreviated as *Pmerg*DNV

4.4. Discussion

The Australian HPV isolate from *Penaeus merguiensis* is the fourth strain of penaeid prawn HPV to be partially sequenced. We have therefore proposed to name this virus *P. merguiensis* densovirus (*Pmerg*DNV), following the convention of the International Committee for the Taxonomy of Viruses. The other three are HPVchin from *P. chinensis* of Korea (Bonami *et al.*, 1995), *Pm*DNV from *P. monodon* of Thailand (Sukhumsirichart *et al.*, 2006) and HPVsemi from *P. semisulcatus* of India (Manjanaik *et al.*, 2005). An additional strain of HPV has been reported in the freshwater prawn *Macrobrachium rosenbergii* (Anderson *et al.*, 1990).

The Australian HPV isolate shared the highest nucleotide similarity (86%) with the Korean isolate and the lowest similarity (83%) with the Thai and Indian HPV isolates. However, nucleotide similarity between strains is indicative since only partial sequences of HPVchin and HPVsemi were analysed and the amount of known sequence from each isolate varies considerably. The phylogenetic analysis suggests the Australian HPV isolate is more closely related to HPVchin than to *Pm*DNV and HPVsemi, respectively. This is probably because *P. merguiensis* is phylogenetically more closely related to *P. chinensis* than to *P. semisulcatus* and *P. monodon*, respectively based on mitochondrial large subunit ribosomal RNA (16S RNA) and cytochrome *c* oxidase subunit I (COI) genes (Lavery *et al.*, 2004). Hepatopancreatic parvovirus strains may be following the phylogenetic relationship of the hosts rather than being geographically, nearest neighbour linked. Nonetheless, a complete understanding of the phylogeny of the arthropod parvoviruses can only be achieved when the analyses are performed using complete genomic sequences.

The phylogenetic analysis in this study was similar to the analysis of Roekring *et al*. (2002), with the addition of *Pmerg*DNV, complete sequence data for *Pm*DNV and the inclusion of partial sequence data for the Australian isolate of IHHNV. The strains of HPV, IHHNV and the insect densoviruses *Aae*DNV and *Aal*DNV remained in the one cluster, suggesting stability. The differences were the insect densovirus *Bm*DNV, *Jc*DNV and the prawn parvovirus SMV. In our phylogenetic analysis, *Bm*DNV and *Jc*DNV grouped with the insect

densoviruses *Pf*DNV, *Gm*DNV and *Ds*DNV (Roekring *et al.*, 2002). In contrast, the insect densoviruses *Aae*DNV and *Aal*DNV and the IHHNV and HPV strains are now grouped together in a separate cluster. Furthermore, SMV is now more likely an outgroup than in the analysis by Roekring *et al.* (2002). Similar to the findings from Sukhumsirichart *et al*. (2006), the phylogenetic tree revealed that the HPV strains are closely related to the Brevidensoviruses (IHHNV and the mosquito densoviruses *Aae*DNV and *Aal*DNV), despite differences in genome organisation (discussed below).

This is the first report of nucleotide information concerning HPV from Australia or from any *P. merguiensis*. The complete genome was sequenced (6299 bp) indicating the total genome length is the same size as (approximately 6.3 kb) as the genome of HPV from *P. monodon* of Thailand (Sukhumsirichart *et al.*, 2006). We report three putative ORFs, in which the two non-structural proteins are overlapping. This is similar to the organisation structure of the *Pm*DNV genome where the two nonstructural proteins are also overlapping but differs from the prawn parvovirus IHHNV and the mosquito densoviruses *Aae*DNV and *Aal*DNV (Afanasiev *et al.*, 1991; Boublike *et al.*, 1994; Shike *et al.*, 2000; Sukhumsirichart *et al.*, 2006). The consensus sequence contains the complete sequence of putative NS1, NS2 and VP1. We were also successfully able to sequence the hairpin like structures on the 3' and 5' end of the genome.

In addition to mortality, HPV reduces the growth rate of infected individuals (Flegel *et al.*, 1999), resulting in production losses for farmers because they continue to spend money on feed and maintenance of ponds where prawns will not grow. To date, HPV has been of little concern and has been overlooked. However, as investigations continue, there is now an impression that HPV is an emerging disease and that there are different strains of HPV associated with different species and/or geographical areas. Consequently, HPV is probably more widespread than previously thought and may therefore have a much wider host range than reported. This study was the first to obtain molecular data for HPV in Australia. Its biology, prevalence and pathogenesis in other species await investigation and should be a focus of further research.

CHAPTER 5

TaqMan Real-time polymerase chain reaction for detection of *Penaeus merguiensis* densovirus

5.1. Introduction

The intensification of prawn farming over the years to keep up with the growing demand for seafood products has been accompanied by an increase in the incidence of disease. Diseases of viral aetiology have contributed to substantial economic losses, estimated to be US$3-4 billion per year, within penaeid prawn aquaculture with all the main species of prawns being affected (Chen *et al.*, 1989; Liao, 1992; Lightner, 1992; Lightner and Redman, 1992; Vega-Villasante and Puente, 1993; Destoumieux-Garzon *et al.*, 2001).

Hepatopancreatic parvovirus is an emerging disease which was first reported in 1984 in wild *Penaeus merguiensis* and *P. indicus* from Singapore (Chong and Loh, 1984). Hepatopancreatic parvovirus has been attributed to mortalities reaching up to 100% during outbreaks and is attributed with the stunting of infected stock (Lightner, 1996; Flegel *et al.*, 1999).

The known host range of hepatopancreatic parvovirus strains encompasses at least ten penaeid prawn species around the world and the freshwater prawn *Macrobrachium rosenbergii* (Lightner and Redman, 1985; Anderson *et al.*, 1990; Lightner, 1996). Its current geographic range is from East Africa to Korea including Australia and the Pacific, and the Atlantic coasts of the Americas (Lightner, 1996). The first Australian record of hepatopancreatic parvovirus came from samples of *P. esculentus* from Moreton Bay and the Gulf of Carpentaria in 1985 (Paynter *et al.*, 1985). In 1988, it was reported in *P. merguiensis* (Owens and Hall-Mendelin, 1988) and it has since been recorded in *P. monodon* and *P. japonicus* (Lightner, 1996; Spann *et al.*, 1997).

To date, the diagnosis of hepatopancreatic parvovirus infection includes histological screening, transmission electron microscopy, *in situ* hybridisation (Pantoja and Lightner, 2001; Phromjai *et al.*, 2002), gene probes (Lightner *et al.*, 1994; Mari *et al.*, 1995), polymerase chain reaction (PCR) (Sukhumsirichart *et al.*, 1999; Pantoja and Lightner, 2000; Phromjai *et al.*, 2001), nested PCR (Manjanaik *et al.*, 2005) and polymerase chain reaction-enzyme linked immunosorbent assay (PCR-ELISA) (Sukhumsirichart *et al.*, 2002). The published OIE primers of Phromjai *et al*. (2001) for hepatopancreatic parvovirus do not work efficiently with the Australian hepatopancreatic parvovirus strain or give the anticipated product size (Chapter 4). However, in order to screen prawns in Australia, a method that is capable of diagnosing low-grade infections needs to be developed.

Real-time TaqMan PCR is an alternative method to conventional PCR which is capable of providing a concise quantitative measure of the number of viral copies in a tissue sample. In addition, the hybridisation of the internal probe provides a validation that the PCR is amplifying the correct sequence. Real-time TaqMan PCR involves sequence specific primers and a fluorescent oligonucleotide probe designed within the target sequence of DNA. During the PCR amplification cycle, the probe is hybridised resulting in a fluorescent signal measuring the number of copies of DNA based on a standard curve generated with known equivalents of the target DNA. In this case, plasmid containing the target sequence of DNA was used. This study describes the first quantitative real-time TaqMan PCR assay for the rapid detection of the *P. merguiensis* strain of hepatopancreatic parvovirus (*Pmerg*DNV).

5.2. Materials and Methods

5.2.1. Source of *Penaeus merguiensis*

Samples of *Penaeus merguiensis* (40) stored frozen at James Cook University, Queensland were used as the source of *Pmerg* DNV. Infected specimens were originally obtained from three commercial prawn farms in northern Queensland as early as June 2003. Wild caught *P. merguiensis* were collected from the north eastern coast of Queensland in 2005 by bottom trawling. Specimens were stored

frozen at James Cook University, Queensland. *Penaeus merguiensis* densovirus was purified from the hepatopancreai of *P. merguiensis* stored frozen at James Cook University, Queensland (Chapter 3.2).

5.2.2. Isolation of nucleic acids

DNA was extracted from 50 µl of the hepatopancreatic parvovirus stock solution using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions.

5.2.3. Oligonucleotide primers and probe

The primers and TaqMan probe were designed to amplify a fragment of the genome encoding the capsid protein of *P. merguiensis* densovirus (DQ458781) (Chapter 4), using the default parameters of Beacon Designer 4.02 (Premier Biosoft International, California, USA). This program selected primer sets and probes with appropriate melting temperatures, base composition and amplicon lengths. The oligonucleotide primers were HPV140F 5'-CTA CTC CAA TGG AAA CTT CTG AGC-3' at positions 4380 bp to 4403 bp and HPV140R 5'-GTG GCG TTG GAA GGC ACT TC-3' at positions 4500 bp to 4519 bp along the genome of *P. merguiensis* densovirus (DQ458781). These primers yield a PCR amplicon of 139 bp. The fluorogenic probe was 5' labelled with FAM (6-carboxyfluoresin) and 3' labelled with the quenching dye TAMRA (6-carboxytetramethylrhodamine). The sequence of the oligonucleotide probe was 5'-FAM TAC CGC CGC ACC GCA GCA GC TAMRA -3' and is situated at positions 4411 bp to 4430 bp. TaqMan primers and probe were synthesised by Sigma Genesis, USA.

5.2.4. Specificity of oligonucleotide primers and probe

The specificity of the primers was tested against the viral genome to ensure multiple products were not amplified from the viral genome or the genome of *P. merguiensis*. DNA was extracted from approximately 50 mg of hepatopancreatic tissue of *P. merguiensis* using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Polymerase chain reaction was performed on extracted DNA from *P. merguiensis* and an aliquot of the hepatopancreatic parvovirus stock solution

(Chapter 5.2.1) using the HPV140 primers. The reaction mixture consisted of 20-50 ng of DNA template, 10 x *Taq* buffer (750mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween20), 2.5 mM $MgCl_2$, 0.75 U *Taq* polymerase (MBI Fermentas), 200 μM of dNTPs, 50 pmol of each primer and nuclease-free water to a total volume of 25 μl. Amplification was performed in an Eppendorf Mastercycler Gradient Thermocycler (Eppendorf, Germany) with a PCR profile consisting of initial denaturation at 94ºC for 7 minutes, 35 cycles at 94ºC for 45 seconds denaturation, 65ºC annealing for 45 seconds and polymerisation at 72ºC for 1 minute. Samples were polymerised for an additional 5 minutes at 72ºC following the last cycle. Amplified products were visualised using gel electrophoresis.

DNA fragments were purified from agarose gels using Wizard®SV Gel and PCR Clean-Up System (Promega, New South Wales, Australia) and cloned into *Escherichia coli* JM 109 cells, using pGEM-T® Easy Vector System (Promega, New South Wales, Australia), according to the manufacture's instructions. Recombinant plasmids were removed using Fast Plasmid™ Mini Kit (Eppendorf, Germany) and digested with *EcoR* I, followed by gel electrophoresis to screen for DNA inserts. Recombinant plasmids containing DNA inserts were sequenced using the Amersham Chemistry DYEnamic ET terminator sequencing kit (Amersham Biosciences) and M13 universal primers. Three forward and three reverse reactions were performed for each of three selected clones. Samples were analysed at the Advanced Analytical Centre at James Cook University, Townsville with a MegaBACE Sequence Analyser (Amersham Biosciences). Sequencher™ software (Gene Codes Corporation) was used to analyse and align overlapping sequences for each clone. Sequences were compared to available sequences using Basic Local Alignment Search Tool (BLAST).

5.2.5. Construction of plasmid standard for quantitation by Taqman polymerase chain reaction

The OIE primers for hepatopancreatic parvovirus amplified an unexpectedly larger 843 bp PCR product located at positions 3971 bp to 4814 bp along the *Pmerg*DNV genome, despite some lack of homology between the primer and target sequences. Serial dilutions from $1x10^9$ to $1x10^0$ copies of the plasmid standard were made using sheared salmon sperm DNA (2 ng/µl) as a diluent.

5.2.6. Optimisation of TaqMan assay and quantitation

Reactions were performed in a total volume of 20 µl using a Rotor-Gene 3000 (Corbett Robotics) 36-well rotor. The cycle pattern consisted of an initial incubation at 95ºC for 10 minutes, followed by a two-step cycle pattern consisting of forty cycles at 95ºC for 10 seconds and 60ºC for 45 seconds. Data acquisition and analysis was performed using Rotor-Gene 3000 (Corbett Robotics) and Microsoft Excel.

5.2.6.1. Optimisation of $MgCl_2$ concentration

$MgCl_2$ concentration was optimised by running 0.5 mM increasing increments of additional $MgCl_2$ to the TaqMan mastermix (Invitrogen, Victoria, Australia) (Table 5.1) against an aliquot of *Pmerg*DNV positive control plasmid. The reaction mixture composed of 10 µl of TaqMan mastermix (Invitrogen, Victoria, Australia), 10 µM of each primer, 10 µM of probe, 2.5 µl of plasmid template at a concentration of $1x10^5$ copies for each reaction and $MgCl_2$. Four reactions were carried out for each concentration of additional $MgCl_2$ from 0 mM to 3.5 mM in increments of 0.5 mM. Nuclease-free water was added so the final reaction volume was 20 µl. Three no-template controls were included in the TaqMan run.

5.2.6.2. Optimisation of probe concentration

A TaqMan PCR run was performed with four replicates of samples containing probe concentrations of 400 nM, 300 nM, 200 nM, 100 nM, 90 nM, 80 nM, 70 nM, 60 nM and 50 nM. The reaction mixture consisted of 10 µl of TaqMan mastermix (Invitrogen, Victoria, Australia), 10 µM of each primer, 0.5 mM of additional $MgCl_2$ (Invitrogen, Victoria, Australia), 2.5 µl of plasmid template at a concentration of $1x10^5$ copies for each reaction, probe and nuclease-free water to

a final volume of 20 µl. Three no-template controls were included in the TaqMan run.

5.2.6.3. Generation of standard curve

Three standard curves were generated using serial dilutions from $1x10^9$ to $1x10^0$ copies of the plasmid standard to determine the sensitivity of the assay. Each real-time TaqMan PCR run contained 10 µl of TaqMan mastermix (Invitrogen, Victoria, Australia), 0.5 mM of additional $MgCl_2$ (Invitrogen, Victoria, Australia), 10 µM of each primer, 50 nM of probe, 2.5 µl of plasmid template and nuclease-free water to a final volume of 20 µl. Each run contained three replicates of each serial dilution and three no template controls. Three standard curves were generated independently on different days up to two weeks apart to determine the sensitivity and reproducibility of the assay and to test whether the higher dilutions of target DNA were influenced by pipetting error or absorption of DNA to tube walls. The sensitivity of the assay was determined by testing serial dilutions from $1x10^9$ to $1x10^0$ copies of plasmid containing the targeted viral sequence.

5.2.7. Clinical specificity of TaqMan assay

The clinical specificity of the TaqMan assay for detecting *Pmerg*DNV was determined by performing the TaqMan assay on other crustacean viruses and other parvoviruses. The TaqMan assay was performed on *P. monodon* DNA samples that had previously been classified positive for infectious hypodermal and haematopoietic necrosis virus (IHHNV) by PCR (unpublished results) and DNA extracted from a purified aliquot of canine parvovirus according to the protocol of the High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany). The specificity of the primers and probe sequence against previously described hepatopancreatic parvovirus strains and other crustacean viruses that are not present in Australia (for example, white spot syndrome virus) were determined using BLAST.

5.2.8. Quantitative detection of *Penaeus merguiensis* densovirus in wild-caught *Penaeus merguiensis*

Twenty-two *Penaeus merguiensis* specimens were screened for hepatopancreatic parvovirus infection using the TaqMan assay described. *Penaeus merguiensis* specimens were obtained off the coast of Townsville from local trawlers. Genomic DNA was extracted from approximately 50 mg of hepatopancreatic tissue according to the protocol of the High Pure PCR Template Preparation Kit (Roche Diagnosis, Germany). Each TaqMan run contained three no template controls and three replicates of 10^3 and 10^6 copies of the plasmid standard. Three reactions were performed independently on different days to determine the specificity and reproducibility of the assay.

5.2.9. Comparisons between histological examination and polymerase chain reaction detection of *Penaeus merguiensis* densovirus

The sensitivity of the assay was assessed by screening 545 *P. merguiensis* from three commercial prawn farms in northern Queensland. All animals were screened for hepatopancreatic parvovirus by PCR (Chapter 5.2.4) and histology. All animals were prepared for histology by fixing half of the cephalothorax in Davidson's fixative for 48 hours and subsequently transferred to 70% ethanol. Tissues were processed routinely for histology by passing through a dehydrating series of ethanol and embedded in paraffin wax. Sections of the gills and hepatopancreas were cut at 5 µm and stained with Mayer's hemotoxylin and eosin (H & E). Sections were viewed by light microscopy.

5.3. Results

5.3.1. Specificity of oligonucleotide primers and probe

A 140 bp amplicon was produced using the set of HPV140 primers (Figure 5.1). A BLAST comparison of the 140 bp product did not have any significant match against any organism but had a 100% sequence identity with the targeted sequence from the genome of *Pmerg*DNV (Chapter 4).

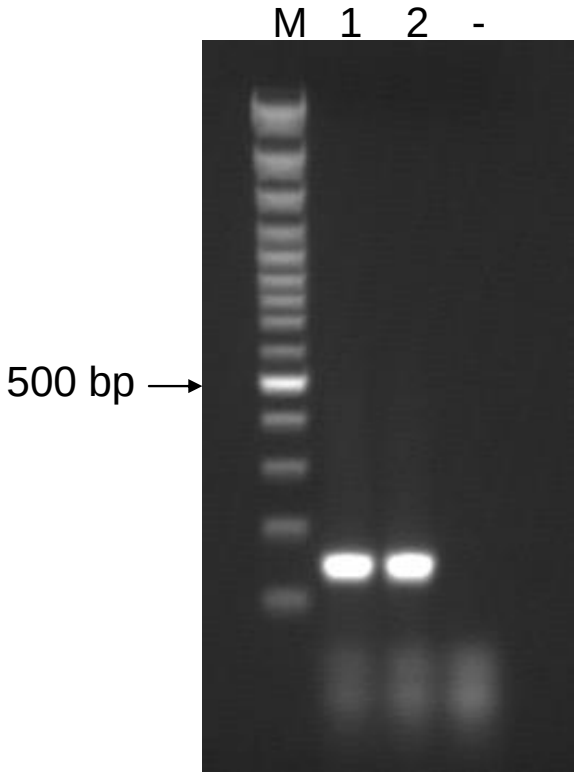


Figure 5.1: Specificity of the oligonucleotide primers. Lane M: 100 bp plus DNA ladder (Fermentas), Lane 1: DNA extracted from infected *Penaeus merguiensis*, Lane 2: DNA extracted from purified hepatopancreatic parvovirus virions, Lane -: Negative control

5.3.2. Optimisation of TaqMan reagents

The optimum TaqMan reaction mixture for the hepatopancreatic parvovirus assay comprises of 10µl of TaqMan mastermix (Invitrogen), 0.5 mM of additional $MgCl_2$ (Invitrogen, Victoria, Australia) (Table 5.1), 10 µM of each primer, 50 nM of probe (Table 5.1), 2.5 µl of DNA template and nuclease-free water to a final volume of 20 µl.

Table 5.1: Optimisation of $MgCl_2$ and probe concentrations for *Penaeus merguiensis* densovirus TaqMan assay. Mean C_T represent four replicates of each concentration

$MgCl_2$			**Probe**		
Concentration	**Mean C_T**	**Standard deviation**	**Concentration**	**Mean C_T**	**Standard deviation**
0 mM	20.43	0.1	400 nM	22.32	0.13
0.5 mM	20.53	0.12	200 nM	22.11	0.18
1.0 mM	21.44	0.33	100 nM	22.3	0.16
1.5 mM	21.43	0.2	90 nM	22.3	0.04
2.0 mM	21.72	0.15	80 nM	22.33	0.13
2.5 mM	22.19	0.34	70 nM	22.18	0.16
3.0 mM	22.13	0.35	60 nM	22.33	0.03
3.5 mM	22.85	0.39	50 nM	22.51	0.1

5.3.3. Standard curve and detection limit

In three independently generated standard curves, linearity between the TaqMan C_T values and target concentration was observed over nine orders of magnitude in a 10x dilution series. The lower limit of detection of hepatopancreatic parvovirus was found to be 10 copies (Figure 5.2) of the plasmid standard for the respective virus, with small (less than 1) mean C_T standard deviations from as low as 0.01. The results demonstrated that the assay was reproducible and that accurate quantification of the target DNA was possible down to 10 copies.

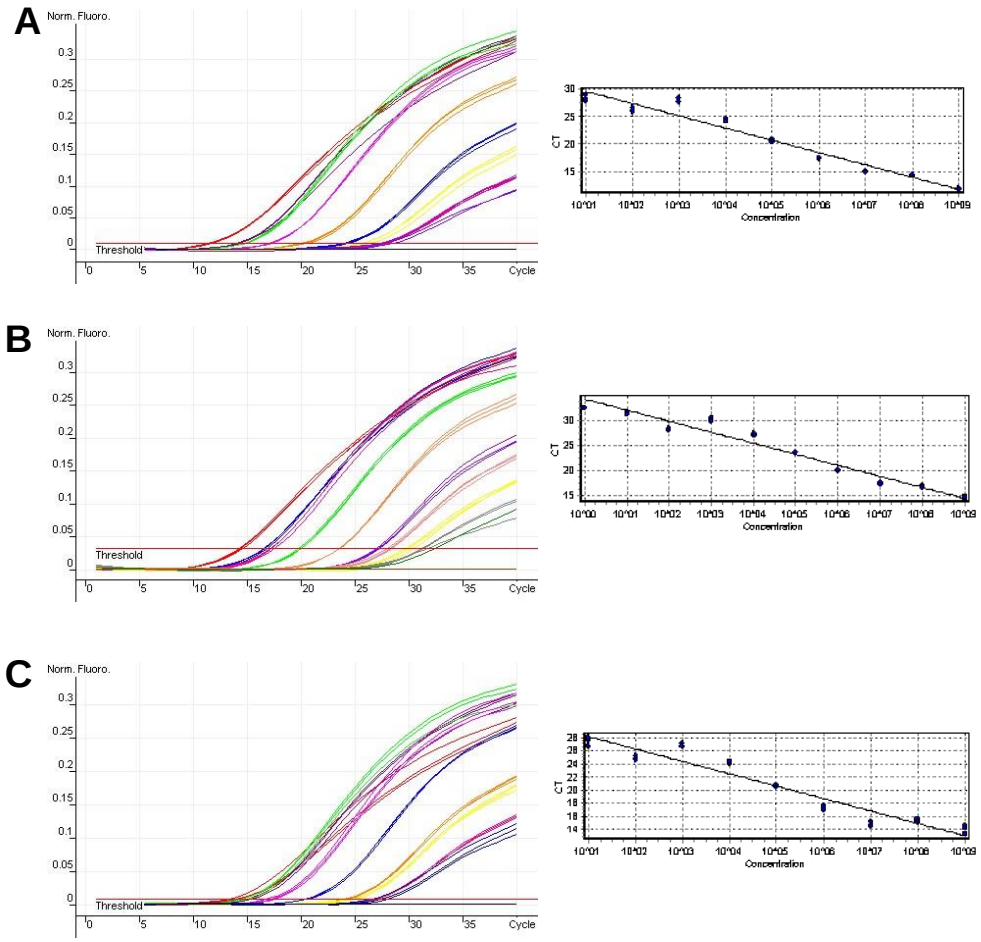


Figure 5.2: Sensitivity of real-time TaqMan assay for *Penaeus merguiensis* densovirus. Real-time analysis of serial 10 folds dilutions of *Penaeus merguiensis* densovirus standard. (A) Flourescence and standard curve generated on the first trial to examine the sensitivity and reproducibility of the assay. (B) Flourescence and standard curve generated on the second run of the assay. (C) Flourescence and standard curve generated on the third run of the assay

5.3.4. Clinical specificity of TaqMan assay

In total, three reactions were performed independently on different days to determine the specificity and reproducibility of the assay. For all three reactions, no fluorescence was observed for all canine parvovirus and IHHNV positive *P. monodon* samples, suggesting high specificity of the assay against other parvoviruses. Since other hepatopancreatic parvovirus strains and crustacean viruses such as white spot syndrome virus (WSSV) are not known to occur in Australia, DNA concerning other crustacean viruses could not be obtained for further examination of the specificity of the TaqMan assay. However, BLAST

searches of the primers and probe did not yield significant matches with published sequences for other crustacean parvoviruses or prawn viruses such as WSSV.

5.3.5. Quantitative detection of hepatopancreatic parvovirus in wild-caught *Penaeus merguiensis*

Genomic DNA extracted from the hepatopancreas of all twenty-two wild-caught *P. merguiensis* fluoresced upon exposure to the real-time PCR assay, and all no template control reaction failed to fluoresce. The number of viral copies varied from 10^4 to 10^{13} copies (Table 5.2).

Table 5.2: Calculated concentrations (number of copies) of *Penaeus merguiensis* densovirus for replicate assays of genomic DNA of wild-caught *Penaeus merguiensis*

***Penaeus merguiensis* sample**	**Number of copies of hepatopancreatic parvovirus**			
	Assay 1	**Assay 2**	**Assay 3**	**Mean number of copies**
1	2.40×10^5	2.46×10^5	2.31×10^5	2.39×10^5
2	5.03×10^6	5.15×10^6	4.67×10^6	4.95×10^6
3	6.24×10^{10}	6.67×10^{10}	6.26×10^{10}	6.39×10^{10}
4	2.16×10^7	2.02×10^7	2.12×10^7	2.10×10^7
5	3.04×10^{11}	3.18×10^{11}	3.11×10^{11}	3.11×10^{11}
6	3.75×10^5	3.70×10^5	3.82×10^5	3.63×10^5
7	1.55×10^{13}	1.84×10^{13}	1.41×10^{13}	1.60×10^{13}
8	2.16×10^{10}	2.76×10^{10}	5.79×10^{10}	3.57×10^{10}
9	2.56×10^7	2.49×10^7	2.51×10^7	2.52×10^7
10	1.10×10^{11}	1.02×10^{11}	1.14×10^{11}	1.09×10^{11}
11	2.04×10^{13}	2.18×10^{13}	2.24×10^{13}	2.15×10^{13}
12	5.59×10^5	5.77×10^5	5.82×10^5	5.73×10^5
13	2.22×10^8	2.40×10^8	2.09×10^8	2.24×10^8
14	4.52×10^9	4.69×10^9	4.93×10^9	4.71×10^9
15	1.04×10^3	1.54×10^3	1.60×10^3	1.39×10^3
16	2.35×10^6	2.02×10^6	1.98×10^6	2.12×10^6
17	1.18×10^6	1.04×10^6	1.16×10^6	1.13×10^6
18	6.62×10^5	8.25×10^5	6.92×10^5	2.18×10^5
19	1.23×10^9	1.12×10^9	1.15×10^9	1.17×10^9
20	8.69×10^6	6.58×10^6	7.98×10^6	7.75×10^6
21	1.52×10^5	1.60×10^5	1.59×10^5	1.57×10^5
22	8.64×10^4	8.79×10^4	9.28×10^4	8.90×10^4

5.3.6. Comparisons between histological examination and polymerase chain reaction detection of *Penaeus merguiensis* densovirus

In total, 545 animals were screened for hepatopancreatic parvovirus infection by histology and PCR. Only 13% of *P. merguiensis* examined were negative for hepatopancreatic parvovirus infection (Figure 5.3). However, of the 87% that were PCR positive for hepatopancreatic parvovirus infection, only 31% of specimens were positive by histological examination.

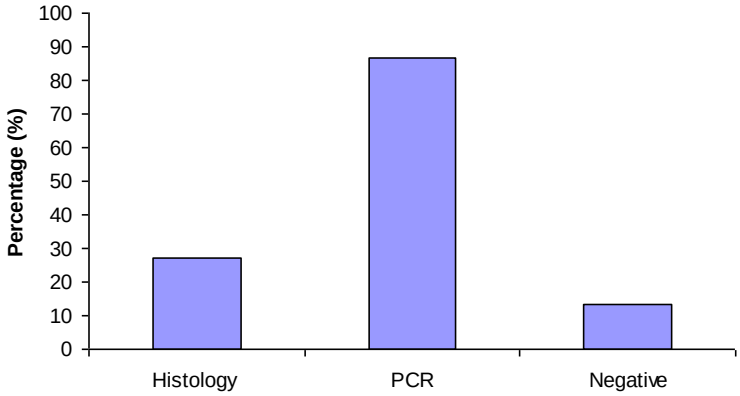


Figure 5.3: Percentage of 545 *Penaeus merguiensis* positive for *Penaeus merguiensis* densovirus infection by histology and polymerase chain reaction and negative by both techniques

5.4. Discussion

Here we describe the first TaqMan based real-time assay for the rapid detection of hepatopancreatic parvovirus. The specificity of the TaqMan PCR primers on the infected tissue of *P. merguiensis* was confirmed by traditional PCR, where the PCR product was sequenced and compared to available databases using BLAST. Furthermore, the specificity of the internal probe was determined by searches for homology in nucleotide databases. There was no significant match with other crustacean or arthropod parvoviruses, suggesting specificity for the Australian hepatopancreatic parvovirus strain (*Pmerg*DNV). The clinical specificity of the assay was partially confirmed by performing the assay on canine parvovirus and another crustacean parvovirus from *P. monodon*, IHHNV, where no fluorescence was recorded. This assay was successfully applied to the quantitation of viral loads in wild *P. merguiensis* and has been applied to

diagnose hepatopancreatic parvovirus infection in farmed and experimentally infected *P. monodon* and *Cherax quadricarinatus*. The analytical sensitivity of this assay suggests its usefulness for the detection of hepatopancreatic parvovirus in diagnostic analyses.

We were successfully able to optimise the concentration of additional $MgCl_2$ added to each reaction down to 0.5 mM. Although 0 mM $MgCl_2$ provided the lowest C_T value, for clinical use we recommended an additional 0.5 mM $MgCl_2$ in each reaction to account for any traces of EDTA that may be left in the DNA samples from DNA extraction. Furthermore, we were able to successfully optimise the assay so that a probe concentration of only 50 nM was required. Consequently, this assay is a cost effective and efficient method for analysing clinical samples and receiving accurate quantitative information on the number of viral copies within a sample.

The prevalence of hepatopancreatic parvovirus based on the PCR results of farmed *P. merguiensis* was 87%. Yet, only 27% of these infections were diagnosed by histological examination. Methods such as PCR are favoured since low grade infections can be detected despite the absence of inclusion bodies in histological examination. However, the economic impact of such a high prevalence of hepatopancreatic parvovirus has not been established. The high prevalence of hepatopancreatic parvovirus infection in our study is not unusual since a prevalence of up to 100% has previously been reported in hatchery-reared postlarvae in the Philippines (Catap *et al.*, 2003). The prevalence of hepatopancreatic parvovirus is highly variable with the prevalence reported to range between 31-62% in wild *P. monodon* from India (Umesha *et al.*, 2003; Manjanaik *et al.*, 2005).

Although the assay developed in this study is specific to the Australian *P. merguiensis* hepatopancreatic parvovirus strain (*Pmerg*DNV), further studies are required for establishing complete sequences of the genome of all currently described hepatopancreatic parvovirus strains so a generic assay can be developed for the detection of all strains by targeting conserved regions within the genome. Hence, the one TaqMan assay would permit the detection and

quantitation of any of the existing hepatopancreatic parvovirus strains and could be applied in the routine screening in all countries around the world.

This study is the first to have devised a rapid, sensitive and specific real-time TaqMan assay for the detection of the Australian strain of hepatopancreatic parvovirus (*Pmerg*DNV) or indeed any strain of hepatopancreatic parvovirus. It permits the simultaneous detection and quantitation of hepatopancreatic parvovirus and can be used for the detection of the virus in culture facilities to prevent economic losses. This would subsequently benefit Australian aquaculturists by allowing them to efficiently exclude potential carriers of the virus from their broodstock and subsequently prevent disease outbreaks.

CHAPTER 6

Effect of *Penaeus merguiensis* densovirus on *Penaeus merguiensis* production in Queensland, Australia

6.1. Introduction

Aquaculture continues to be an important component of Australian fisheries production. After declining for several years, the gross value of Australian aquaculture increased by 18 percent in 2005-06 to $748 million (ABARE, 2007). Although this increase was driven largely by an increase in the value of finfish aquaculture (rising from $315 million in 2004-05 to $451 million in 2005-06), marine prawns are a key aquaculture species in Australia, valued at $56 million (8%) in 2003/04 (Hanna *et al.*, 2005). Prawns are the dominant crustacean species farmed in Australia and include the most commonly farmed species, the black tiger prawn (*Penaeus monodon*), banana prawn (*P. merguiensis*), brown tiger prawn (*P. esculentus*), and kuruma prawns (*P. japonicus*) (Lobegeiger and Wingfield, 2007). The value of prawn aquaculture production has increased steadily since 1990, estimated at $35 million in 1996-97 (ABARE, 1997), rising to $65 million in 2001-02 (ABARE, 2003) and falling to close to $50 million in 2005-06 (ABARE, 2007). The value of the Queensland component of the aquacultured prawn industry alone was estimated to be approximately 93 percent of the whole aquacultural prawn industry at $46.5 million (ABARE, 2007). However, the impact of diseases is recognised as one of the problems in prawn farming in Queensland.

The impacts of disease have been estimated in socio-economic terms (eg. losses in production, income, employment, market access or market share, investment and consumer confidence, food shortages and industry failure or closure of business or industry). Analysis of economic impacts of aquatic animal diseases is very much a grey area in the literature. However, due to the frequency of occurrence and the magnitude of spread and effects, many countries are now providing some estimates of disease impacts. At a global level, combined estimated losses in production value due to prawn disease from 11 countries for

the period 1987-1994 were on the order of US$3.02 billion (Lundin, 1997; Israngkura and Sae-Hae, 2002).

Some of the major socio-economic and other impacts of diseases in prawn aquaculture include losses of US$280.5 million in Ecuador in 1999 due to white spot syndrome virus (WSSV), resulting in closing of hatchery operations, 13% laying off of labour force (26,000 people) and a 68% reduction in sales and production of feed mills and packing plants. In 1994, Thailand experienced losses of US$650 million (excluding losses in related businesses such as feed production, processing and exporting and loss of income for labourers) due to yellow head disease and WSSV. Production declined 12% from 250,000 tonnes to 220,000 tonnes within 12 months and losses for 1997 almost reached 50% of the value of total farm output (Bondad-Reantaso, 2004). During the period 1994-1998, the Australian prawn aquaculture sector experienced *P. monodon* production losses worth US$32.5 million due to mid-crop mortality syndrome (MCMS) including gill-associated virus (GAV) (Bondad-Reantaso, 2004).

There are at least three major viruses affecting penaeid prawn production in Queensland: GAV, infectious hypodermal and hematopoietic necrosis virus (IHHNV) and *Penaeus merguiensis* densovirus (*Pmerg*DNV).
Penaeus merguiensis densovirus currently occurs on several Queensland prawn farms culturing *P. merguiensis*. However, the impact of this disease on the Australian penaeid aquaculture industry has not been estimated in terms of production losses. Here we attempt to determine the impact of *Pmerg*DNV disease on three *P. merguiensis* culture facilities in northern Queensland to determine if *Pmerg*DNV warrants further control methods.

6.2. Materials and Methods

6.2.1. Source of larvae

Ten day old postlarvae (PL10) of *P. merguiensis* were obtained from three commercial prawn farms in northern Queensland. Larvae were transported to James Cook University in single vials of 95% methylated spirits (95% ethanol, 5% methanol) labelled with the farm's pond number from which they were stocked. Hence, only the hatchery had records of which farm or pond each vial of larvae were collected (i.e. they were processed blind).

Two groups of larvae were collected and provided for analysis. The first group of larvae was sourced from 55 ponds stocked between January and June, 2007. The second group of larvae was sourced from 135 ponds stocked between August, 2007 and March, 2008.

6.2.2. Sample preparation of ten day old postlarvae of *Penaeus merguiensis*

Penaeus merguiensis PL10s were divided into ten replicates of fifteen larvae from each pond (total of 150 larvae). The hepatopancreas was removed from larvae by dissecting below the eyestalks and behind the cephalothorax region using a sterile scalpel blade and stored in 95% ethanol for processing.

6.2.3. Nucleic acid extraction

Total DNA was extracted from tissues using Promega Wizard SV Genomic DNA Purification System (New South Wales, Australia), according to the manufacturer's instructions.

6.2.4. Nested polymerase chain reaction

6.2.4.1. Outer nest polymerase chain reaction

The outer nest PCR primers had previously been used to sequence the genome of *Pmerg*DNV from Australian *P. merguiensis* (GenBank Accession Number: DQ458781) (Chapter 4). The forward and reverse primers were at base pair positions 3449 and 4645 of the *Pmerg*DNV genome, respectively. Primer sequences were HPV605F 5'- AGCATTAGGGCAAGAGGAAAG-3' and HPVconfirmR 5'- TGACTTTCTTTGCCATTACCA-3'. These primers yielded a 1176 bp fragment within the capsid protein of *Pmerg*DNV.

One microlitre of template DNA (Chapter 6.2.3) was added to 24 µl of reaction mixture containing 1 x *Taq* buffer (750 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween20), 2.5 mM $MgCl_2$, 0.75 U *Taq* polymerase (MBI Fermentas), 200 µM each dNTP, 50 pmol of each primer and nuclease-free water. The reaction was carried out for 35 cycles of 94^oC for 45 seconds denaturation, 55^oC annealing for 45 seconds and polymerisation at 72^oC for 1 minute in an Eppendorf Mastercycler Gradient Thermocycler (Eppendorf, Germany). Prior to the first cycle, there was an additional heat denaturation of 94^oC for 7 minutes. Following the last cycle, samples were polymerised for an additional 5 minutes at 72^oC. Amplified products were stored at four degrees and used as the DNA template for the innernest PCR (Chapter 6.2.4.2).

6.2.4.2. Inner nest polymerase chain reaction

Inner nest PCR primers were the same primers used for real-time PCR (Chapter 5); HPV140F and HPV140R situated within the capsid protein at positions 4380 and 4500 along the *Pmerg*DNV genome. Amplification was performed using the same concentration of reagents and under the same cycling conditions as the outer nest PCR (Chapter 6.2.4.1).

6.2.5. Real-time polymerase chain reaction

Real-time PCR was performed using RotorGene 3000 (Corbett Research, Sydney, Australia) (Chapter 5.3.2). However, the 10 µl of Platinum® qPCR Supermix-UDG (Invitrogen) was replaced with 10 µl of Immomix (Bioline, Australia) and triplicate samples of 1 x 10^4 and 1 x 10^8 copies of the plasmid standard were included in each run. Real-time PCR was performed using DNA template from both the outer nest and inner nest PCR from each pond in duplicate. Data acquisition and subsequent data analyses were performed using RotorGene 3000 and Microsoft Excel.

6.2.6. Statistical analyses

Data was analysed to determine the maximum attributable risk by comparing above and below mean survival of the lightly infected ponds against the above and below mean survival of the moderately to heavily infected ponds. The maximum attributable risk {[a(c+d)/c(a+b)-1]/a(c+d)/c(a+b) (refer to Table 4.1 for cell labels) (Cockcroft and Holmes, 2003)} was estimated in a sequential manner. The attributable risk was maximised by considering ponds with zero replicates positive for *Pmerg*DNV categorised as uninfected and ponds with one or more replicates positive for *Pmerg*DNV categorised as having a load of *Pmerg*DNV. Then the attributable risk was calculated considering ponds with one replicate positive for *Pmerg*DNV categorised as lightly infected and ponds with two or more replicates positive for *Pmerg*DNV categorised as being moderately to heavily infected and so fourth, until the attributable risk was maximised and further analyses resulted in a decrease in the attributable risk. At maximum attributable risk, the odds ratio (ad/bc) (Cockcroft and Holmes, 2003) was calculated.

6.3. Results

6.3.1. Ponds stocked between January and June, 2007

In total, 550 individual replicates (8,250 larvae from 55 ponds) were screened for *Pmerg*DNV infection (Table 6.1). Three ponds were excluded from the analysis due to incomplete data corresponding to those prawns provided by the farms. The mean survival of the 51 ponds at harvest was 60.1%. The maximum attributable risk (0.29) was obtained when ponds with less than two replicates were considered as being lightly infected with *Pmerg*DNV and those with two or more replicates positive for *Pmerg*DNV having moderate to heavy *Pmerg*DNV loads. The odds for below the mean survival when more heavily infected with *Pmerg*DNV were 2.12 (95% CI=0.6-7.5) times more likely than when lightly or not infected with *Pmerg*DNV.

Table 6.1: Summary of the number of ponds with below and above mean survival compared against heavy exposure to *Penaeus merguiensis* densovirus during January and June, 2007

	Survival < mean	**Survival > mean**	**Totals**
Exposed to heavy loads of *Penaeus merguiensis* densovirus	9 (a)	5 (b)	14 (a + b)
Not exposed to heavy loads of *Penaeus merguiensis* densovirus	17 (c)	20 (d)	37 (c + d)
Totals	26 (a + c)	20 (b + d)	51 (a + b+ c+ d)

6.3.2. Ponds stocked between August, 2007 and March, 2008

In total, 1350 individual replicates (20, 250 larvae from 135 ponds) were screened for *Pmerg*DNV infection (Table 6.2). Seventeen ponds were excluded from the analysis due to incomplete data corresponding to those samples provided by the farms. The mean survival of the 118 ponds at harvest was 54%. The maximum attributable risk (0.28) was obtained when ponds with less than four replicates were considered as being lightly infected with *Pmerg*DNV and those with four or more replicates positive for *Pmerg*DNV having moderate to heavy *Pmerg*DNV loads. The odds for below the mean survival when heavily infected with *Pmerg*DNV were 1.8 (95% CI=0.74-4.4) times more likely than when lightly or not infected with *Pmerg*DNV.

Table 6.2: Summary of the number of ponds with below and above mean survival compared against exposure to *Penaeus merguiensis* densovirus during August 2007 and March 2008

	Survival < mean	**Survival > mean**	**Totals**
Exposed to heavy loads of *Penaeus merguiensis* densovirus	13 (a)	12 (b)	25 (a + b)
Not exposed to heavy loads of *Penaeus merguiensis* densovirus	35 (c)	58 (d)	93 (c + d)
Totals	48 (a + c)	70 (b + d)	118 (a + b+ c+ d)

6.4. Discussion

This study was the first to analyse the impact of *Pmerg*DNV disease on production of *Penaeus merguiensis* in Queensland aquaculture and is the first of its kind to determine the extent of the impact that strains of hepatopancreatic parvovirus may have on penaeid production.

There was a consistent pattern observed between the two data sets. The odds ratios for the samples received in 2007 and 2008 were 2.12 and 1.8, respectively. These values indicate that the odds are approximately 2:1 that ponds with moderate to heavy loads of *Pmerg*DNV in the postlarvae will have below mean survival at harvest. Although the mean survival differed between the two data sets (60.1% for 2007 samples and 54% for 2008 samples), and therefore what was considered to be moderately or heavily infected slightly differed between the two data sets (greater than or equal 20% and 40% of replicates moderate or heavily infected with *Pmerg*DNV from 2007 and 2008 data respectively), the odds ratio remained at approximately 2:1.

Since the first reports of HPV disease in the early 1980's, there have been several studies documenting the prevalence of HPV disease among prawn farms. The impact of this disease on survival and how this could equate to losses in production has been discussed. The first of these were in 1984, where Chong and Loh reported a prevalence of 50% (on average) across two penaeid farms in Singapore. Interestingly, one of these farms had reported reduced feed intake and moderate mortalities one month prior to the study. Accumulative mortality rates in epizootics have been reported in *P. merguiensis* and *P. semisulcatus* as high as 50-100%, respectively (Lightner and Redman, 1985). Aside from mortalities, HPV infection has been associated with stunting of infected prawns. However, it wasn not until the late 1990s that a statistical negative correlation was reported between the length and weight of infected prawns and severity of infection (Flegel *et al.*, 1999). Out of a total harvest of 5000 kg, one farm in Thailand had reported 200 kg of stunted prawns, which would equate to an increase in profit of 20% if these prawns had grown to the average size of normal prawns at harvest (Flegel *et al.*, 1999). Hence, taking into account previous reports and the results

from this study, there is mounting evidence that HPV can result in substantial economic losses due to decreased production.

Attributable risk values were also consistent between the two data sets (0.29 and 0.28 for 2007 and 2008 samples, respectively) suggesting 28-29% of ponds with below average survival will have at least average survival following the removal or decreased levels of *Pmerg*DNV. Hence, if survival only increases to match the mean survival, there would be an expected increase in production across the farm of 14%. However, it would be expected that production would increase by a minimum of 14% since survival in ponds that had above average survival with *Pmerg*DNV (and consequently production) would also be likely to increase. The Queensland prawn industry is worth approximately $46.5 million (ABARE, 2007). In terms on monetary value, the Queensland penaeid industry would be worth an additional $6.5 million in the first year and hence, would be expected to further increase in production in the following years if it was solely stocked with species susceptible to *Pmerg*DNV (*P. merguiensis*, *P. esculentus*, *P. japonicus*).

The results from this study are the predicted outcomes based on the removal or reduction of *Pmerg*DNV within the farms. However, the culture of penaeid prawns is a complex system influenced by a number of extrinsic and intrinsic factors. Water temperature is considered the most important environmental variable because it directly affects metabolism, oxygen consumption, growth, moulting and survival (Chen *et al.*, 1995; Hennig and Andreatta, 1998). However, other extrinsic factors include salinity, dissolved oxygen, season, stocking densities, environmental pollutants and differences in pond ecology between ponds can influence management and pond production (Chen *et al.*, 1990a; Chen *et al.*, 1990b; Allan and Maguire, 1991; Bray *et al.*, 1994; Le Moullac *et al.*, 1998; Cheng and Chen, 2001; Schuur, 2002). Intrinsic factors such as moult cycle, disease, nutrition, species, sex, life stage and stocking density also influence penaeid production (Benzie, 1998; Cheng and Chen, 2001; Menasveta, 2002; Schuur, 2002). Whatever the case, under unhealthy conditions when infected with a viral infection, the stress provoked by less than optimal extrinsic and intrinsic factors often intensifies the effects produced by the infection (Bray *et al.*, 1994). Nevertheless, this study provides the first data on

the potential impacts *Pmerg*DNV has on production of *P. merguiensis* in Queensland culture facilities.

Australian aquaculture production of prawns has remained relatively stable over the four years to 2005-06. However the value per kilogram has continued to decline from $16.66 per kilogram in 2002-03 to $14.12 per kilogram in 2005-06 (ABARE, 2007). This decline in value of more than 15% is a result of the large increase in imports of the cheaper, vannamei prawn (*P. vannamei*), from south east Asian countries. For example, the weighted average value per kilogram of imported prawns from Vietnam, Thailand and China during 2002-03 was $16.22, $13.07 and $10.48 respectively. Imports from Asia have since increased and the weighted average value per kilogram of prawns has decreased by up to 60% ($10.55, $5.05 and $6.59 per kilogram from Vietnam, Thailand and China, respectively) (ABARE, 2007). These low value imports have led to increased competition for Australian prawn farmers both in the domestic and export market (Hanna *et al.*, 2005).

The viability of prawn farming in Australia will depend on developments in world prawn prices and the competitiveness of the local industry relative to those of the main producing countries in Asia. Hence, reducing or eliminating the effects of disease on the local industry, will increase sustainability of the Australian industry and will be better equipped to survive against increased competition in the domestic and export markets.

Research and development is being undertaken to ensure prawn farming in Australia is both environmentally and economically sustainable, which includes feeding prawns on low protein diets, improved feed management strategies, and cost effective treatment of waste water before it leaves the farm. However, unless appropriate health management measures are maintained and effectively implemented, prawn aquaculture sectors will face more costs in terms of production and the efforts needed to contain and eradicate diseases. This study suggests that Queensland *P. merguiensis* culture facilities should have at least a 14% increase in production, following the removal or reduction of *Pmerg*DNV in

their ponds. Hence, focussing efforts on prevention, better management practices and on maintaining healthy stock should be of top priority.

CHAPTER 7

Insects as a bioassay for *Penaeus merguiensis* densovirus

7.1. Introduction

More than forty species of aquatic animals are produced commercially; however there are only five main species that contribute to more than 90 percent of the gross value of aquaculture production. These include Atlantic salmon, southern blue fin tuna, oysters, pearls and prawns (ABARE, 2007). Aquaculture has intensified considerably to keep up with an increasing worldwide demand for seafood. However, the intensification of aquaculture has been accompanied by an increase in the incidence of viral diseases. Therefore, the availability of biotechnological tools to study viral diseases in Crustacea is crucial for aquaculture facilities which experience disease problems exaggerated by intensive culture methods.

The availability of cellular tools is important in this endeavor since viruses require a living cell to replicate. Tissue culture is currently being used in a variety of circumstances and in many scientific disciplines as important tools for experimentation and the development of diagnostic reagents and probes. Cells under *in vitro* conditions are also used as alternative tools for animal experimentation, for biotechnological application and pathological investigation. Although numerous attempts have been undertaken, no established cell lines of marine crustaceans have been developed, despite primary cell cultures of several crustacean species being obtained from various species, culture conditions and tissues (Toullec *et al.*, 1996; Fraser and Hall, 1999; Mulford *et al.*, 2001; Gao *et al.*, 2003). Hence, novel techniques need to be developed to assist in the study of crustacean diseases of viral aetiology.

Penaeus merguiensis densovirus (*Pmerg*DNV) is the fourth penaeid prawn strain of hepatopancreatic parvovirus to be partially sequenced worldwide, but the first in Australia. Its known host range includes captive, wild and hatchery-reared species including penaeid prawns (Chapter 4, Chapter 5 and Chapter 6) and the sand crab *Portunus pelagicus* (La Fauce, 2005). It is logistically difficult to use these animals in bioassays. Furthermore, they are naturally infected with *Pmerg*DNV which can interfere with the results of infection studies. Therefore, it is necessary to find an alternative bioassay species. Initially, a search of the literature for insects that had been reported to naturally be infected with densovirus was performed, as these may have the cellular receptors and biosynthetic machinery to grow *Pmerg*DNV. The list of shortened by removing the hard to source species leaving three candidates for experimentation, namely crickets (*Acheta domesticus*), mealworms (*Tenebrio molitor*) and silkworms (*Bombyx mori*). *Bombyx mori* could not be sourced for this study due to difficulties in purchasing the number of animals required for the experiment. Hence, this study reports *A. domesticus* and *T. molitor* experimentally exposed to *Pmerg*DNV.

7.2. Materials and Methods

7.2.1. Preparation of inoculum

Infectious material containing *Pmerg*DNV was prepared via ultracentrifigation directly from the hepatopancreas of specimens of *Penaeus merguiensis* stored frozen at James Cook University, Queensland (Chapter 3.2). Infected prawns were obtained from three commercial farms in northern Queensland as early as June 2003 and originated from the same outbreak. Infected specimens were classified as *Pmerg*DNV-positive by real-time PCR (Chapter 5.3.2). The inoculum was considered free from additional bacterial, fungal and viral pathogens since more than 1000 prawns from the same *P. merguiensis* population were screened by routine histology, before prawns were used as the source of *Pmerg*DNV. Prawns were additionally screened for spawner isolated mortality virus, gill associated virus (GAV) and infectious hypodermal and haematopoietic necrosis virus (IHHNV) by PCR (L. Owens pers.comm.).

Furthermore, penaeid viral diseases such as Mourilyan virus, GAV and IHHNV are not known to occur naturally in *P. merguiensis* or in the hepatopancreas.

7.2.2. Insects

Infection experiments were conducted on the house cricket (*Acheta domesticus*) and mealworm beetle larvae (*Tenebrio molitor*) purchased from Pisces Enterprises (Queensland, Australia). Mature *Acheta domesticus* were housed in plastic aquaria and supplied with dried dog food and carrots (water source) *ad libitum*. *Tenebrio molitor* larvae were also housed in plastic aquaria and supplied with unprocessed bran and carrots (water source).

7.2.3. Host range studies

Insects were randomly distributed between four experimental treatments: (1) unhandled controls, (2) controls injected with Insect Xpress (TropBio Pty. Ltd., Townsville, Australia) insect medium, (3) injected with 1 x 10^6 virions of *Pmerg*DNV suspended in 20 µl Insect Xpress insect medium, and (4) injected with 1 x 10^6 virions of *Pmerg*DNV suspended in 20 µl Insect Xpress insect medium supplemented with 10 mM $MgCl_2$. Experimental treatments contained three replicates of ten insects each. All injections were performed using sterile Terumo (1 ml) syringes and a 30-gauge needle, and were discarded after each inoculation to minimize cross infections. *Acheta domesticus* were injected by inserting the needle into the membrane below the pronotum. *Tenebrio molitor* larvae were injected by inserting the syringe through the lateral mid-abdominal cuticle. *Tenebrio molitor* larvae were temporarily immobilized on ice prior to injection. Control insects were injected with the same volume of insect medium. The experimental period began on the day of infection (day 0) and concluded on day 30, or until insects in all treatments were deceased.

Experimental aquaria were monitored daily. Dead animals were removed, recorded and prepared for real-time PCR. Approximately 50 mg of tissue was removed from *A. domesticus* (flight muscle, gut and reproductive tissue) by longitudinal ventral dissection and from *T. molitor* by dissecting the insect along the fourth and sixth abdominal segments (larvae) or by dissecting the pupa and beetle longitudinally (haematopoietic organs). Tissues were stored frozen until processed. At the end of the experimental period, all remaining insects were sacrificed and the tissue removed for real-time PCR.

7.2.4. *Acheta domesticus* time course experiment

Acheta domesticus were randomly distributed between two experimental treatments: (1) controls injected with Insect Xpress insect medium, and (2) injected with *Pmerg*DNV inoculum (prepared as described in Chapter 3.2). The plan was for two crickets from both control and *Pmerg*DNV inoculated treatments to be sacrificed every day for ten days (including day 0), followed by two crickets every second day until day 30, or until the crickets in all treatments were deceased. Crickets were inoculated by inserting the needle into the membrane below the pronotum (Chapter 7.2.3). Dead crickets were removed, recorded and all insects were prepared for real-time PCR (Chapter 5.3.2).

7.2.5. Effect of viral concentration of *Acheta domesticus* survival

Acheta domesticus were randomly distributed between eight experimental treatments: (1) controls injected with Insect Xpress, and seven treatments receiving *Pmerg*DNV inoculum containing 1×10^0 through to 1×10^6 virions. Five animals were included in each treatment. Crickets were inoculated as described above (Chapter 7.2.3). The experiment commenced on day 0 and concluded on day 30 or until crickets in all treatments were deceased.

7.2.6. Nucleic acid extraction

Total DNA was extracted from approximately 50 mg of tissue using High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Sometimes only the exoskeleton was found, presumably from cannibalism and DNA extraction from the exoskeleton was not practical.

7.2.7. Real-time polymerase chain reaction

Real-time PCR was performed using RotorGene 3000 (Corbett Research, Sydney, Australia) (Chapter 5.3.2).

7.2.8. Statistical analyses

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) Version 14. The effect of treatment and replicate number was determined by a univariate analysis of variance (ANOVA). Data that was not normally distributed was transformed using log10. The effect of treatment, replicate number, survival and viral titres were determined by univariate ANOVA. Post-hoc comparisons between significant means were performed using least significant differences (LSD).

7.3. Results

7.3.1. Host range studies

7.3.1.1. *Acheta domesticus*

Mortalities in the *Pmerg*DNV challenged treatments began four days post-infection (Figure 7.1). Treatment had a significant effect on percentage mortality ($F=18.015$, $df=3,11$, $p<0.05$) which was significantly greater in the *Pmerg*DNV - inoculated treatment (80%), followed by *Pmerg*DNV + $MgCl_2$ treatment (63.3%) (Figure 7.1). Mortalities in the placebo control treatment were largely due to cannibalism, with many animals partially cannibalized below the head. No mortalities were recorded in the unhandled controls.

Penaeus merguiensis densovirus was not detected in tissues from crickets in the placebo or the unhandled controls. Tissues from over 86.6% of crickets challenged with *Pmerg*DNV were positive for *Pmerg*DNV by real-time PCR. Viral titres in crickets from the *Pmerg*DNV -challenged treatments ranged from 10^2 to 10^6 copies per 2.5 µl, most often less than the inoculum dosage (1 x 10^6 per 2.5 µl) (Figure 7.2). *Penaeus merguiensis* densovirus was not detected in nine animals (three from *Pmerg*DNV inoculated and six animals from *Pmerg*DNV + $MgCl_2$ treatment) but all of these had been cannibalised leaving only the exoskeleton.

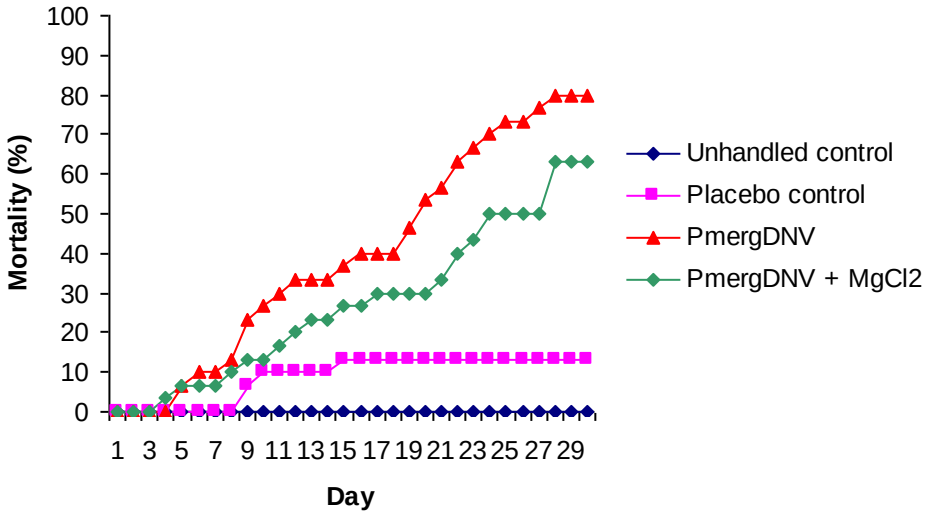


Figure 7.1: Percentage mortality of *Acheta domesticus* inoculated with 1 x 10^6 virions of *Penaeus merguiensis* densovirus during a 30-day experimental trial

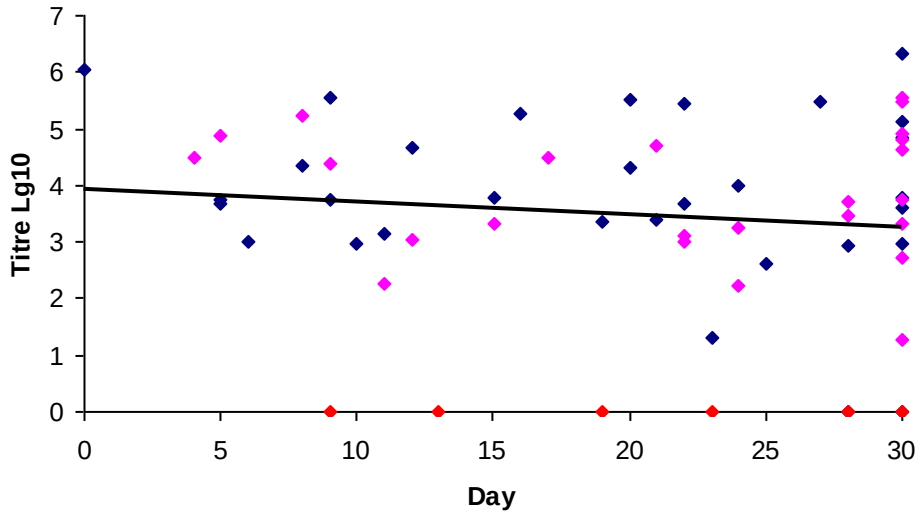


Figure 7.2: Concentration of *Penaeus merguiensis* densovirus in tissues of *Acheta domesticus*, following their death after being challenged with approximately 1 x 10^6 virions (blue) and 1 x 10^6 virions supplemented with 10 mM $MgCl_2$ (pink). A*cheta domesticus* represented by a red diamond were insects where due to cannibalism, only the exoskeleton of the cricket could be obtained for DNA extraction. The treadline of all data points including the zeros is the best fit using Microsoft® Excel 2003

7.3.1.2. *Tenebrio molitor*

Mortalities in the *Pmerg*DNV challenged treatments began three days post-infection (Figure 5.3). Treatment had a significant effect on percentage mortality ($F=4.714$, $df=3,11$, $p<0.05$) which was significantly greater in the *Pmerg*DNV challenged treatment (10%) and *Pmerg*DNV + $MgCl_2$ treatment (20%) compared to the placebo (0%) and unhandled control (0%) treatments but the inoculated treatments were not significantly different from each other. There were no mortalities in the placebo and unhandled control treatments.

Penaeus merguiensis densovirus was detected in only five mealworms (16.6%) from the *Pmerg*DNV inoculated treatment and seven (23.3%) mealworms from the *Pmerg*DNV + $MgCl_2$ (Figure 7.4). Viral titres did not exceed 10^2 copies per 2.5 µl except in one mealworm from each *Pmerg*DNV-challenged treatment where the viral titre was as high as 10^4 copies per 2.5 µl on day 3 (Figure 7.4).

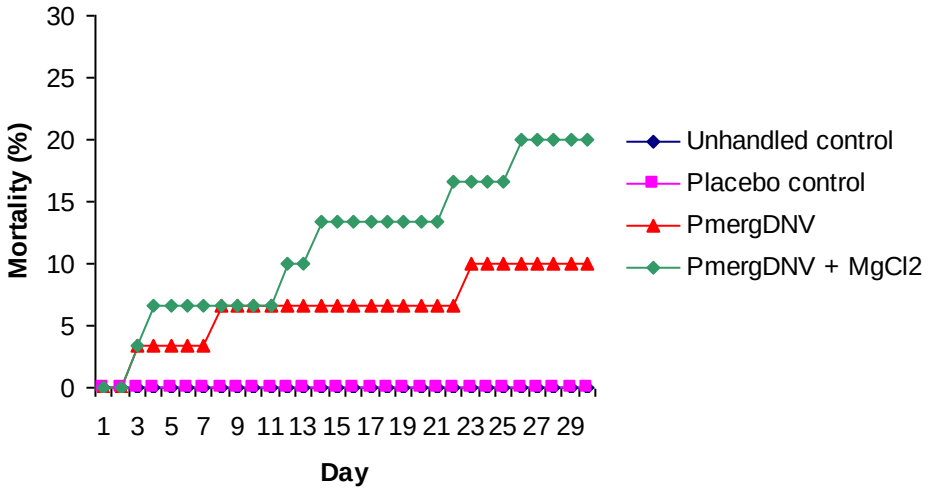


Figure 7.3: Percentage mortality of *Tenebrio molitor* inoculated with 1 x 10^6 virions of *Penaeus merguiensis* densovirus during a 30-day experimental trial

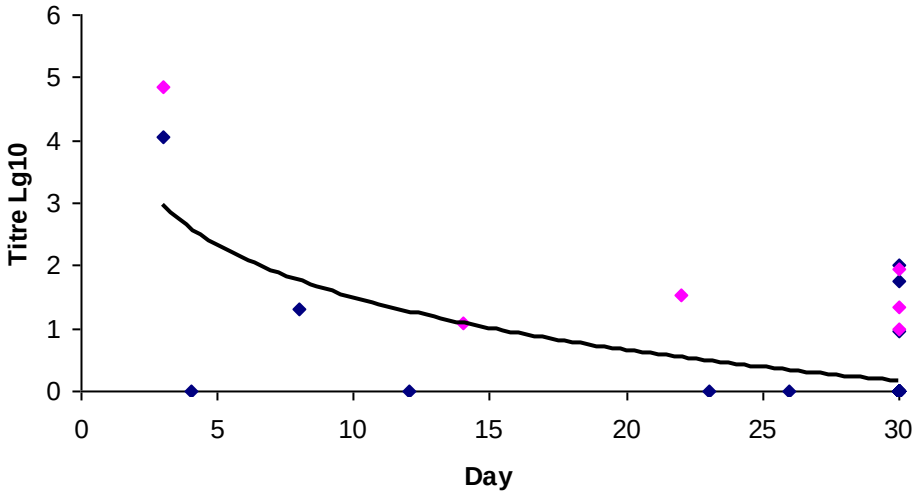


Figure 7.4: Concentration of *Penaeus merguiensis* densovirus in tissues of *Tenebrio molitor*, following their death after being challenged with approximately 1 x 10^6 virions (blue) and 1 x 10^6 virions supplemented with 10 mM $MgCl_2$ (pink). The treadline is best fit using Microsoft® Excel 2003

7.3.2. *Acheta domesticus* time course experiment

Penaeus merguiensis densovirus titres in challenged crickets sacrificed on day 0 were approximately 1 x 10^5 copies per 2.5 µl. Of the crickets that were sacrificed, *Pmerg*DNV titres remained around 10^4 to 10^5 copies per tissue sample by real-time PCR for the first six days of the experiment with possibly a peak on day 5 (Figure 7.5). Following day 6, there possibly was an apparent decay in *Pmerg*DNV titres through to day 20 where the *Pmerg*DNV titre in tissues was approximately 1.3 x 10^3 per PCR reaction volume (Figure 7.5) but this was not significant ($F=1.45$, $df=1,15$, $p>0.05$). Approximately 48% (29/60) of *A. domesticus* challenged with *Pmerg*DNV died naturally during the course of the experiment. Hence, the last cricket was sacrificed on day 20 and the experiment was concluded ten days earlier than planned. When all crickets (sacrificed and non-sacrificed) were included in the analysis, there was still a slight decay in viral titres over the 20 day experimental period (Figure 7.5). Interestingly, the titre of *Pmerg*DNV was either similar or greater in tissues of crickets that died throughout the experimental period compared to those that were sacrificed (Figure 7.5). *Penaeus merguiensis* densovirus was not detected using real-time PCR in tissues from control crickets.

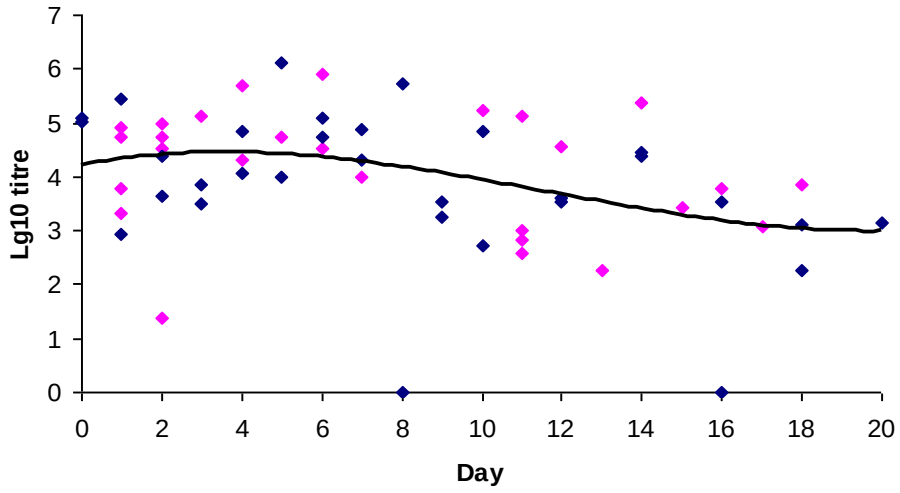


Figure 7.5: *Penaeus merguiensis* densovirus concentration in tissues of *Acheta domesticus* over a 20 day time-course experiment. Two animals (blue) were sacrificed every day for the first 10 days, followed by every second day for the following 10 days. Crickets that died naturally (pink) are also included in the analysis. Crickets on day 8 and day 16 were insects where due to cannibalism, only the exoskeleton of the animal could be obtained for DNA extraction. The treadline of all titres including the zeros is the best fit using Microsoft® Excel 2003

7.3.3. Effect of viral dosage on *Acheta domesticus* survival

During this study, there was a threshold of infection between the concentration of the *Pmerg*DNV inoculate and percentage mortality. Percentage mortality in treatments decreased as the initial inoculation dosage received by each treatment decreased. A threshold of 10^3 virions was required to get any mortality (Figure 7.6). Crickets injected with the highest concentration of *Pmerg*DNV had the greatest percentage mortality (100%), whereas 100% survival was observed in treatments where crickets were challenged with less than or equal to 10^2 *Pmerg*DNV virions (Figure 7.6). However, the speed at which the maximum mortality occurred was not dose dependent with those doses equal to or greater than 10^4 virions (determined by real-time PCR), reaching the maximum mortality rate at approximately two weeks post-infection. This is contrary to the effect of a proteinaceous cytotoxin where the time taken to reach maximum mortality would increase as the number of virions in the viral dose decreases.

Penaeus merguiensis densovirus was not detected by real-time PCR in the control inoculate for *A. domesticus*, nor in crickets inoculated with that preparation. *Penaeus merguiensis* densovirus was only detected in tissues from crickets in treatments challenged initially with inoculate containing at least 10^3 virions (Figure 7.7). This might support the idea of a threshold level of 10^3 virions for infection with the virus. Viral concentrations in *Pmerg*DNV-positive crickets ranged from one to three logs less than the challenge inoculate, depending on the day of mortality (Figure 7.7). However, one titre (12,100 virions) from a cricket that died on day 4 in the treatment initially injected with approximately 10^4 *Pmerg*DNV virions was not reduced (Figure 7.7). Viral concentrations were always greater in tissues from crickets that died earlier in the experiment compared to those that died closer to the conclusion of the experiment (Figure 7.7). *Penaeus merguiensis* densovirus infection was present in all of the crickets that died naturally.

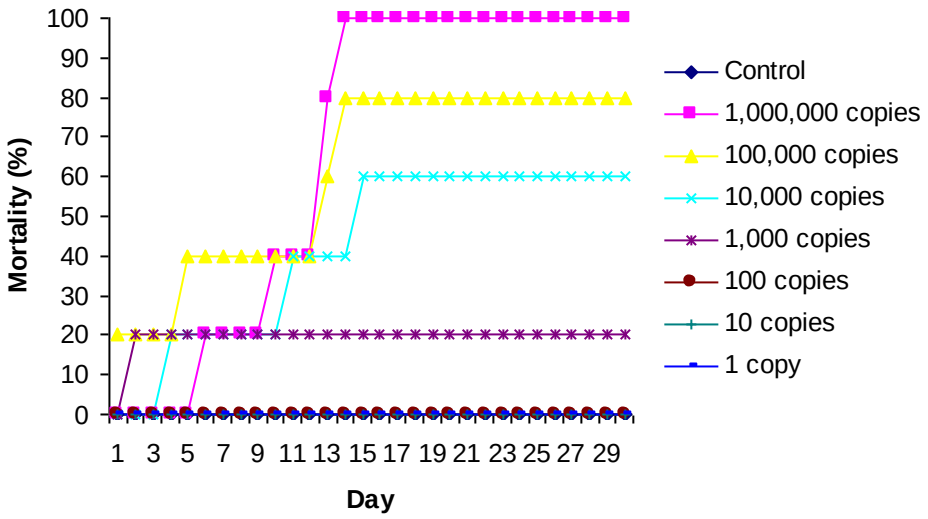


Figure 7.6: Percentage mortality of *Acheta domesticus*. Crickets were divided into eight treatments, each challenged with *Penaeus merguiensis* densovirus ranging in concentration from 10^0 virions to 10^6 virions. Control animals were inoculated with insect medium

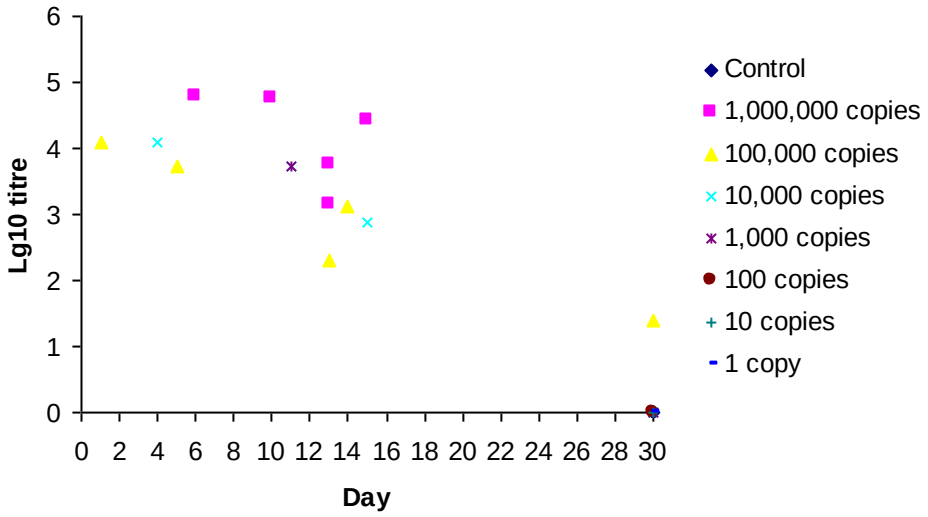


Figure 7.7: *Penaeus merguiensis* densovirus concentration in tissues of *Acheta domesticus* challenged with *Penaeus merguiensis* densovirus ranging in concentration from 10^0 to 10^6 virions. Control crickets were inoculated with insect medium

7.4. Discussion

The results from this study describe the potential use of insect models for viral diseases of crustaceans. *Tenebrio molitor* (mealworms) and *Acheta domesticus* (house cricket) were tested as potential alternate bioassays for *Penaeus merguiensis* densovirus (*Pmerg*DNV). When challenged with *Pmerg*DNV, virus was not commonly detected in tissue from *T. molitor* by real-time PCR with twelve *Pmerg*DNV challenged animals (20%) infected. Therefore, it was not investigated any further. Conversely, the majority (86.6%) of *Pmerg*DNV challenged *A. domesticus* tissues were positive for *Pmerg*DNV by real-time PCR with viral titres close to or lower than the initial dosage of 10^6 virions. However, given the dilution factor of placing the inoculum (20 µl) into the body of the cricket, the titres were not unexpected. This suggested *A. domesticus* has the capability of being used as an insect model to study *Pmerg*DNV.

The addition of $MgCl_2$ has previously been reported to increase baculovirus infection levels by 80-100% (Becnel *et al.*, 2001) and cypovirus infections in mosquitoes (Shapiro *et al.*, 2005). The addition of $MgCl_2$ to the *Pmerg*DNV inoculum increased infection mortality rates in *T. molitor* but not in *A. domesticus* and therefore, similar treatments were not included in the experiments that followed the initial challenge.

The time course experiment confirmed *A. domesticus* may be a permissive host for *Pmerg*DNV since qPCR titres did not significantly decrease, as would be expected if animals were unable to be a host for the virus. With Taura syndrome virus in non-permissive primate cells, titres fell two logs in seven days (Pantoja *et al.*, 2004). Very rapid decrease in titres similar to the case of *T. molitor* was seen when crayfish primary cell lines were infected with the Australian strain of infectious hypodermal and haematopoietic necrosis virus (K. Claydon, James Cook University pers. comm.). These rapid declines are clearly different to the stable relationship in Figure 7.2 and the possible peak and slow decline seen herein (Figure 7.5 and Figure 7.7) with *A. domesticus*.

Penaeus merguiensis densovirus titres were found in all moribund crickets, suggesting the inoculum containing at least 10^3 virions was the threshold of inducing infection in challenged crickets. Clinically relevant thresholds of Herpes simplex virus and cytomegalovirus DNA have been correlated with the severity of outcome where high viral loads above a determined threshold were associated with mortalities, whereas, viral loads below the determined threshold were not associated with mortality (Chemaly *et al.*, 2004; Gooskens *et al.*, 2007). Similarly, high levels of adenovirus (AdV) DNA in serum samples after a 100-fold dilution have been correlated with fatal disseminated AdV disease (Schilham *et al.*, 2002). An infectious viral dose has also been established for latent western equine encephalomyelitis virus in mosquitoes (Mahmood *et al.*, 2006). Hence, quantitative detection of *Pmerg*DNV DNA in cricket tissue after infection is a potential diagnostic tool for differentiating between infections associated with mortalities and those in which the virus is present in tissues but not associated with active infection; possibly a latent infection.

Although the results from this study cannot disprove the theory of a proteinaceous cytotoxic effect from the virions being responsible for the mortalities observed, it is considered unlikely since there are currently no known reports of a toxin being produced by parvoviruses. Furthermore, as the titre of virions increased, the time to maximum mortality did not decrease as would occur with a toxin. Most toxins act quickly within at least four days. In addition, there may have been an initial increase in viral titre during the time course experiment suggesting some amplification, which would not have been observed if there was an effect from a toxin. However, further confirmatory experiments investigating mRNA of *Pmerg*DNV levels during the course of infection, need to be performed to confirm viral amplification during infection.

Based on this information, we suggest *A. domesticus* is a potential model to study *Pmerg*DNV. This is the first evidence that insects may be used to study viral diseases in crustaceans. However, mosquito densoviruses have been used to model viral accommodation in prawns (Roekring *et al.*, 2006). The concept of some similarity between insects and crustaceans is not surprising since it has been reported in Taura syndrome virus is most similar genetically to those of the genus Cricket paralysis-like viruses (*Picornaviridae*) (Robles-Sikisaka *et al.*, 2001; Mari *et al.*, 2002). Since there are currently no available cellular tools such as cell lines to study diseases of viral aetiology in crustaceans, insects may be a viable alternate method. These insect models can be used for the induction of antiviral immunity against *Pmerg*DNV by RNA interference (RNAi). Recently, several papers have demonstrated the potential of RNAi for providing protection of *P. monodon* against yellow head virus and white spot syndrome virus (Robalino *et al.*, 2004; Tirasophon *et al.*, 2005; Westenberg *et al.*, 2005; Xu *et al.*, 2007). The use of alternative animals circumvents the lack of specific pathogen free (SPF) crustaceans which hampers viral research in countries where import restrictions do not allow the importation of live SPF crustaceans.

CHAPTER 8

In vivo down regulation of *Penaeus merguiensis* densovirus gene expression in a cricket model using small interfering RNAs

8.1. Introduction

The commercial importance of prawn farming has resulted in an increased interest in the response of prawns to viral infections. However, the lack of information regarding the genome of prawns, the lack of tools for its genetic manipulation, and the unavailability of clonal long-term cell lines for *in vitro* studies have been the main constraints on gaining an insight into the molecular basis for antiviral immunity in these animals (Argue *et al.*, 2002). Progress has been reported but unsubstantiated in culturing cells from the freshwater crayfish, *Orconectes limosus*, using neuronal tissue (Neumann *et al.*, 2000) and from *Pacifastacus leniusculus* from primary haematopoietic stem cells to study interactions between the host and its viral pathogen (Soderhall *et al.*, 2005; Jiravanichpaisal *et al.*, 2006). In marine crustacea, short-term primary cell cultures have been used to study host-virus interactions (Tapay *et al.*, 1997; Itami *et al.*, 1999; Tirasophon *et al.*, 2005). No established long-term or permanent cell lines have been established from marine prawns.

Most organisms have evolved protective defence mechanisms against viruses and other pathogens. Vertebrates rely on a both an 'adaptive' immune system that specifically targets pathogens as well as a broad-action, non-specific 'innate' immune response (Silverstein, 1989). On the other hand, invertebrates lack the protein-based adaptive immune response found in vertebrates but are still capable of effectively fighting viral infections (Brennan and Anderson, 2004).

RNA interference (RNAi) is an evolutionary conserved gene regulatory mechanism. It can be triggered by the recognition of intracellular long double stranded RNA, which can be transcribed from nuclear genes, replicating viruses or in the form of synthetic RNAs, resulting in sequence-specific degradation of the homologous RNA (post-transcriptional gene silencing) (Hannon, 2002; McCown *et al.*, 2003; Hammond, 2005). Pioneering observations regarding RNA interference were reported in the nematode worm (Fire *et al.*, 1998), plants (Waterhouse *et al.*, 1998) and *Drosophila* (Kennerdell and Carthew, 1998). However, RNAi-related events have since been recognised in almost all eukaryotic organisms including protozoans (Bastin *et al.*, 2001; Malhotra *et al.*, 2002; Cottrell and Doering, 2003), fungi (Raoni and Arndt, 2003), algae (Wu-Scharf *et al.*, 2000), nematodes (Fire *et al.*, 1998; Caplen *et al.*, 2001; Schott *et al.*, 2005), plants (Fagard and Vaucheret, 2000), insects (Misquitta and Paterson, 1999; Hughes and Kaufman, 2000), fish (Nasevicuis and Ekker, 2000; Dang *et al.*, 2008) and mammals (Wianny and Zernicka-Goetz, 2000; Caplen *et al.*, 2001). Plants and insects encode multiple Dicer enzymes that recognize distinct precursors of small RNAs (Fire *et al.*, 1998; Berstein *et al.*, 2001; Lee *et al.*, 2004; Xie *et al.*, 2004; Tomari and Zamore, 2005). Hence, they are capable of initiating the small RNA-guided RNA interference antiviral immunity (Hamilton and Baulcombe, 1999; Li *et al.*, 2002a) and have been recognised as an important defence against viruses in invertebrates, particularly insects (van Rij *et al.*, 2006; Wang *et al.*, 2006; Zambon *et al.*, 2006).

Penaeus merguiensis densovirus (*Pmerg*DNV) a member of the family Parvoviridae, is non-enveloped and contains a linear, single stranded DNA genome approximately 6 kb in size encoding one structural and two non-structural proteins (Siegl *et al.*, 1985; Berns *et al.*, 2000). Infected prawns exhibit a reduction in their growth rate (Flegel *et al.*, 1999). Consequently, economic losses are a result from the stunting of infected prawns since farmers are unable to sell their stock at full market value. There is currently no effective strategy to treat or prevent *Pmerg*DNV. Hence, development of a strategy to control *Pmerg*DNV is of high priority for the aquaculture industry.

Our previous work has indicated *Acheta domesticus* may be used as an animal model for *Pmerg*DNV (Chapter 7). The cricket model for *Pmerg*DNV was developed because it was impossible to get *Pmerg*DNV-free prawns for *in vivo* experiments. Here, we investigate whether RNAi provides protection against the crustacean virus *Pmerg*DNV using an insect model, *Acheta domesticus*.

8.2. Materials and Methods

8.2.1. Preparation of inoculum

Infectious material containing *Pmerg*DNV was prepared via ultracentrifigation directly from the hepatopancreai of *Penaeus merguiensis* stored frozen at James Cook University, Queensland (Chapter 3.2).

8.2.2. Insects

Adult *Acheta domesticus* (house crickets) were purchased from Pisces Enterprises (Queensland, Australia). *Acheta domesticus* were housed in plastic aquaria and supplied with dried dog food and carrots (water source) *ad libitum.*

8.2.3. Stealth RNAi assay against the capsid protein

The 25-nucleotide stealth small interfering RNAs (siRNA) against the capsid protein of *Pmerg*DNV (DQ458781) and stealth RNAi negative control duplexes were designed online using BLOCK-iT™ RNAi Designer (rnaidesigner.invitrogen.com) and synthesised by Invitrogen (Victoria, Australia) (Table 8.1).

Table 8.1: Sequence of stealth RNAi and control stealth RNAi targeting the capsid proteins of *Penaeus merguiensis* densovirus, designed using BLOCK-iT™ RNAi Designer (Invitrogen, Australia). Base pair differences are underlined, italicised letters indicate an additional base pair not in the stealth RNAi target sequence and the letter d indicates a base pair deletion from the stealth RNAi target sequence

Stealth RNAi	Sequence (5'-3')
*Pmerg*DNV capsid target	CCUAC<u>A</u>AGAAGAGGAG<u>G</u>AAAU<u>U</u>AUU
*Pmerg*DNV capsid control	CCUA<u>G</u>AAGAdGAGGAG<u>U</u>AAAU<u>C</u>*A*AUU

Acheta domesticus were randomly distributed between six experimental treatments (Table 8.2) with three replicates of ten insects in each. Crickets in each experimental treatment received two injections: the first on Day 0 and the second 24 hours later. All injections were performed using sterile Terumo (1 ml) syringes and a 30-gauge needle, and were discarded after each inoculation to minimize cross-infections. *Acheta domesticus* were injected by inserting the needle into the membrane below the pronotum. Crickets in the appropriate treatments were challenged with 5 µg of the stealth RNAis and 5 x 10^4 virions of *Pmerg*DNV. Control crickets were injected with the same volume of Grace's insect medium (Invitrogen, Victoria, Australia). The experimental period began on the day of the second injection and concluded on day 14. The knockdown of *Pmerg*DNV was confirmed by real-time reverse-transcriptase PCR (RT-PCR) (Chapter 8.2.6).

Table 8.2: Experimental design for the knockdown of *Penaeus merguiensis* densovirus in *Acheta domesticus* targeting the capsid protein

Treatment	First Injection (0 hours)	Second Injection (24 hours)
Unhandled control	N/A	N/A
Placebo control	Insect medium	Insect medium
Stealth RNA target only	Stealth RNAi for target gene	Insect medium
Target stealth RNA + *Pmerg*DNV	Stealth RNAi for target gene	*Pmerg*DNV
Control stealth RNA + *Pmerg*DNV	Stealth RNAi control	*Pmerg*DNV
*Pmerg*DNV	Insect medium	*Pmerg*DNV

8.2.4. Stealth RNAi assay targeting non-structural protein two

As part of the Australia-India strategic research fund through the Queensland government, the stealth RNAi assay was repeated by the author in conjunction with a post-doctoral scientist from India, targeting the NS2 protein of *Pmerg*DNV.

The 25-nucleotide stealth siRNAs against NS2 protein of *Pmerg*DNV (DQ458781) and stealth RNAi negative control duplexes were designed online using BLOCK-iT™ RNAi Designer (rnaidesigner.invitrogen.com) and synthesised by Invitrogen (Victoria, Australia) (Table 8.3). *Acheta domesticus* were randomly distributed between four experimental treatments (Table 8.4) with three replicates of ten insects in each. The experiment was performed as described above (Chapter 8.2.3). However, crickets were challenged with a approximately 1 x 10^5 virions of *Pmerg*DNV.

Table 8.3: Sequence of stealth RNAi and control stealth RNAi targeting non-structural two protein *Penaeus merguiensis* densovirus, designed using BLOCK-iT™ RNAi Designer (Invitrogen, Australia). Base pair differences are underlined, italicised letters indicate an additional base pair not in the stealth RNAi target sequence and the letter d indicates a base pair deletion from the stealth RNAi target sequence

Stealth RNAi	Sequence (5'-3')
*Pmerg*DNV NS2 target	CCGCCGCAGUGUAUUUAUCAGUUAU
*Pmerg*DNV NS2 control	CCGGACGAUdGUAUUUGAC*U*UCCUAU

Table 8.4: Experimental design for the knockdown of *Penaeus merguiensis* densovirus in *Acheta domesticus* targeting non-structural two protein

Treatment	First Injection (0 hours)	Second Injection (24 hours)
Stealth RNA target only	Stealth RNAi for target gene	Insect medium
Target stealth RNA + *Pmerg*DNV	Stealth RNAi for target gene	*Pmerg*DNV
Control stealth RNA + *Pmerg*DNV	Stealth RNAi control	*Pmerg*DNV
*Pmerg*DNV	Insect medium	*Pmerg*DNV

8.2.5. RNA extraction

Total RNA was extracted from tissues using Promega SV® Total RNA Isolation System (New South Wales, Australia). A DNase step is included in the protocol to exclude contaminating DNA. Sometimes only the exoskeleton was found, presumably from cannibalism and RNA extraction from the exoskeleton was not productive.

8.2.6. Reverse-transcriptase, real-time polymerase chain reaction

Reverse transcriptase, real-time PCR was performed using a modified protocol described in Chapter 5.2.3 using RotorGene 3000 (Corbett Research, Sydney, Australia).

Sensimix-one step kit allows reverse transcription to cDNA synthesis and subsequent polymerisation to be performed in a single step, using specific primers, fluorescent probe and total RNA. The reaction mixture consisted of 1 x Sensimix One-Step (Quantace, New South Wales, Australia), 0.5 mM of $MgCl_2$, 10 µM of each primer (HPV140F and HPV140R (Chapter 5), 50 nM of probe (Chapter 5), 2.5 µl of DNA template and nuclease-free water to a final volume of 20 µl.

The cycling pattern consisted of a reverse transcriptase step of an initial 30 minute incubation at 49ºC, an enzyme activation step of 95ºC for 10 minutes, followed by a two-step cycle pattern consisting of thirty five cycles at 95ºC for 15 seconds and 60ºC for 1 minute. Each run contained three no template controls and triplicate samples of 1×10^4 and 1×10^8 copies of the plasmid standard for the virus. Duplicate samples from each cricket were independently quantified and statistically analysed (Chapter 8.2.7). Following the completion of each run, a standard curve (Chapter 5.3.3) was imported into the run file to obtain accurate quantitative information regarding the number of viral copies in each tissue sample. Data acquisition and subsequent data analyses were performed using RotorGene 3000 and Microsoft Excel.

8.2.7. Statistical analyses

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) Version 14. The effect of treatment on viral titres and survival were both determined by oneway analysis of variance (ANOVA). Data that was not normally distributed was transformed using log10. Post-hoc comparisons between significant means were performed using least significant differences (LSD).

8.3. Results

8.3.1. Mortality

8.3.1.1. Capsid protein assay

Cumulative percentage mortality was significantly greater in treatments exposed to *Pmerg*DNV ($F = 30.0$, $df = 7,5$, $p<0.05$) than those not exposed, but was not significantly different between inoculated treatments. The non-significant trends are as follows. The greatest cumulative percentage mortality was recorded in the treatment of crickets receiving the control RNAi + *Pmerg*DNV (Figure 8.1). Mortality continued to increase from day one until day 4, followed by another increase again at day 10, reaching maximum mortality (approximately 33%) on day 12. Mortality in the group that only received *Pmerg*DNV followed a similar pattern with two peaks in mortality at day 4 and at day 11, reaching maximum mortality (25%) on day 11. Percentage mortality in the target RNAi + *Pmerg*DNV had the lowest percentage mortality (11.5%) of the treatments exposed to *Pmerg*DNV, despite mortality being 4% in the first two days of the experiment. No mortalities were recorded in treatments that were not challenged with *Pmerg*DNV (Figure 8.1).

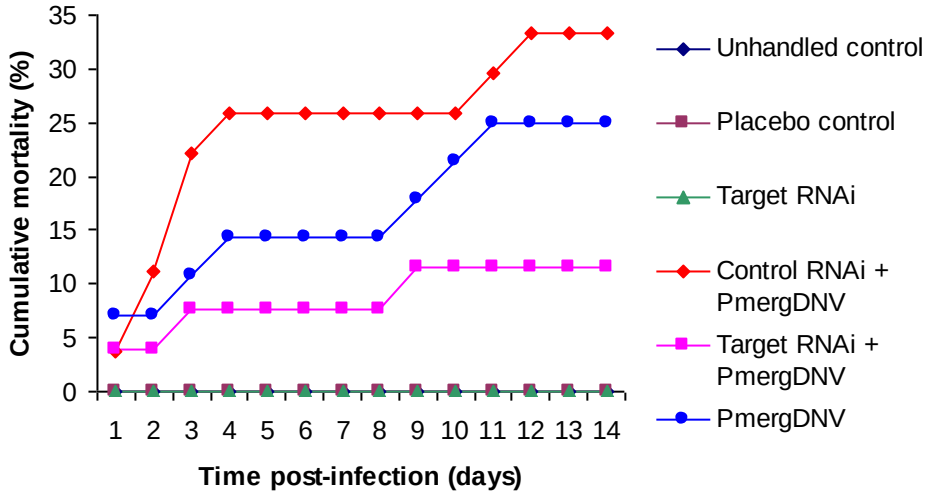


Figure 8.1: Percentage mortality of *Acheta domesticus* during a 14-day experimental trial assessing if stealth interfering RNAs targeting the capsid protein could inhibit *Penaeus merguiensis* densovirus

8.3.1.2. Non-structural protein two assay

Cumulative percentage mortality was significantly greater in the target RNAi + *Pmerg*DNV treatment ($F = 2.0$, $df = 3,1$, $p<0.05$), but was not significantly different between remaining treatments. Mortalities in treatments exposed to *Pmerg*DNV began on day one of the experiment and continued to increase throughout the progression of the experiment (Figure 8.2). The greatest cumulative percentage mortality (70%) was recorded in the target RNAi + *Pmerg*DNV treatment, peaking at day 10. Cumulative percentage mortality was 50% and 46.5% in the control RNAi + *Pmerg*DNV and *Pmerg*DNV only treatments, respectively. The lowest cumulative percentage mortality (43%) was in the target RNAi only treatment (Figure 8.2).

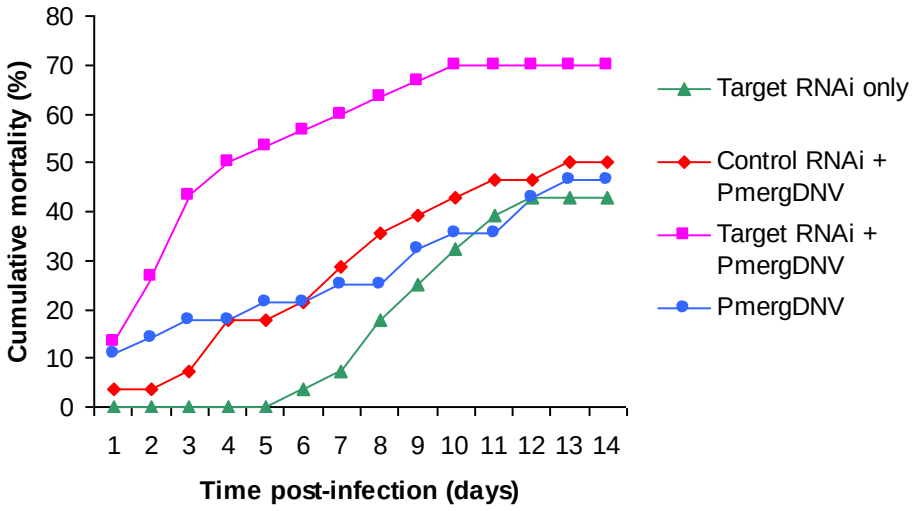


Figure 8.2: Percentage mortality of *Acheta domesticus* during a 14-day experimental trial assessing if stealth interfering RNAs targeting non-structural protein two could inhibit *Penaeus merguiensis* densovirus replication

8.3.2. Reverse-transcriptase, real-time polymerase chain reaction

8.3.2.1. Capsid protein assay

Tissues from approximately 82% of crickets challenged with *Pmerg*DNV were positive by real-time RT-PCR. Tissues from crickets that were negative for *Pmerg*DNV had been cannibalised, leaving only the exoskeleton. There was no significant difference between duplicate *Pmerg*DNV titres from each cricket ($F = 0.652$, $df = 1,342$, $p>0.05$). The *Pmerg*DNV only treatment had the greatest range of *Pmerg*DNV copies detected in tissues, ranging from 0 (cannibalised animals) to 3.8×10^4. *Penaeus merguiensis* densovirus titres ranged from 0 (cannibalised animals) to 3.05×10^3 copies per 2.5 µl in tissues from crickets from the control RNAi + *Pmerg*DNV treatment which were similar to the 0 (cannibalised animals) to 3.21×10^3 per 2.5 µl in tissues from crickets from the target RNAi + *Pmerg*DNV treatment.

The greater level of *Pmerg*DNV in positive control crickets receiving no dsRNA was statistically significant compared to the stealth RNAi treatments ($F = 42.2$, $df = 5, 342$, $p<0.05$) and all other treatments. The titres of treatments receiving both target and control siRNA and *Pmerg*DNV were not statistically different from each other but were statistically different from all other treatments. The average number of copies in tissues from the *Pmerg*DNV-challenged treatment was 3.42×10^3. Interestingly, the average number of copies of *Pmerg*DNV from crickets in the control RNAi treatment and target RNAi treatments (3.95×10^2 and 3.58×10^2, respectively) was approximately one log less than the virus alone treatment. *Penaeus merguiensis* densovirus was not detected in tissues from crickets in the unhandled controls, placebo control or crickets injected with the stealth RNAi target for *Pmerg*DNV.

8.3.2.2. Non-structural protein two assay

Tissues from approximately 94% of crickets challenged with *Pmerg*DNV were positive by real-time RT-PCR. Tissues from crickets that were negative for *Pmerg*DNV had been cannibalised, leaving only the exoskeleton. There was no significance difference between duplicate *Pmerg*DNV titres from each cricket ($F = 0.297$, $df = 1,228$, $p>0.05$).The *Pmerg*DNV only treatment had the greatest

range of *Pmerg*DNV copies detected in tissues, ranging from 0 (cannibalised animals) to 3.2 x 10^6. *Penaeus merguiensis* densovirus titres ranged from 0 (cannibalised animals) to 5.2 x 10^5 copies per 2.5 µl in tissues from crickets from the control RNAi + *Pmerg*DNV treatment which were similar to the 0 (cannibalised animals) to 1.1 x 10^5 per 2.5 µl in tissues from crickets from the target RNAi + *Pmerg*DNV treatment.

The average number of copies of *Pmerg*DNV in tissues of crickets was significantly different between all treatments ($F = 115.59$, $df = 3,228$, $p<0.05$). The average number of copies in tissues from the *Pmerg*DNV-challenged treatment was 9.85 x 10^4. Interestingly, the average number of copies of *Pmerg*DNV from crickets in the control RNAi treatment + *Pmerg*DNV and target RNAi + *Pmerg*DNV treatments (2.2 x 10^4 and 8.1 x 10^3, respectively) was less than the virus alone treatment. *Penaeus merguiensis* densovirus was not detected in tissues from crickets injected with the stealth RNAi target only.

8.4. Discussion

RNA interference is increasingly being investigated as a preventative treatment for viral infections in a variety of eukaryotic systems ranging from fungi (Raoni and Arndt, 2003), algae (Misquitta and Paterson, 1999; Hughes and Kaufman, 2000; Wu-Scharf *et al.*, 2000) to invertebrate (Schott *et al.*, 2005) and vertebrate (Caplen *et al.*, 2001; Morissey *et al.*, 2005; Dang *et al.*, 2008) systems. This approach was used in an insect model, *Acheta domesticus*, to induce RNAi against the crustacean virus *Pmerg*DNV.

The delivery of stealth RNAs into *Acheta domesticus* was done by injection. Similarly, direct injection of dsRNA in other invertebrates such as *Anopheles gambiae* (Keen *et al.*, 2004), *Drosophila melanogaster* (Goto *et al.*, 2003), and *Tenebrio molitor* (Valdes *et al.*, 2003), have led to successful and efficient gene silencing. Hence, the sequence-specific binding of the siRNA to its target mRNA triggering the nucleolytic activity of the RISC complex, resulting in sequence specific RNAi can act in invertebrate systems.

Injection of stealth RNAs targeting the capsid protein of *Pmerg*DNV resulted in a 10-fold reduction in the average viral titre detected in tissues, compared to crickets challenged with *Pmerg*DNV. Interestingly, tissues from crickets that received the control stealth RNAi injection also showed a 10-fold reduction in *Pmerg*DNV titres. This ten-fold reduction is significant as it drops the average infective dose below the threshold level needed for *Pmerg*DNV infection (Chapter 7) and therefore on average, it should reduce the spread of the virus. Similarly, the injection of stealth RNAs targeting the NS2 protein of *Pmerg*DNV resulted in a reduction in the average viral titre detected in tissues, compared to crickets challenged with *Pmerg*DNV. However, only the target stealth interfering RNAs produced a 10-fold reduction in titres.

Given that the number of copies of mRNA was analysed during this study, the capsid and NS2 proteins must have been converted to mRNA for the technology to have worked, thus proving that *Pmerg*DNV was indeed replicating in crickets and the assay was not just the detection of virus that had been inoculated (Chapter 7).

The control stealth RNA for the capsid protein only differed in sequence by three base pairs and the rearrangement of a base pair from position 10 to position 22 in the control RNAi, suggesting this was not enough difference to stop the RNAi system from working. Similar results have also been observed in *P. monodon* where unrelated dsRNA also had partial inhibitory effect on yellow head virus (YHV) replication, whereas YHV-specific dsRNA resulted in a greater inhibitory effect (Yodmuang *et al.*, 2006). Similarly, injection of unrelated dsRNA has also been shown to result in some protection against white spot syndrome virus in low level infections in *P. vannamei* and *P. monodon* (Robalino *et al.*, 2004; Westenberg *et al.*, 2005). Alternatively, the introduction of the gene-specific siRNAs resulted in off-target effects, which was still seen through the downregulation of *Pmerg*DNV. The dsRNA may have cross-reacted with targets of limited sequence similarity due to partial sequence similarity between the target mRNA and dsRNA. Even regions consisting of only 11-15 similar nucleotides of sequence similarity can be sufficient to induce gene-silencing (Jackson *et al.*, 2003). The higher mortality in the NS2 target RNAi +

*Pmerg*DNV treatment may have been due to the target siRNAs affecting a vital cell function in the crickets. This hypothesis should be an area of focus for future investigation.

Off-target effects may also occur at the level of translation by blocking the translation of transcripts with partial homology, affecting unintended targets at the level of translation. A three to four mismatch between the siRNA and the targeted gene resulted in downregulation primarily at the protein level (Saxena *et al.*, 2003). However, the explanation for the downregulation of *Pmerg*DNV in the control stealth RNAi treatment requires further investigation. Non-specific knockdown of *Pmerg*DNV could potentially pose some problems delivering the stealth interfering RNAs to prawns.

RNA interference is a promising strategy for viral disease control as demonstrated in a number of organisms and operating systems, including prawns. However, many questions such as applying the method at the farm level need to be addressed. These include the stability of dsRNA in prawns, if it induces long term inhibition and if the immunity can be passed onto offspring. Furthermore, a more efficient delivery method needs to be developed for the delivery of dsRNA for large scale operations such as in aquaculture. Ideally, a feeding method similar to those adopted for RNAi application in *C. elegans* (Timmons and Fire, 1998; Kamath *et al.*, 2000; Timmons *et al.*, 2001) would be ideal.

Finally, to exploit good RNAi targeting and to get more insight into the RNAi mechanism and efficiency against *Pmerg*DNV, this experiment should ideally be repeated in prawns. An innate antiviral defence triggered by sequence independent dsRNA/siRNA has been previously reported to provide partial protection in prawns against YHV and WSSV (Robalino *et al.*, 2004; Robalino *et al.*, 2005; Tirasophon *et al.*, 2005; Westenberg *et al.*, 2005; Yodmuang *et al.*, 2006; Kim *et al.*, 2007; Tirasophon *et al.*, 2007; Xu *et al.*, 2007). Hence, the innate immune system of prawns is capable of recognising virus sequence-specific dsRNA, triggering the antiviral response of the prawn through the RNAi pathway. RNA interference provides an exciting new perspective for treating and combating *Pmerg*DNV and other crustacean viral pathogens in aquaculture.

CHAPTER 9

Penaeus merguiensis densovirus in the mud crab *Scylla serrata* of Australia

9.1. Introduction

Portunid crabs (Family Portunidae) are common inhabitants in intertidal flats and mangrove habitats throughout the tropical and sub-tropical Indo-West Pacific (IWP) region (Varley and Greenway, 1992). Over eighty species of portunid crabs have been reported from eastern Australia alone (Shields, 1992). Of the various species, *Scylla serrata*, the mud or mangrove crab is commonly found in shallow coastal waters, lagoons, brackishwater lakes, estuaries, intertidal swamp, and mangrove areas (Marichamy and Rajapackiam, 2001) and commands a higher price in the export market due to its large size, high meat yield and delicate flavour. Hence, they are sought after as a quality food item and consequently, becoming increasingly popular for aquaculture, capture fisheries and trade. Their high value makes them an important source of income for small-scale fishers through the Asia-Pacific region (Hudson and Lester, 1994; Leung *et al.*, 2000; Quinitio *et al.*, 2001; Christensen *et al.*, 2004).

The market for mud crabs has been reported as being promising resulting from high market prices and high growth rates in crab consumption being experienced in many countries in Asia, America, Europe and Australia (Aldon and Dagoon, 1997; Cholik, 1999). Within Australia, the culture of mud crabs is not extensive with only a few operators having produced marketable quantities (McCormack, 1989).

Pathogens and diseases have been reported as being the major constraint preventing the establishment of a successful hatchery production (Cholik, 1999; Fielder and Heasman, 1999). A thraustochytrid-like organism has been associated with egg mortality in *S. serrata* (Kvingedal *et al.*, 2006) and from a number of vibrio species, *Vibrio harveyi* was found to be the major contributor to disease (Quinitio *et al.*, 2001; Williams and Primavera, 2001). Other larval stages where mortalities occur known as moulting death syndrome (Fielder and

Heasman, 1999) have been reported as being around the time when zoea-metamorphosed to megalopa. The underlying reason for the high mortalities associated with this particular larval phase is unclear. However, nutritional deficiency has been regarded as a factor (Zeng and Li, 1999).

Yet to be reported as major disease agents in larval S. *serrata*, are the viruses. However, viruses have been found in adult mud crabs. A non-occluded rod-shaped nuclear virus has been reported in *S. serrata* from Darwin, Northern Territory, Australia (Anderson and Prior, 1992). These infections were observed in juvenile, subadult and adult specimens but did not cause clinical disease at any stage. On the contrary, a reovirus has been reported in cultured *S. serrata* from southern China that caused 80% mortality in cohabitation studies of juvenile to subadult *S. serrata* (Weng *et al.*, 2007).

During attempts to increase the success rate of the hatchery production of *S. serrata* by a better understanding of disease agents, basophilic to magenta hepatopancreatic intranuclear inclusions were discovered first in locally caught broodstock and then progeny larvae from those broodstock (L. Owens pers.comm.). The aim of this study was to investigate if *Pmerg*DNV had any role on these inclusions from broodstock and larvae of *S. serrata*.

9.2. Materials and Methods

9.2.1. Origin of *Scylla serrata*

Wild-caught adult *Scylla serrata* were obtained from Ross River, Townsville, northern Queensland (n=10) and from the Burdekin River, northern Queensland (n=9) in 2005.

Scylla serrata larvae were obtained from aquaculture facilities at James Cook University, Townsville, Queensland. Mud crab larvae had been obtained from the spawning of wild-caught adults obtained from Ross River, Townsville and were preserved in 70% ethanol. Four batches of larvae (Day 0 and Day 12) including two batches with inclusion bodies and two batches without inclusion bodies were examined by real-time PCR (Chapter 5.3.2) for *Pmerg*DNV.

9.2.2. Nucleic acid extraction

As the broodstock crabs were needed for breeding experiments and could not be sacrificed, DNA was extracted from the haemolymph of the broodstock and the total tissues of the larvae using the High Pure Template Preparation Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Haemolymph (500 µl-1 ml) was collected from the adults coxa joint membrane using a 29-gague needle and was immediately placed directly into ice-cold 0.06M sodium citrate (Appendix A) at a ratio of 1:1 to prevent coagulation.

9.2.3. Screening for *Penaeus merguiensis* densovirus by TaqMan real-time polymerase chain reaction

All *S. serrata* larvae and adults were screened for *Pmerg*DNV infection using real-time PCR (Chapter 5.3.2).

9.2.4. Polymerase chain reaction amplification

Seven sets of PCR primers (Table 9.1) were used to sequence part of the genome of *Pmerg*DNV from real-time PCR positive samples. All primers were synthesised by Sigma-Genosys, Australia and had previously been used to sequence the genome of *Pmerg*DNV from Australian *Penaeus merguiensis* (Chapter 4).

The PCR reaction mixture contained 1 x *Taq* buffer (750 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween20), 2.5 mM $MgCl_2$, 0.75 U *Taq* polymerase (MBI Fermentas), 200 µM each dNTP, 50 pmol of each primer and 20-50 ng of DNA template. The PCR reaction volume was adjusted with nuclease-free water to a final volume of 25 µl. Amplification was performed in an Eppendorf Mastercycler Gradient Thermocycler (Eppendorf, Germany) with a PCR profile consisting of an initial 94°C for 7 minutes, 35 cycles at 94°C for 45 seconds denaturation, 55°C annealing for 45 seconds and polymerisation at 72°C for 1 minute and an additional 5 minutes at 72°C following the last cycle. Amplified products were analysed in 1% agarose gels containing ethidium bromide at a concentration of 0.5 µg ml^{-1} and visualised under ultraviolet light. Amplified product size was determined by comparison with a 100 bp DNA ladder (MBI Fermentas).

Table 9.1: Overview of the primers used to sequence *Penaeus merguiensis* densovirus from *Scylla serrata*

Primer name	Primer sequence 5'-3'	Product length (bp)	Nucleotide position along 2475 bp
HPV823F	CATAGCCAAGAGCGAGGACAG	381	1-381
HPV500compR	CTTGTCGGGTTAGTTGAATCT		
HPVconfirmF	CACCGCAACAAACCTAT	495	159-653
HPVconfirmR	TGACTTTCTTTGCCATTACCA		
HPV140F	CTACTCCAATGGAAACTTCTGAGC	632	159-790
HPVconfirmR	TGACTTTCTTTGCCATTACCA		
HPV605F	AGCATTAGGGCAAGAGGAAAG	706	651-1356
HPV140R	GTGGCGTTGGAAGGCACTTC		
HPV823R	TGTCTGAAAATCCTGATGCGT	972	1103-2074
HPV623R	CGCCACCAGTAAAGTAATTGA		
HPV415F	TATTGGCAAGCATGCAGTATG	367	1708-2074
HPV623R	CGCCACCAGTAAAGTAATTGA		
HPVbegF	CGCTTTGACAGGATTAGTAA	768	1708-2475
HPV623R	CGCCACCAGTAAAGTAATTGA		

9.2.5. Cloning and sequencing

DNA was purified from agarose gel bands using Promega Wizard® SV Gel and PCR Clean-Up System (New South Wales, Australia) and cloned into *Escherichia coli* JM 109 cells, using pGEM-T® Easy Vector System (Promega, Australia), according to the manufacture's instructions. Blue/white screening was used to screen for recombinant plasmids. Recombinant plasmids were purified from at least three white colonies using Wizard® Plus SV Minipreps DNA Purification System (Promega, Australia), according to the manufacturer's instructions. Recombinant plasmids were digested with *Spe* I, followed by electrophoresis on 1% agarose gel to screen for DNA inserts. Recombinant plasmids containing DNA inserts were sent to Macrogen Inc (Seoul, Korea) for sequencing using M13 universal primers. Three forward and three reverse sequencing reactions were performed for each clone. Sequencher™ software (Gene Codes Corporation) was used to analyse and align overlapping sequences for each clone. Sequence results were compared to available databases using Basic Local Alignment Search Tool (BLAST) via the National Centre for Biotechnology Information (NCBI) homepage.

9.3. Results

9.3.1. Real-time polymerase chain reaction

Fourteen adult *S. serrata* (approximately 74%) and the two larval samples that had previously been reported containing basophilic, magenta inclusion bodies were positive for *Pmerg*DNV by real-time PCR. Larvae at Day 12 had substantially higher viral titres than those at Day 0 (1.95×10^4 and 6.03×10^2, respectively). *Pmerg*DNV numbers in positive animals were between 10^2 and 10^5 copies (Table 9.2).

Table 9.2: Calculated concentrations (number of copies) of *Penaeus merguiensis* densovirus in *Scylla serrata* adult and larvae tissues

	Sample number	Number of copies
Scylla serrata adults	1	1.43×10^3
	2	1.26×10^4
	3	1.64×10^4
	4	1.80×10^4
	5	5.39×10^4
	6	1.59×10^4
	7	0
	8	4.15×10^4
	9	1.21×10^3
	10	0
	11	3.73×10^3
	12	1.10×10^4
	13	0
	14	4.01×10^4
	15	0
	16	6.81×10^3
	17	2.38×10^4
	18	1.51×10^5
	19	0
Scylla serrata larvae inclusion body positive	Batch 1: Day 0	6.03×10^2
	Batch 2: Day 12	1.95×10^4
Scylla serrata larvae inclusion body negative	Batch 3: Day 0	0
	Batch 4: Day 12	0

9.3.2. Sequencing

The size of the *Pmerg*DNV genome from *S. serrata* that was sequenced was 2475 bp (Appendix E); just under half the genome. Nucleotide sequence comparisons revealed 99% homology with the genome of *Pmerg*DNV from Australian *P. merguiensis* (DQ458781) (Chapter 4). The sequence data of *Pmerg*DNV from *S. serrata* is lodged in Genbank with assession number EU073937.

9.4. Discussion

The results of this study indicate that in addition to penaeid prawns, the host range of *Pmerg*DNV includes cultured and wild *S. serrata*. The *Pmerg*DNV signal was found in the haemolymph of *S. serrata* demonstrating that the virus had crossed the gut/haemolymph barrier and that the signal was not just from ingested *P. merguiensis* (the common host of *Pmerg*DNV in Australia) in the gut of the crab. However, it remains unanswered to whether the virus is replicating or just being sequestered in *S. scylla* and are therefore asymptomatic hosts for *Pmerg*DNV. *Scylla serrata* titres (10^2-10^5) were lower than in *P. merguiensis* (10^3-10^{13}) (Chapter 5) but higher than experimentally infected juvenile *Cherax quadricarinatus* (10^1-10^3) (La Fauce and Owens, 2007) and *Tenebrio molitor*, in which there was a rapid decay in *Pmerg*DNV qPCR titres (Chapter 7). In non-permissive hosts, quantitative PCR titres decayed over time with titres approaching those in *C. quadricarinatus* which is not what appears to be happening with *S. serrata*. Of considerable interest was the fact the *Pmerg*DNV signal in the larvae was found at day 0, the day of hatch before they had begun to feed, and therefore the virus must have come from the parents.

The ecology of mud crabs and banana prawns are so intertwined that sharing of viruses should not be a surprise. Both species have their postlarval and juvenile life stages in the mangrove lined estuaries and the adults move offshore to breed and broadcast their eggs for dispersal. Postlarval prawns would possibly feed on megalopa of mudcrabs and later stage mudcrabs would feed on juvenile and adult prawns. Any virus like *Pmerg*DNV that mutated to grow in both species would be selected for over a monospecific-host variant as the chances of transmission would be greatly enhanced. These findings of this study complicate the epidemiology of *Pmerg*DNV, particularly in terms of controlling the disease at the farm level for both host species. Not only will production of virus-free stock be necessary, but exclusion of the alternative host will also be required.

CHAPTER 10

General Discussion

Worldwide, it is acknowledged that supplies from traditional capture fisheries cannot keep up with global population growth and therefore, there is an increasing dependence on aquaculture to help satisfy the growing world's demand for fisheries products. The current trend in aquaculture development is towards increased intensification of production. Unfortunately, like other farming sectors, the likelihood of major disease problems occurring increases as aquaculture activities intensify and expand. Thus, aquaculture has been faced with its share of diseases, attributed to a variety of multi-faceted and highly interconnected factors such as the increased globalization of trade in live aquatic animals and their products, poor or lack of effective biosecurity measures, intensification of aquaculture, slow awareness of human-mediated movements of commodities and emerging diseases.

Prawns are the dominant crustacean species farmed in Australia and in Queensland alone, the penaeid culture industry is estimated to be worth over $45 million (ABARE, 2007). Unfortunately, the development and sustainability of the aquaculture sector is facing increased pressure due to cheap imports from south-east Asian countries and infectious diseases, resulting in restrictions in trade, increased operating costs and production losses (Hanna *et al.*, 2005).

Hepatopancreatic parvovirus (HPV) from Australian *Penaeus merguiensis* is the fourth penaeid prawn strain to be described and has been named *Penaeus merguiensis* densovirus (*Pmerg*DNV). This is the first report of nucleotide information regarding HPV from Australia or from *P. merguiensis*. It shares a similar genome size (approximately 6.3 kb) and organisation to the HPV isolate from *P. monodon* of Thailand (Sukhumsirichart *et al.*, 2006), with two overlapping non-structural proteins and a larger capsid protein. A specific, rapid and sensitive real-time TaqMan assay targeting the capsid protein was developed and optmised to to permit the simultaneous detection and quantitiation of

*Pmerg*DNV to as low as 10 virions per sample. The assay detected a prevalence of 87% among farmed *P. merguiensis*, in which only 27% were positive for *Pmerg*DNV infection after histological examinantion. Hence, this assay can be utilised to exclude potential carriers from broodstock and subsequently reduce the economic impact of this disease in Australian penaeid culture facilities.

Penaeus merguiensis densovirus has a prevalence of approximately 80% (unpublished data) in culture facilities in northern Queensland and wild *P. merguiensis* have been reported to carry heavy infections of the virus (Chapter 5). However, the findings of this study complicate the epidemiology of *Pmerg*DNV since *Pmerg*DNV was detected in the haemolymph of adult *S. serrata* and in larvae that had not yet begun to feed, indicating the virus must have been obtained from the parents. Since mud crabs and banana prawns share the same ecology, controlling the disease at the farm level must exclude both wild host species.

Despite the high prevalence of *Pmerg*DNV in Queensland *P. merguiensis*, the extent to which the industry is suffering from production losses due to the effects of this virus was unknown. Hence, this study was to determine the effect of this disease on the culture of *P. merguiensis* on farms in northern Queensland and determine if this virus warranted further control methods. An analysis of 190 ponds over a two year period revealed approximately 28.5% of the ponds with moderate to heavy loads of *Pmerg*DNV have below average survival. Furthermore, if you remove *Pmerg*DNV or reduce the *Pmerg*DNV levels in the moderately to heavily infected ponds to lighter levels, approximately 28-29% of the drop in survival below the mean will be removed. Hence, production should increase by at least 14% across farms. Based on the Australian Fisheries Statistics and if *P. merguiensis*, *P. esculentus* and *P. japonicus* were the only species farmed, this would equate to an increase in production of $6.5 million across Queensland. Consequently, removal and prevention of *Pmerg*DNV outbreaks will result in an increase in production and aid in developing a more sustainable industry.

Crustaceans have an innate immune response and therefore lack the protein-based adaptive immune response found in vertebrates (Brennan and Anderson, 2004). Consequently, they cannot be vaccinated against viral diseases. Unlike bacterial and parasitic disease that can be treated through the use of antibiotics, the control of viral diseases in culture facilities rests largely on preventative measures. RNA interference is an evolutionary conserved natural gene regulatory mechanism, triggered by the recognition of intracellular long double stranded RNA that inhibits gene expression at the level of translation or by hindering gene transcription (Hammond, 2005). It is therefore a vital part of the immune response to foreign genetic material. Since the phenomenon was first described in the early 1990's RNA interference antiviral immunity has since been recognised as an important defence against viruses in plants (Fagard and Vaucheret, 2000; Wu-Scharf *et al.*, 2000), vertebrates (Wianny and Zernicka-Goetz, 2000; Caplen *et al.*, 2001) and invertebrates (Fire *et al.*, 1998; Hughes and Kaufman, 2000; van Rij *et al.*, 2006). Numerous studies have documented the potential of RNAi for providing partial protection in prawns against white spot syndrome virus and yellow head virus (Robalino *et al.*, 2004; Robalino *et al.*, 2005; Tirasophon *et al.*, 2005; Westenberg *et al.*, 2005; Yodmuang *et al.*, 2006; Kim *et al.*, 2007; Tirasophon *et al.*, 2007; Xu *et al.*, 2007). Hence, RNAi may be used to induce antiviral immunity against *Pmerg*DNV.

Unfortunately, in Australia it is logistically difficult to use *P. merguiensis* in bioassays and natural infections of *Pmerg*DNV would interfere with the results of infection studies. Therefore, it was necessary to find an alternate animal model for *Pmerg*DNV. Here, we report the use of the house cricket *Acheta domesticus* as an alternate bioassay species for *Pmerg*DNV. Furthermore, RNAi was successfully induced against *Pmerg*DNV in *A. domesticus*. Injection of stealth RNAs (control and target) for the capsid protein and target stealth RNAs for NS2 of *Pmerg*DNV resulted in a 10-fold reduction in the average viral titre detected in tissues of *A. domesticus*, compared to crickets challenged with *Pmerg*DNV. The final titre was below the threshold level (10^3 virions) needed for *Pmerg*DNV infection and should therefore, on average, reduce the spread of the virus.

RNA interference is therefore a promising strategy for the control of *Pmerg*DNV. To obtain a greater insight into the mechanism and efficiency of RNAi againt *Pmerg*DNV, a similar experiment needs to be repeated in prawns. Furthermore, successful application of RNAi at the farm level requires further investigations into the stability of dsRNA in prawns, if dsRNA induces long term inhibition and if the immunity can be passed onto offspring. More importantly, an efficient method needs to be developed for the delivery of dsRNA such as through the food pellets for large scale operations as in aquaculture. This is where future research should be targeted.

For sustainable prawn farming, the industry should aim at undertaking ecologically sound management practices. Intensification of aquaculture and disproportionate growth of the industry relative to infrastructure development can lead to both environmental and social problems. Good management practices and responsible health management can minimise the risk of disease.

There is no doubt the aquaculture sector will continue to intensify and as such, the risk of major disease incursions and newly emerging diseases will keep on threatening the sector. Unless appropriate health management measures are maintained and effectively implemented, the sectors will face more costs in terms of production and the efforts needed to contain and eradicate diseases, funds that would have been better spend in preventing their entry into the system.

Maintaining healthy stock through better management practices and focusing efforts disease prevention and control should be of top priority. RNAi therapeutics provides a novel approach to control *Pmerg*DNV and other viral disease of penaeid prawns that are otherwise unable to be controlled by existing methods of control such as vaccines.

In summary, this project was the first to investigate the effect on production *Pmerg*DNV has on the Queensland penaeid culture industry; the first anywhere in the world to use insects as an alternate bioassay species and the first to inhibit *Pmerg*DNV replication via RNA interference. *Penaeus merguiensis* densovirus is prevalent among *P. merguiensis* culture facilities in northern Queensland. The results from this study suggest control of this disease can potentially increase production across the relevant farms by at least 14%. However, control of *Pmerg*DNV at a farm level is more complicated than expected since mud crabs are now known to be carriers of the disease. Hence, preventative control methods through the use of RNAi show great promise as a method to control and prevent outbreaks of *Pmerg*DNV. Following the successful application of RNAi against *Pmerg*DNV in prawns, the same technology can subsequently be applied against other penaeid viral diseases such as white spot syndrome virus, that are known to cause substantial losses among crustacean culture facilities around the world.

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APPENDIX A

Buffers and solutions

A.1. Reagents for viral purification

A.1.1. TN Buffer

0.02 M Tris-HCl	1.2 g
0.4 M NaCl	11.69 g

Dissolve in distilled water, adjust to pH 7.4 and make up to a final volume of 500 ml. Store at 4^{o}C.

A.1.2. 20% Sucrose

20% (w/v) sucrose	2.0 g

Dissolve in distilled water to make a final volume of 10.0 ml.

A.1.3. 30% Sucrose

30% (w/v) sucrose	3.0 g

Dissolve in distilled water to make a final volume of 10.0 ml.

A.1.4. 40% Sucrose

40% (w/v) sucrose	4.0 g

Dissolve in distilled water to make a final volume of 10.0 ml.

A.2. Sodium citrate

0.45 M Sodium chloride	13.15 g
0.1 M Glucose	9.0 g
0.03 M Trisodium citrate	4.41 g
0.026 M Citric acid	2.73 g

Dissolve in distilled water and make up to a final volume of 500 ml

A.3. Luria-Bertani medium

Bacto®-tryptone	10.0 g
Bacto®-yeast extract	5.0 g
Sodium chloride	5.0 g

Dissolve in distilled water, adjust to pH 7.0 and make up to a final volume of one litre. Store at 4^{o}C.

A.4. Sodium citrate

0.45 M Sodium chloride	13.15 g
0.1 M Glucose	9.0 g
0.03 M Trisodium citrate	4.41 g
0.026 M Citric acid	2.73 g

Dissolve in distilled water and make up to a final volume of 500 ml.

APPENDIX B

Survival and diagnostic data of *Acheta domesticus* and *Tenebrio molitor* after experimentally infected with *Penaeus merguiensis* densovirus

Table B.1: Survival and diagnostic data for *Tenebrio molitor*

Treatment	Tank replicate	Animal number	Day collected	P*merg*DNV copy number
Unhandled control	1	1	30[a]	0.00E+00
		2	30[a]	0.00E+00
		3	30[a]	0.00E+00
		4	30[a]	0.00E+00
		5	30[a]	0.00E+00
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
	2	1	30[a]	0.00E+00
		2	30[a]	0.00E+00
		3	30[a]	0.00E+00
		4	30[a]	0.00E+00
		5	30[a]	0.00E+00
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
	3	1	30[a]	0.00E+00
		2	30[a]	0.00E+00
		3	30[a]	0.00E+00
		4	30[a]	0.00E+00
		5	30[a]	0.00E+00
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
Placebo control	1	1	30[a]	0.00E+00
		2	30[a]	0.00E+00
		3	30[a]	0.00E+00
		4	30[a]	0.00E+00

		5	30[a]	0.00E+00
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
	2	1	30[a]	0.00E+00
		2	30[a]	0.00E+00
		3	30[a]	0.00E+00
		4	30[a]	0.00E+00
		5	30[a]	0.00E+00
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
	3	1	30[a]	0.00E+00
		2	30[a]	0.00E+00
		3	30[a]	0.00E+00
		4	30[a]	0.00E+00
		5	30[a]	0.00E+00
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
*Pmerg*DNV	1	1	3	1.09E+04
		2	8	1.94E+01
		3	23	0.00E+00
		4	30[a]	0.00E+00
		5	30[a]	0.00E+00
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
	2	1	30[a]	0.00E+00
		2	30[a]	0.00E+00
		3	30[a]	0.00E+00
		4	30[a]	0.00E+00
		5	30[a]	0.00E+00
		6	30[a]	5.48E+01
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
	3	1	30[a]	0.00E+00
		2	30[a]	1.04E+02

		3	30[a]	0.00E+00
		4	30[a]	8.23E+00
		5	30[a]	0.00E+00
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
*Pmerg*DNV + $MgCl_2$	1	1	3	7.09E+04
		2	30[a]	0.00E+00
		3	30[a]	0.00E+00
		4	30[a]	0.00E+00
		5	30[a]	0.00E+00
		6	30[a]	8.71E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	2.11E+01
	2	1	12	0.00E+00
		2	30[a]	0.00E+00
		3	30[a]	0.00E+00
		4	30[a]	0.00E+00
		5	30[a]	0.00E+00
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
	3	1	4	0.00E+00
		2	14	1.07E+01
		3	22	3.27E+01
		4	26	0.00E+00
		5	30[a]	8.70E+01
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00

[a] Alive at the end of the experimental period

Table B.2: Survival and diagnostic data for *Acheta domesticus*

Treatment	Tank replicate	Animal number	Day collected	P*merg*DNV copy number
Unhandled control	1	1	30[a]	0.00E+00
		2	30[a]	0.00E+00
		3	30[a]	0.00E+00
		4	30[a]	0.00E+00
		5	30[a]	0.00E+00
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
	2	1	30[a]	0.00E+00
		2	30[a]	0.00E+00
		3	30[a]	0.00E+00
		4	30[a]	0.00E+00
		5	30[a]	0.00E+00
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
	3	1	30[a]	0.00E+00
		2	30[a]	0.00E+00
		3	30[a]	0.00E+00
		4	30[a]	0.00E+00
		5	30[a]	0.00E+00
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
Placebo control	1	1	30[a]	0.00E+00
		2	30[a]	0.00E+00
		3	30[a]	0.00E+00
		4	30[a]	0.00E+00
		5	30[a]	0.00E+00
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
	2	1	30[a]	0.00E+00
		2	30[a]	0.00E+00
		3	30[a]	0.00E+00
		4	30[a]	0.00E+00

		5	30[a]	0.00E+00
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
	3	1	30[a]	0.00E+00
		2	30[a]	0.00E+00
		3	30[a]	0.00E+00
		4	30[a]	0.00E+00
		5	30[a]	0.00E+00
		6	30[a]	0.00E+00
		7	30[a]	0.00E+00
		8	30[a]	0.00E+00
		9	30[a]	0.00E+00
		10	30[a]	0.00E+00
*Pmerg*DNV	1	1	9	5.46E+03
		2	9	3.52E+05
		3	11	1.38E+03
		4	12	4.71E+04
		5	19	2.33E+03
		6	19	0.00E+00
		7	22	4.89E+03
		8	25	4.17E+02
		9	30[a]	9.14E+02
		10	30[a]	0.00E+00
	2	1	5	5.31E+03
		2	5	4.93E+03
		3	10	9.19E+02
		4	15	5.93E+03
		5	20	3.33E+05
		6	23	1.99E+01
		7	28	8.86E+02
		8	30[a]	5.97E+03
		9	30[a]	4.06E+03
		10	30[a]	2.21E+06
	3	1	6	1.03E+03
		2	8	2.19E+04
		3	9	0.00E+00
		4	16	1.88E+05
		5	20	2.01E+04
		6	21	2.46E+03
		7	22	2.77E+05
		8	24	1.01E+04
		9	27	3.08E+05
		10	30[a]	1.32E+05
*Pmerg*DNV + $MgCl_2$	1	1	4	3.11E+04
		2	5	7.71E+04

		3	13	0.00E+00
		4	15	2.02E+03
		5	28	2.83E+03
		6	28	0.00E+00
		7	30[a]	6.95E+04
		8	30[a]	6.48E+04
		9	30[a]	5.26E+02
		10	30[a]	3.65E+05
	2	1	11	1.90E+02
		2	12	1.09E+03
		3	17	2.99E+04
		4	22	1.30E+03
		5	23	0.00E+00
		6	24	1.74E+02
		7	28	0.00E+00
		8	30[a]	8.09E+04
		9	30[a]	1.80E+01
		10	30[a]	3.00E+05
	3	1	8	4.27E+04
		2	9	1.71E+05
		3	21	2.37E+04
		4	22	5.04E+04
		5	24	1.04E+03
		6	28	1.81E+03
		7	30[a]	5.21E+03
		8	30[a]	0.00E+00
		9	30[a]	2.19E+03
		10	30[a]	5.82E+03

[a] Alive at the end of the experiment

Table B.3: *Acheta domesticus* time course experiment

Treatment	Animal number	Day	Sacrificed or moribund	P*merg*DNV copy number
Placebo control	1	0	Sacrificed	0.00E+00
	2	0	Sacrificed	0.00E+00
	3	1	Sacrificed	0.00E+00
	4	1	Sacrificed	0.00E+00
	5	2	Sacrificed	0.00E+00
	6	2	Sacrificed	0.00E+00
	7	3	Sacrificed	0.00E+00
	8	3	Sacrificed	0.00E+00
	9	4	Sacrificed	0.00E+00
	10	4	Sacrificed	0.00E+00
	11	5	Sacrificed	0.00E+00
	12	5	Sacrificed	0.00E+00
	13	6	Sacrificed	0.00E+00
	14	6	Sacrificed	0.00E+00
	15	7	Sacrificed	0.00E+00
	16	7	Sacrificed	0.00E+00
	17	8	Sacrificed	0.00E+00
	18	8	Sacrificed	0.00E+00
	19	9	Sacrificed	0.00E+00
	20	9	Sacrificed	0.00E+00
	21	10	Sacrificed	0.00E+00
	22	10	Sacrificed	0.00E+00
	23	12	Sacrificed	0.00E+00
	24	12	Sacrificed	0.00E+00
	25	14	Sacrificed	0.00E+00
	26	14	Sacrificed	0.00E+00
	27	16	Sacrificed	0.00E+00
	28	16	Sacrificed	0.00E+00
	29	18	Sacrificed	0.00E+00
	30	18	Sacrificed	0.00E+00
	31	20	Sacrificed	0.00E+00
	32	20	Sacrificed	0.00E+00
*Pmerg*DNV	1	0	Sacrificed	1.27E+05
	2	0	Sacrificed	1.03E+05
	3	1	Moribund	5.48E+04
	4	1	Moribund	6.11E+03
	5	1	Moribund	8.47E+04
	6	1	Moribund	2.04E+03
	7	1	Sacrificed	8.27E+02
	8	1	Sacrificed	2.90E+05
	9	2	Moribund	5.51E+04
	10	2	Moribund	9.68E+04
	11	2	Moribund	2.35E+01
	12	2	Sacrificed	4.26E+03
	13	2	Moribund	3.25E+04

	14	2	Sacrificed	2.45E+04
	15	3	Moribund	1.39E+05
	16	3	Sacrificed	3.24E+03
	17	3	Sacrificed	7.14E+03
	18	4	Moribund	1.99E+04
	19	4	Moribund	5.04E+05
	20	4	Sacrificed	7.17E+04
	21	4	Sacrificed	1.13E+04
	22	5	Moribund	5.32E+04
	23	5	Sacrificed	1.26E+06
	24	5	Sacrificed	1.02E+04
	25	6	Moribund	3.47E+04
	26	6	Moribund	8.39E+05
	27	6	Sacrificed	1.22E+05
	28	6	Sacrificed	5.64E+04
	29	7	Moribund	9.64E+03
	30	7	Sacrificed	7.61E+04
	31	7	Sacrificed	2.08E+04
	32	8	Sacrificed	5.13E+05
	33	8	Sacrificed	0.00E+00
	34	9	Sacrificed	1.74E+03
	35	9	Sacrificed	3.33E+03
	36	10	Moribund	1.69E+05
	37	10	Sacrificed	7.11E+04
	38	10	Sacrificed	5.33E+02
	39	11	Moribund	6.53E+02
	40	11	Moribund	1.02E+03
	41	11	Moribund	1.35E+05
	42	11	Moribund	3.91E+02
	43	12	Moribund	3.56E+04
	44	12	Sacrificed	3.92E+03
	45	12	Sacrificed	3.48E+03
	46	13	Moribund	1.76E+02
	47	14	Moribund	2.29E+05
	48	14	Sacrificed	2.52E+04
	49	14	Sacrificed	2.73E+04
	50	15	Moribund	2.60E+03
	51	16	Moribund	6.00E+03
	52	16	Sacrificed	3.37E+03
	53	16	Sacrificed	0.00E+00
	54	17	Moribund	1.22E+03
	55	18	Moribund	7.06E+03
	56	18	Sacrificed	1.33E+03
	57	18	Sacrificed	1.91E+02
	58	20	Sacrificed	1.37E+03

[a] Alive at the end of the experiment

Table B.4: *Acheta domesticus* viral titre experiment

Treatment	Animal number	Day collected	P*merg*DNV copy number
Control	1	30	0.00E+00
	2	30	0.00E+00
	3	30	0.00E+00
	4	30	0.00E+00
	5	30	0.00E+00
1000000	6	6	6.26E+04
	7	10	5.70E+04
	8	15	2.69E+04
	9	13	5.68E+03
	10	13	1.43E+03
100000	11	1	1.21E+04
	12	5	5.37E+03
	13	13	2.04E+02
	14	14	1.36E+03
	15	30	2.30E+01
10000	1	4	1.21E+04
	2	11	5.37E+03
	3	30	0.00E+00
	4	30	0.00E+00
	5	15	7.82E+02
1000	6	2	5.37E+03
	7	30	0.00E+00
	8	30	0.00E+00
	9	30	0.00E+00
	10	30	0.00E+00
100	11	30	0.00E+00
	12	30	0.00E+00
	13	30	0.00E+00
	14	30	0.00E+00
	15	30	0.00E+00
10	1	30	0.00E+00
	2	30	0.00E+00
	3	30	0.00E+00
	4	30	0.00E+00
	5	30	0.00E+00
1	6	30	0.00E+00
	7	30	0.00E+00
	8	30	0.00E+00
	9	30	0.00E+00
	10	30	0.00E+00

[a] Alive at the end of the experiment

APPENDIX C

Results of *in vivo* down regulation of *Penaeus merguiensis* densovirus gene expression in *Acheta domesticus* using small interfering RNAs targeting the capsid protein

Table C.1: Survival and diagnostic data for *Acheta domesticus*

Treatment	Tank replicate	Animal number	Day collected	P*merg*DNV copy number (1)	P*merg*DNV copy number (2)
Unhandled control	1	1	14[a]	0.00E+00	0.00E+00
		2	14[a]	0.00E+00	0.00E+00
		3	14[a]	0.00E+00	0.00E+00
		4	14[a]	0.00E+00	0.00E+00
		5	14[a]	0.00E+00	0.00E+00
		6	14[a]	0.00E+00	0.00E+00
		7	14[a]	0.00E+00	0.00E+00
		8	14[a]	0.00E+00	0.00E+00
		9	14[a]	0.00E+00	0.00E+00
		10	14[a]	0.00E+00	0.00E+00
	2	1	14[a]	0.00E+00	0.00E+00
		2	14[a]	0.00E+00	0.00E+00
		3	14[a]	0.00E+00	0.00E+00
		4	14[a]	0.00E+00	0.00E+00
		5	14[a]	0.00E+00	0.00E+00
		6	14[a]	0.00E+00	0.00E+00
		7	14[a]	0.00E+00	0.00E+00
		8	14[a]	0.00E+00	0.00E+00
		9	14[a]	0.00E+00	0.00E+00
		10	14[a]	0.00E+00	0.00E+00
	3	1	14[a]	0.00E+00	0.00E+00
		2	14[a]	0.00E+00	0.00E+00
		3	14[a]	0.00E+00	0.00E+00
		4	14[a]	0.00E+00	0.00E+00
		5	14[a]	0.00E+00	0.00E+00
		6	14[a]	0.00E+00	0.00E+00
		7	14[a]	0.00E+00	0.00E+00
		8	14[a]	0.00E+00	0.00E+00
		9	14[a]	0.00E+00	0.00E+00
		10	14[a]	0.00E+00	0.00E+00
Placebo control	1	1	14[a]	0.00E+00	0.00E+00

		2	14[a]	0.00E+00	0.00E+00
		3	14[a]	0.00E+00	0.00E+00
		4	14[a]	0.00E+00	0.00E+00
		5	14[a]	0.00E+00	0.00E+00
		6	14[a]	0.00E+00	0.00E+00
		7	14[a]	0.00E+00	0.00E+00
		8	14[a]	0.00E+00	0.00E+00
		9	14[a]	0.00E+00	0.00E+00
		10	14[a]	0.00E+00	0.00E+00
	2	1	14[a]	0.00E+00	0.00E+00
		2	14[a]	0.00E+00	0.00E+00
		3	14[a]	0.00E+00	0.00E+00
		4	14[a]	0.00E+00	0.00E+00
		5	14[a]	0.00E+00	0.00E+00
		6	14[a]	0.00E+00	0.00E+00
		7	14[a]	0.00E+00	0.00E+00
		8	14[a]	0.00E+00	0.00E+00
		9	14[a]	0.00E+00	0.00E+00
		10	14[a]	0.00E+00	0.00E+00
	3	1	14[a]	0.00E+00	0.00E+00
		2	14[a]	0.00E+00	0.00E+00
		3	14[a]	0.00E+00	0.00E+00
		4	14[a]	0.00E+00	0.00E+00
		5	14[a]	0.00E+00	0.00E+00
		6	14[a]	0.00E+00	0.00E+00
		7	14[a]	0.00E+00	0.00E+00
		8	14[a]	0.00E+00	0.00E+00
		9	14[a]	0.00E+00	0.00E+00
		10	14[a]	0.00E+00	0.00E+00
Target RNAi	1	1	14[a]	0.00E+00	0.00E+00
		2	14[a]	0.00E+00	0.00E+00
		3	14[a]	0.00E+00	0.00E+00
		4	14[a]	0.00E+00	0.00E+00
		5	14[a]	0.00E+00	0.00E+00
		6	14[a]	0.00E+00	0.00E+00
		7	14[a]	0.00E+00	0.00E+00
		8	14[a]	0.00E+00	0.00E+00
		9	14[a]	0.00E+00	0.00E+00
		10	14[a]	0.00E+00	0.00E+00
	2	1	14[a]	0.00E+00	0.00E+00
		2	14[a]	0.00E+00	0.00E+00
		3	14[a]	0.00E+00	0.00E+00
		4	14[a]	0.00E+00	0.00E+00
		5	14[a]	0.00E+00	0.00E+00
		6	14[a]	0.00E+00	0.00E+00
		7	14[a]	0.00E+00	0.00E+00
		8	14[a]	0.00E+00	0.00E+00

		9	14[a]	0.00E+00	0.00E+00
		10	14[a]	0.00E+00	0.00E+00
	3	1	14[a]	0.00E+00	0.00E+00
		2	14[a]	0.00E+00	0.00E+00
		3	14[a]	0.00E+00	0.00E+00
		4	14[a]	0.00E+00	0.00E+00
		5	14[a]	0.00E+00	0.00E+00
		6	14[a]	0.00E+00	0.00E+00
		7	14[a]	0.00E+00	0.00E+00
		8	14[a]	0.00E+00	0.00E+00
		9	14[a]	0.00E+00	0.00E+00
		10	14[a]	0.00E+00	0.00E+00
Control RNAi + *Pmerg*DNV	1	1	1	0.00E+00	4.44E+01
		2	2	1.84E+02	1.12E+02
		3	2	1.03E+03	5.52E+02
		4	12	0.00E+00	3.98E+01
		5	14[a]	0.00E+00	2.37E+01
		6	14[a]	1.39E+03	0.00E+00
		7	14[a]	0.00E+00	3.61E+02
		8	14[a]	0.00E+00	0.00E+00
		9	14[a]	1.74E+02	2.14E+01
		10	14[a]	N/A	N/A
	2	1	3	0.00E+00	0.00E+00
		2	3	7.61E+01	1.24E+01
		3	11	1.15E+02	6.65E+01
		4	14[a]	0.00E+00	0.00E+00
		5	14[a]	5.18E+02	7.19E+00
		6	14[a]	3.98E+02	2.37E+02
		7	14[a]	1.62E+03	2.21E+02
		8	14[a]	1.02E+03	4.63E+02
		9	14[a]	0.00E+00	0.00E+00
		10	14[a]	N/A	N/A
	3	1	3	0.00E+00	3.64E+01
		2	4	1.48E+02	0.00E+00
		3	14[a]	7.59E+02	7.96E+01
		4	14[a]	3.56E+02	4.13E+01
		5	14[a]	4.32E+03	1.77E+03
		6	14[a]	1.13E+03	8.27E+02
		7	14[a]	3.62E+01	5.80E+01
		8	14[a]	0.00E+00	6.50E+00
		9	14[a]	1.94E+03	1.14E+03
		10	14[a]	N/A	N/A
Target RNAi + *Pmerg*DNV	1	1	1	8.90E+02	5.11E+02
		2	14[a]	3.14E+01	3.35E+01

		3	14[a]	0.00E+00	1.32E+01
		4	14[a]	2.67E+02	2.34E+02
		5	14[a]	0.00E+00	0.00E+00
		6	14[a]	5.65E+02	3.24E+02
		7	14[a]	0.00E+00	0.00E+00
		8	14[a]	4.88E+03	1.54E+03
		9	14[a]	7.73E+02	2.44E+02
		10	14[a]	N/A	N/A
	2	1	11	4.77E+02	1.48E+02
		2	14[a]	0.00E+00	2.18E+01
		3	14[a]	1.07E+02	2.06E+01
		4	14[a]	8.02E+01	6.66E+00
		5	14[a]	0.00E+00	1.68E+01
		6	14[a]	1.44E+02	6.54E+01
		7	14[a]	5.60E+01	4.57E+01
		8	14[a]	1.39E+03	9.27E+02
		9	14[a]	N/A	N/A
		10	14[a]	N/A	N/A
	3	1	1	1.71E+02	1.62E+02
		2	14[a]	0.00E+00	0.00E+00
		3	14[a]	0.00E+00	0.00E+00
		4	14[a]	9.31E+01	0.00E+00
		5	14[a]	1.68E+03	8.27E+01
		6	14[a]	6.29E+02	2.23E+02
		7	14[a]	4.53E+02	7.23E+01
		8	14[a]	4.78E+02	3.09E+02
		9	14[a]	3.53E+02	1.13E+02
		10	14[a]	N/A	N/A
*Pmerg*DNV	1	1	1	0.00E+00	0.00E+00
		2	4	N/A	N/A
		3	10	6.35E+02	2.05E+02
		4	14[a]	8.87E+02	5.76E+02
		5	14[a]	4.33E+03	2.58E+01
		6	14[a]	0.00E+00	2.32E+03
		7	14[a]	1.95E+03	1.18E+01
		8	14[a]	1.34E+04	8.36E+02
		9	14[a]	0.00E+00	1.19E+03
		10	14[a]	N/A	N/A
	2	1	11	0.00E+00	1.45E+02
		2	3	6.37E+02	6.68E+01
		3	9	9.90E+02	2.24E+02
		4	14[a]	2.68E+03	2.60E+02
		5	14[a]	0.00E+00	4.34E+02
		6	14[a]	0.00E+00	1.37E+01
		7	14[a]	1.18E+04	0.00E+00
		8	14[a]	2.69E+02	3.55E+03
		9	14[a]	7.82E+03	1.69E+02
		10	14[a]	1.20E+03	2.28E+03

	3	1	1	3.34E+03	2.07E+02
		2	14[a]	7.59E+04	1.88E+02
		3	14[a]	5.07E+03	2.42E+04
		4	14[a]	9.23E+03	1.15E+03
		5	14[a]	2.22E+03	2.84E+03
		6	14[a]	2.37E+03	3.46E+02
		7	14[a]	2.74E+03	8.93E+02
		8	14[a]	0.00E+00	8.25E+02
		9	14[a]	9.67E+02	4.93E+01
		10	14[a]	0.00E+00	9.74E+01

[a] Alive at the end of the experimental period

N/A Cricket absent due to cannibalism

APPENDIX D

Results of *in vivo* down regulation of *Penaeus merguiensis* densovirus gene expression in *Acheta domesticus* using small interfering RNAs targeting non-structural protein two

Table D.1: Survival and diagnostic data for *Acheta domesticus*

Treatment	Tank replicate	Animal number	Day collected	P*merg*DNV copy number (1)	P*merg*DNV copy number (2)
Target RNAi	1	1	6	0.00E+00	0.00E+00
		2	9	0.00E+00	0.00E+00
		3	10	0.00E+00	0.00E+00
		4	14[a]	0.00E+00	0.00E+00
		5	14[a]	0.00E+00	0.00E+00
		6	14[a]	0.00E+00	0.00E+00
		7	14[a]	0.00E+00	0.00E+00
		8	14[a]	0.00E+00	0.00E+00
		9	14[a]	0.00E+00	0.00E+00
		10	14[a]	0.00E+00	0.00E+00
	2	1	7	0.00E+00	0.00E+00
		2	8	0.00E+00	0.00E+00
		3	8	0.00E+00	0.00E+00
		4	9	0.00E+00	0.00E+00
		5	10	0.00E+00	0.00E+00
		6	11	0.00E+00	0.00E+00
		7	14[a]	0.00E+00	0.00E+00
		8	14[a]	0.00E+00	0.00E+00
		9	14[a]	0.00E+00	0.00E+00
		10	14[a]	0.00E+00	0.00E+00
	3	1	8	0.00E+00	0.00E+00
		2	11	0.00E+00	0.00E+00
		3	12	0.00E+00	0.00E+00
		4	14[a]	0.00E+00	0.00E+00
		5	14[a]	0.00E+00	0.00E+00
		6	14[a]	0.00E+00	0.00E+00
		7	14[a]	0.00E+00	0.00E+00
		8	14[a]	0.00E+00	0.00E+00
		9	N/A	N/A	N/A
		10	N/A	N/A	N/A
Control RNAi +	1	1	3	1.19E+03	1.71E+04

*Pmerg*DNV					
		2	4	1.97E+03	3.18E+04
		3	4	1.45E+01	0.00E+00
		4	7	5.19E+01	0.00E+00
		5	8	2.93E+02	1.95E+03
		6	10	9.62E+01	0.00E+00
		7	12	0.00E+00	0.00E+00
		8	14[a]	8.80E+02	2.08E+04
		9	14[a]	2.68E+03	3.00E+04
		10	14[a]	5.16E+03	7.43E+04
	2	1	1	4.63E+02	6.44E+03
		2	6	0.00E+00	0.00E+00
		3	9	3.61E+01	0.00E+00
		4	11	1.10E+03	1.09E+04
		5	14[a]	3.85E+01	0.00E+00
		6	14[a]	0.00E+00	0.00E+00
		7	14[a]	3.73E+01	0.00E+00
		8	14[a]	5.72E+02	1.07E+04
		9	N/A	N/A	N/A
		10	N/A	N/A	N/A
	3	1	4	4.20E+01	0.00E+00
		2	7	1.80E+04	3.10E+05
		3	8	2.31E+02	6.98E+03
		4	14[a]	7.86E+02	1.29E+04
		5	14[a]	5.14E+03	7.17E+04
		6	14[a]	6.90E+02	1.65E+04
		7	14[a]	5.44E+01	1.87E+03
		8	14[a]	4.04E+02	3.08E+03
		9	14a	8.42E+01	1.58E+03
		10	14[a]	2.69E+04	5.22E+05
Target RNAi + *Pmerg*DNV	1	1	1	0.00E+00	0.00E+00
		2	1	7.63E+01	2.30E+01
		3	2	3.63E+04	2.28E+04
		4	2	1.27E+04	1.03E+04
		5	3	4.51E+02	1.11E+02
		6	3	1.04E+04	7.60E+03
		7	4	2.61E+03	1.32E+03
		8	9	3.99E+02	2.37E+02
		9	14[a]	7.34E+02	2.49E+02
		10	14[a]	7.57E+03	3.50E+03
	2	1	2	1.09E+05	4.77E+04
		2	3	9.89E+02	2.46E+02
		3	3	4.52E+01	0.00E+00
		4	4	1.69E+03	6.81E+02
		5	5	8.53E+03	1.07E+03
		6	14[a]	1.24E+04	5.11E+03

		7	14[a]	2.17E+04	1.03E+04
		8	14[a]	4.80E+03	2.88E+03
		9	14[a]	1.38E+02	7.63E+01
		10	14[a]	1.36E+04	1.03E+04
	3	1	1	2.50E+04	8.35E+03
		2	1	1.93E+03	7.17E+02
		3	2	2.56E+03	8.16E+02
		4	3	3.94E+02	1.42E+02
		5	6	1.15E+03	7.46E+02
		6	7	3.28E+03	6.19E+02
		7	8	1.48E+03	1.06E+03
		8	10	1.76E+03	5.49E+02
		9	14[a]	2.12E+04	1.06E+04
		10	14[a]	2.43E+04	8.64E+03
*Pmerg*DNV	1	1	1	4.87E+03	3.58E+03
		2	1	7.64E+03	2.66E+03
		3	2	7.82E+03	3.97E+03
		4	3	1.76E+05	1.21E+05
		5	5	5.91E+02	2.37E+02
		6	7	2.02E+04	1.06E+04
		7	13	1.50E+04	2.37E+03
		8	14[a]	3.21E+06	1.16E+06
		9	14[a]	2.19E+04	8.32E+03
		10	14[a]	6.56E+03	5.17E+03
	2	1	1	1.62E+05	5.35E+04
		2	9	1.38E+04	7.38E+03
		3	10	9.15E+04	4.82E+04
		4	12	2.25E+04	1.59E+04
		5	14[a]	4.42E+02	3.78E+02
		6	14[a]	2.91E+03	2.01E+03
		7	14[a]	3.07E+03	2.63E+03
		8	14[a]	1.01E+03	5.37E+02
		9	N/A	N/A	N/A
		10	N/A	N/A	N/A
	3	1	9	2.33E+03	6.10E+02
		2	12	1.14E+03	2.93E+02
		3	14[a]	2.99E+04	1.05E+04
		4	14[a]	1.82E+04	1.03E+04
		5	14[a]	8.18E+03	5.45E+03
		6	14[a]	1.26E+04	1.08E+04
		7	14[a]	7.64E+04	4.70E+04
		8	14[a]	3.35E+03	1.30E+03
		9	14[a]	0.00E+00	0.00E+00
		10	14[a]	4.08E+04	2.27E+04

[a] Alive at the end of the experimental period

N/A Cricket absent due to cannibalism

Appendix E

Nucleotide sequence of *Penaeus merguiensis* densovirus from the mud crab *Scylla serrata* (Genbank accession number: EU073937)

1	cgctttgaca	ggattagtaa	atacagcagt	tatgacaaat	gtcggtgatg
51	gtggaacatt	ccatttcagt	aacataacag	agatgagtac	cattgtggta
101	gggaatgaga	ctaagattag	aactcagaca	attgaacagt	ggaagggatt
151	atgtggagga	gagaatgtaa	caatgcctat	gaagtataag	gagcataaga
201	cacatatgtt	caggaagcct	gtgtttttga	ccaaccagca	tcatccactg
251	gtagatattt	cacattatga	tgacaggagg	gctatagaga	ataggagttt
301	catgtataaa	gtagagttag	gaagtgaggc	agtaaatgca	catataaagt
351	ttcctaataa	gatgattcca	ataaagaaga	acccagaact	aacacagttt
401	gtattggcaa	gcatgcagta	tgttcattca	aactatatgg	acaagccaga
451	caggaagttt	aagattgggt	ttttcaacaa	gctttatgat	atgctgtttg
501	agaataacta	aatatatgta	caccaatgtt	tgagtttccc	ggcatcaata
551	aagtgataag	ataagatcgt	gtgtttgaat	atccacgtca	cctatacagt
601	cagtatacga	tgtctctagc	atgggagcag	tcgtatcagc	agtagctgca
651	gtcatagctg	cagtaactga	agttgtggag	ttcatagtaa	atgtcgtgga
701	agcagctttc	gtggtggcag	agactgttca	ggtcgttgca	gacacagtca
751	attactttac	tggtggcgat	agtgctgcga	agggcgatca	gagcaatccg
801	caggcggaag	gtatttacga	aatcaacgac	agatcgacag	caggagaagt
851	aggagtgaga	cagggccata	tgctaggacc	tgatcagttg	gaagattacc
901	tggagagagc	cacagatata	gactaatgtc	acctacaaga	agaggaggaa
951	attattttgc	aagtaagcat	tttcaaggta	aacgaaagag	taaactacag
1001	agagtgaagg	atttactggc	aagtaagaaa	aaggacataa	agttcaaagg
1051	aaagggaaat	actttaagtg	aggagccaag	tacatcaggg	tggagagatc
1101	cagtaagaca	gagatttcca	gcattagggc	aagaggaaag	aaatacattt
1151	gcaggattat	tggcaataga	agcagcacca	gaccaaagac	aattgggacg
1201	tgacagtaac	aatcaattag	cactagtaca	gagagataca	agagtagcag
1251	taagacagag	tacaaacaga	agagaagcat	tagaggtagt	aagaacagct
1301	aacgaagcaa	taagaagtgg	tggagataga	ttatcagagt	tagtacaagc
1351	atacgcatca	ggattttcag	acagtacaga	aatagtagaa	acaagacaag
1401	aagatagagt	acagagagat	atattccaag	cagagggaca	gaatctacta
1451	gctatagaga	tcgcattaga	gggaccgagc	agtgtaacac	agcagtttga
1501	tcaagagaga	actctagcag	tcaagagagc	tttagaatta	actcaagaag

1551	aagaacggat	agagcagata	gagaacgcca	agaagtatat	agaagaagtc
1601	atagacgaga	cgaatagaga	actagccacg	caagagcgac	aagatataag
1651	tgcggcggcg	gaagatacga	tgaacagtgc	cgcacctact	ccaatggaaa
1701	cttctgagcc	agggactacc	gccgcaccgc	agcagctatc	tgcagcgggt
1751	ggtggcggtg	gtggcggagg	cggaggtgaa	acagcagggt	acgggaaaaa
1801	tacaggaagt	gccttccaac	gccaccgcaa	caaacctatc	gacctacaac
1851	acattggaga	caatgtatac	gtagctcaga	gagtttacaa	agtagaggcc
1901	gagtgtaagc	tagtaaataa	caagttaaca	tggcaaacag	cagaggacaa
1951	tccgtttaca	cgaagattaa	tgggactgtc	tagcgccagt	aacaaaggaa
2001	acttcaagta	cagctttaag	tcaatgctaa	atggaagtgt	caatttggga
2051	aatcttagcc	tatccaacta	cataaatgca	tggggcatag	acaacatagc
2100	caagagtgaa	gacagttggg	ctattttatg	tacgagaggc	aagatgaacc
2151	atttacaggc	atttgagatg	attccacaac	ttcagggaga	aactgtgata
2201	ggatacacaa	gtgcaccagt	acagtttggt	aaacttttgg	ggcacatata
2251	ttatccagat	ccaaagggag	aagagaccat	aaaagttgca	ggcattggta
2301	atggcaaaga	aagtcaggta	tttgagggag	cacaggaagg	ttatctgatg
2351	gacgacgaca	tgagccaaaa	gaagatcaca	tcagaagccc	atccagtata
2401	catgttcaca	gacttacgag	atgcgccgat	gatcagtgaa	gtaacagcat
2451	acctagattc	aactaacccg	acaag		

Figure E.1: Nucleotide sequence of the 2475 bp amplified from the genome of *Penaeus merguiensis* densovirus from Australian *Scylla serrata*.

APPENDIX F

Animal Ethics Approval

JAMES COOK UNIVERSITY
Townsville Qld 4811 Australia

Tina Langford, Ethics Administrator, Research Office. Ph: 07 4781 4342; Fax: 07 4781 5521

ETHICS REVIEW COMMITTEE **Animal Ethics Committee** *APPROVAL FOR ANIMAL BASED RESEARCH OR TEACHING*						
PRINCIPAL INVESTIGATOR	**Ms Kathy La Fauce**					
SUPERVISOR	A/Prof Leigh Owens (Veterinary and Biomedical Sciences)					
SCHOOL	Veterinary and Biomedical Sciences					
PROJECT TITLE	**RNA interference (RNAi) as an antiviral mechanism against hepatopancreatic parvovirus (HPV)**					
APPROVAL DATE	5 Sep 2006	**EXPIRY DATE**	5 Sep 2009		**CATEGORY**	**4**

This project has been allocated Ethics Approval Number with the following conditions:	**A**	**1142**

1. All subsequent records and correspondence relating to this project must refer to this number.
2. That there is NO departure from the approved protocols unless prior approval has been sought from the Animal Ethics Committee.
3. The Principal Investigator is to advise the responsible Ethics Monitor appointed by the Ethics Review Committee:
   - periodically of the progress of the project;
   - when the project is completed, suspended or prematurely terminated for any reason.
4. In compliance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*, and the *Queensland Animal Care and Protection Act 2001*, it is **MANDATORY** that you provide an annual report on the progress of your project. This report must also detail animal usage, and any unexpected event or serious adverse effect that may have occurred during the study.

NAME OF RESPONSIBLE MONITOR	Summers, Prof Phillip
EMAIL ADDRESS	phillip.summers@jcu.edu.au
ASSESSED AT MEETING **APPROVED** **Professor Phillip Summers** **Chair, Animal Ethics Committee**	**Date:** 5 Sep 2006 **Date:** 5 Sep 2006
Tina Langford Ethics Officer **Research Office** Tina.Langford@jcu.edu.au	Date: 11 September 2006

APPENDIX G

List of publications, exhibitions and presentations

Presentations

The use of insects as a bioassay for *Penaeus merguiensis* densovirus (*Pmerg*DNV). FRDC Aquatic Animal Health Conference. July 2007, Cairns.

Publications

La Fauce, K. and Owens, L. (2007). Investigation into the pathogenicity of *Penaeus merguiensis* densovirus (*Pmerg*DNV) to juvenile *Cherax quadricarinatus*. Aquaculture **271**: 31-38.

La Fauce, K. A., Elliman, J. and Owens, L. (2007). Molecular characterisation of hepatopancreatic parvovirus (*Pmerg*DNV) from Australian *Penaeus merguiensis*. Virology **362**: 397-403.

La Fauce, K. A., Layton, R. and Owens, L. (2007). TaqMan real-time PCR for detection of hepatopancreatic parvovirus from Australia. Journal of Virological Methods **140**: 10-16.

La Fauce, K. A. and Owens, L. (2008). The use of insects as a bioassay for *Penaeus merguiensis* densovirus (*Pmerg*DNV). Journal of Invertebrate Pathology **98**: 1-6.

Submissions

La Fauce, K.A. and Owens, L. RNA interference reduces *Pmerg*DNV expression and replication in an in vivo cricket model. Submitted to Journal of Invertebrate Pathology.

Owens, L., La Fauce, K, Nguyen, T. and Zeng, C. Intranuclear bacilliform virus and hepatopancreatic parvovirus (*Pmerg*DNV) in the mud crab *Scylla serrata* (Forskal) of Australia. Sumbitted to Journal of Fish Diseases.

Owens, L., La Fauce, K., Juntunen, K., Hayakijkosol, O, Endo, K. and Zeng, C. The occurrence of endemic *Macrobrachium rosenbergii* nodavirus disease (white tailed disease) in Australia. Submitted to Diseases of Aquatic Organisms.

Workshops

PhD Smart State 'Saying it in a minute workshop'. June 2006, Brisbane.

APPENDIX H

Publications

Available online at www.sciencedirect.com
ScienceDirect
Journal of Virological Methods 140 (2007) 10–16
Journal of Virological Methods

TaqMan real-time PCR for detection of hepatopancreatic parvovirus from Australia

Kathy A. La Fauce*, Ramon Layton, Leigh Owens
School of Veterinary and Biomedical Sciences, Solander Drive, James Cook University, Townsville, Qld 4811, Australia

Received 11 July 2006; received in revised form 28 September 2006; accepted 19 October 2006
Available online 21 November 2006

Available online at www.sciencedirect.com
ScienceDirect
Virology 362 (2007) 397–408
VIROLOGY
www.elsevier.com/locate/yviro

Molecular characterisation of hepatopancreatic parvovirus (*Pmerg*DNV) from Australian *Penaeus merguiensis*

Kathy A. La Fauce*, Jennifer Elliman, Leigh Owens

Microbiology and Immunology, James Cook University, Townsville, QLD, 4811, Australia

Received 20 October 2006; accepted 29 November 2006
Available online 1 February 2007

Available online at www.sciencedirect.com
ScienceDirect
Aquaculture 271 (2007) 31–38
Aquaculture
www.elsevier.com/locate/aqua-online

Investigation into the pathogenicity of *Penaeus merguiensis* densovirus (*Pmerg*DNV) to juvenile *Cherax quadricarinatus*

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Available online at www.sciencedirect.com
ScienceDirect
Journal of Invertebrate Pathology 98 (2008) 1–6
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

The use of insects as a bioassay for *Penaeus merguiensis* densovirus (*Pmerg*DNV)

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Received 29 May 2007; accepted 20 November 2007
Available online 3 December 2007

APPENDIX I

Journal of Invertebrate Pathology submitted manuscript

RNA interference reduces *Pmerg*DNV expression and replication in an *in vivo* cricket model

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Abstract

RNA interference (RNAi) is an attractive antiviral preventative because it allows interference with the expression of a viral gene in a highly sequence-specific manner. Thus, essential viral genes can be targeted by design, with little or no risk of undesired off-target effects. To investigate if stealth RNAis can mediate a sequence specific anti-viral effect against *Pmerg*DNV, adult *Acheta domesticus* were injected with 5 μg of stealth RNAi or control stealth RNAi, targeting the capsid protein. Twenty-four hours post-injection, crickets were challenged with *Pmerg*DNV. Mortality was monitored for 14 days and real-time reverse transcriptase PCR was used to enumerate the number of copies of *Pmerg*DNV in cricket tissues. Crickets injected with RNAi targeting *Pmerg*DNV had the lowest mortality rate (11.5%) compared to crickets injected with control dsRNAi (33%) and *Pmerg*DNV alone (25%). Crickets challenged with specific dsRNAi significantly reduced *Pmerg*DNV production by one log (3.58×10^2) compared to crickets challenged with *Pmerg*DNV alone (3.42×10^3). Interestingly, even the control dsRNAi was capable of reducing *Pmerg*DNV production by one log (3.95×10^2), but did not produce an inhibitory effect quite as strong as the targeted dsRNAi for the capsid protein of *Pmerg*DNV. The introduction of dsRNAi corresponding to the capsid protein of *Pmerg*DNV, was effective in reducing viral replication in *Acheta domesticus*. Administration of *Pmerg*DNV-specific dsRNAis may provide an efficient counter measure against *Pmerg*DNV in prawns.

Keywords: RNA interference; RNAi; *Acheta domesticus*; *Penaeus merguiensis* densovirus; *Pmerg*DNV; gene silencing

1. Introduction

The worldwide production of marine prawns has substantially increased to keep pace with the worldwide demand for seafood products. However, this huge increase in cultured prawn production has not been without its setbacks. One of the most serious impediments of farming prawns is the incidence of disease. Disease epizootics of economic significance are a major constraint to the industry, not only because they affect the quantity of harvest, but because disease also affects the quality and regularity of production.

Most organisms have evolved protective defence mechanisms against viruses and other pathogens. Vertebrates rely on a both an 'adaptive' immune system that specifically targets pathogens as well as a broad-action, non-specific 'innate' immune response (Silverstein, 1989). On the other hand, invertebrates lack the protein-based adaptive immune response found in vertebrates but are still capable of effectively fighting viral infections (Brennan and Anderson, 2004).

RNA interference (RNAi) is an evolutionary conserved gene regulatory mechanism. It can be triggered by the recognition of intracellular long double stranded RNA, which can be transcribed from nuclear genes, replicating viruses or in the form of synthetic RNAs, resulting in sequence-specific degradation of the homologous RNA (post-transcriptional gene silencing) (Hannon, 2002; McCown et al., 2003; Hammond, 2005). Pioneering observations regarding RNA interference were reported in the nematode worm (Fire et al., 1998), plants (Waterhouse et al., 1998) and *Drosophila* (Kennerdell and Carthew, 1998). However, RNAi-related events have since been recognised in almost all

61 eukaryotic organisms including protozoans (Bastin et al., 2001; Malhotra et al.
62 2002; Cottrell and Doering, 2003), fungi (Raoni and Arndt, 2003), algae (Wu-
63 Scharf et al., 2000), nematodes (Fire et al., 1998, Caplen et al., 2001; Schott et
64 al., 2005), plants (Fagard and Vaucheret, 2000), insects (Misquitta and Paterson,
65 1999; Hughes and Kaufman, 2000), fish (Nasevicuis and Ekker, 2000; Dang et
66 al., 2008) and mammals (Wianny and Zernicka-Goetz, 2000; Caplen et al.,
67 2001). Plants and insects encode multiple Dicer enzymes that recognize distinct
68 precursors of small RNAs (Fire et al., 1998; Berstein et al., 2001; Lee et al.,
69 2004; Xie et al. 2004; Tomari and Zamore, 2005). Hence, they are capable of
70 initiating the small RNA-guided RNA interference antiviral immunity (Hamilton
71 and Baulcombe, 1999; Li et al., 2002) and have been recognised as an important
72 defence against viruses in invertebrates, particularly insects (van Rij et al., 2006;
73 Wang et al., 2006; Zambon et al., 2006).
74
75 *Penaeus merguiensis* densovirus (*Pmerg*DNV), a member of the family
76 Parvoviridae, is non-enveloped and contains a linear, single stranded DNA
77 genome approximately 6 kb in size encoding one structural and two non-
78 structural proteins (Siegl et al., 1985; Berns et al., 2000; La Fauce et al., 2007a).
79 It has a wide host range including marine and freshwater prawns (Anderson et
80 al., 1990; Lightner, 1996), crayfish (La Fauce and Owens, 2007) and crabs.
81 Infected prawns exhibit a reduction in their growth rate (Flegel et al., 1999).
82 Consequently, economic losses are a result from the stunting of infected prawns
83 since farmers are unable to sell their stock at full market value. There is currently
84 no effective strategy to treat or prevent *Pmerg*DNV. Hence, development of a
85 strategy to control *Pmerg*DNV is of high priority for the aquaculture industry.

Our previous work has indicated *Acheta domesticus* may be used as an animal model for *Pmerg*DNV (La Fauce and Owens, 2008). The cricket model for *Pmerg*DNV was developed because it was impossible to get *Pmerg*DNV-free prawns for *in vivo* experiments. Here, we investigate whether RNAi provides protection against the crustacean virus *Pmerg*DNV using an insect model, *Acheta domesticus*.

2. Materials and methods

2.1. Preparation of inoculum

Infectious material containing *Pmerg*DNV was prepared via ultracentrifigation directly from the hepatopancreai of *Penaeus merguiensis* stored frozen at James Cook University, Queensland, according to the protocol described by La Fauce et al. (2007a) and La Fauce and Owens (2008).

2.2. Insects

Adult *Acheta domesticus* (house crickets) were purchased Pisces Enterprises (Queensland, Australia). *Acheta domesticus* were housed in plastic aquaria and supplied with dried dog food and carrots (water source) *ad libitum*.

2.3. Stealth RNAi assay in crickets in vivo

The 25-nucleotide stealth small interference RNAs (siRNA) against the capsid protein of the *Pmerg*DNV (DQ458781) and stealth RNAi negative control duplexes were designed online using BLOCK-iT™ RNAi Designer

111 (rnaidesigner.invitrogen.com) and synthesised by Invitrogen (Victoria, Australia)
112 (Table 1).
113
114 *Acheta domesticus* were randomly distributed between six experimental
115 treatments (Table 2) with three replicates of ten insects in each. Crickets in each
116 experimental treatment received two injections: the first on Day 0 and the second
117 24 hours later. All injections were performed using sterile Terumo (1 ml)
118 syringes and a 30-gauge needle, and were discarded after each inoculation to
119 minimize cross-infections. *Acheta domesticus* were injected by inserting the
120 needle into the membrane below the pronotum. Crickets in the appropriate
121 treatments were challenged with 5 µg of the stealth RNAis and 5 x 10^4 virions of
122 *Pmerg*DNV. Control crickets were injected with the same volume of Grace's
123 insect medium (Invitrogen, Victoria, Australia). The experimental period began
124 on the day of the second injection and concluded on day 14. The knockdown of
125 *Pmerg*DNV was confirmed by real time reverse-transcriptase PCR (RT-PCR).
126
127 Experimental aquaria were monitored daily. Deceased crickets were removed,
128 recorded and prepared for real-time RT-PCR. Approximately 50 mg of tissue
129 was removed from *Acheta domesticus* (flight muscle, gut and reproductive
130 tissue) by longitudinal ventral dissection. Tissues were stored frozen at -20°C
131 until processed. At the end of the experimental period, all remaining insects were
132 sacrificed and the tissue removed for real-time RT-PCR.
133
134 *2.4. RNA extraction*

Total RNA was extracted from tissues using Promega SV® Total RNA Isolation System (New South Wales, Australia). Sometimes only the exoskeleton was found, presumably from cannibalism and RNA extraction from the exoskeleton was not productive.

2.5. Real time RT-PCR

Real-time RT-PCR was performed using a modified protocol described by La Fauce et al. (2007b) using RotorGene 3000 (Corbett Research, Sydney, Australia).

The reaction mixture consisted of 1 x Sensimix One-Step (Quantace, New South Wales, Australia), 0.5 mM of $MgCl_2$, 10 µM of each primer (HPV140F and HPV140R (La Fauce et al., 2007b)), 50 nM of probe (La Fauce et al., 2007b), 2.5 µl of DNA template and nuclease-free water to a final volume of 20 µl.

The thermocycling pattern consisted of an initial incubation at 49ºC for 30 minutes, followed by the run profile described by La Fauce *et al*. (2007b) of an initial incubation at 95ºC for 10 minutes, followed by a two-step cycle pattern consisting of forty cycles at 95ºC for 10 seconds and 60ºC for 45 seconds. Each run contained three no template controls and triplicate samples of 1×10^4 and 1×10^8 copies of the plasmid standard for the virus. Duplicate samples from each cricket were quantified and statistically analysed. Following the completion of each run, a standard curve generated by La Fauce *et al*. (2007b) was imported into the run file to obtain accurate quantitative information regarding the number

of viral copies in each tissue sample. Data acquisition and subsequent data analyses were performed using RotorGene 3000 and Microsoft Excel.

2.6. Statistical analyses

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) Version 14. The effect of treatment and survival was determined by oneway analysis of variance (ANOVA). Data that was not normally distributed was transformed using log10. Post-hoc comparisons between significant means were performed using least significant differences (LSD).

3. Results

3.1. Mortality

Cumulative percentage mortality was significantly greater in treatments exposed to *Pmerg*DNV ($F = 30.0$, $df = 7,5$, $p<0.05$), but was not significantly different from each other. The greatest cumulative percentage mortality was recorded in the treatment of crickets receiving the control RNAi + *Pmerg*DNV (Fig. 1). Mortality continued to increase from day one until day 4, followed by another increase again at day 10, reaching maximum mortality (approximately 33%) on day 12. Mortality in the group that only received *Pmerg*DNV followed a similar pattern with two peaks in mortality at day 4 and at day 11, reaching maximum mortality (25%) on day 11. Percentage mortality in the target RNAi + *Pmerg*DNV had the lowest percentage mortality (11.5%) of the treatments exposed to *Pmerg*DNV, despite mortality being 4% in the first two days of the

184 experiment. No mortalities were recorded in treatments that were not challenged
185 with *Pmerg*DNV (Fig. 1).
186
187 *3.2. Quantitative real-time RT-PCR*
188 Tissues from approximately 82% of crickets challenged with *Pmerg*DNV were
189 positive by real-time RT-PCR. Tissues from crickets that were negative for
190 *Pmerg*DNV had been cannibalised, leaving only the exoskeleton. The
191 *Pmerg*DNV only treatment had the greatest range of *Pmerg*DNV copies detected
192 in tissues, ranging from 0 (cannibalised animals) to 3.8×10^4. Similarly,
193 *Pmerg*DNV titres ranged from 0 (cannibalised animals) to 3.05×10^3 copies per
194 2.5 µl in tissues from crickets from the control RNAi treatment and 0
195 (cannibalised animals) to 3.21×10^3 per 2.5 µl in tissues from crickets from the
196 target RNAi treatment. The level of *Pmerg*DNV in positive control crickets
197 receiving no dsRNA was significantly greater than those in the stealth RNAi
198 treatments ($F = 42.2$, $df = 11, 342$, $p<0.05$). The average number of copies in
199 tissues from the *Pmerg*DNV-challenged treatment was 3.42×10^3. Interestingly,
200 the average number of copies of *Pmerg*DNV from crickets in the control RNAi
201 treatment and target RNAi treatments was approximately one log less than the
202 virus alone treatment at 3.95×10^2 and 3.58×10^2, respectively. *Pmerg*DNV was
203 not detected in tissues from crickets in the unhandled controls, placebo control or
204 crickets injected with the stealth RNAi target for *Pmerg*DNV.
205
206 **4. Discussion**
207 RNAi is increasingly being investigated as a preventative treatment for viral
208 infections in a variety of eukaryotic systems ranging from fungi (Raoni and

209 Arndt, 2003) and algae (Misquitta and Paterson, 1999; Hughes and Kaufman,
210 2000; Wu-Scharf et al., 2000) to invertebrate (Schott et al., 2005) and vertebrate
211 (Caplen et al., 2001; Morissey et al., 2005; Dang et al., 2008) systems. In this
212 paper, we take this approach to use an insect model, *Acheta domesticus*, to
213 induce RNAi against the crustacean virus *Pmerg*DNV.
214
215 The delivery of stealth RNAs into *Acheta domesticus* was done by injection.
216 Similarly, direct injection of dsRNA in other invertebrates such as
217 *Anopheles gambiae* (Keen et al., 2004), *Drosophila melanogaster* (Goto et al.,
218 2003), and *Tenebrio molitor* (Valdes et al., 2003), have led to successful and
219 efficient gene silencing. Hence, the sequence-specific binding of the siRNA to its
220 target mRNA triggering the nucleolytic activity of the RISC complex, resulting
221 in sequence specific RNAi can act in invertebrate systems.
222
223 Injection of stealth RNAs targeting the capsid protein of the *Pmerg*DNV resulted
224 in a 10-fold reduction in the average viral titre detected in tissues, compared to
225 crickets challenged with *Pmerg*DNV. Interestingly, tissues from crickets that
226 received the control stealth RNAi injection also showed a 10-fold reduction in
227 *Pmerg*DNV titres. This ten-fold reduction is significant as it drops the average
228 infective dose below the threshold level needed for *Pmerg*DNV infection (La
229 Fauce and Owens, 2008) and therefore on average, it should reduce the spread of
230 the virus.
231
232 Given that the number of copies of mRNA was analysed during this study, the
233 capsid protein must have been converted to mRNA for the technology to have

234 worked, thus proving that *Pmerg*DNV was indeed replicating in crickets in the
235 study of La Fauce and Owens (2008) and the assay was not just the detection of
236 virus that had been inoculated.
237
238 The control stealth RNA only differed in sequence by three base pairs and the
239 rearrangement of a base pair from position 10 to position 22 in the control RNAi,
240 suggesting this was not enough difference to stop the RNAi system from
241 working. Similar results have also been observed in *P. monodon* where unrelated
242 dsRNA also had partial inhibitory effect on Yellow Head Virus (YHV)
243 replication, whereas YHV-specific dsRNA resulted in a greater inhibitory effect
244 (Yodmuang et al., 2006). Similarly, injection of unrelated dsRNA has also been
245 shown to result in some protection against white spot syndrome virus in low
246 level infections in *P. vannamei* and *P. monodon* (Robalino et al., 2004;
247 Westenberg et al., 2005). Alternatively, the introduction of the gene-specific
248 siRNAs resulted in off-target effects, which was still seen through the
249 downregulation of *Pmerg*DNV. The dsRNA may have cross-reacted with targets
250 of limited sequence similarity due to partial sequence similarity between the
251 target mRNA and dsRNA. Even regions consisting of only 11-15 similar
252 nucleotides of sequence similarity can be sufficient to induce gene-silencing
253 (Jackson et al., 2003). Off-target effects may also occur at the level of translation
254 by blocking the translation of transcripts with partial homology, affecting
255 unintended targets at the level of translation. A three to four mismatch between
256 the siRNA and the targeted gene resulted in downregulation primarily at the
257 protein level (Saxena et al., 2003). However, the explanation for the

downregulation of *Pmerg*DNV in the control stealth RNAi treatment requires further investigation.

RNAi is a promising strategy for viral disease control as demonstrated in a number of organisms and operating systems, including prawns. However, many questions such as applying the method at the farm level need to be addressed. These include the stability of dsRNA in prawns, if it induces long term inhibition and if the immunity can be passed onto offspring. Furthermore, a more efficient delivery method needs to be developed for the delivery of dsRNA for large scale operations such as in aquaculture. Ideally, a feeding method similar to those adopted for RNAi application in *C. elegans* (Timmons et al., 2001) would be ideal.

Finally, to exploit good RNAi targeting and to get more insight into the RNAi mechanism and efficiency against *Pmerg*DNV, this experiment should ideally be repeated in prawns. An innate antiviral defence triggered by sequence independent dsRNA/siRNA has been previously reported to provide partial protection in prawns against YHV and WSSV (Robalino et al., 2004; Robalino et al., 2005; Tirasophon et al., 2005; Westenberg et al., 2005; Yodmuang et al., 2006; Kim et al., 2007; Tirasophon et al., 2007; Xu et al., 2007). Hence, the innate immune system of prawns is capable of recognising virus sequence-specific dsRNA, triggering the antiviral response of the prawn through the RNAi pathway. RNAi provides an exciting new perspective for treating and combating *Pmerg*DNV and other crustacean viral pathogens in aquaculture.

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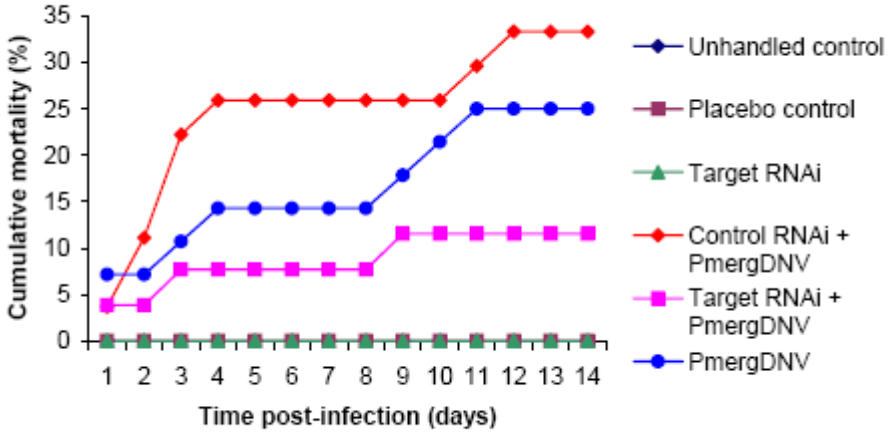

Fig. 1: Percentage mortality of *Acheta domesticus* during a 14-day experimental trial assessing whether stealth RNAi could inhibit *Pmerg*DNV replication.

Table 1: Sequence of stealth RNAi and control stealth RNAi targeting the capsid protein of *Pmerg*DNV, designed using BLOCK-iT™ RNAi Designer (Invitrogen, Australia). Base pair differences are underlined, italicised letters indicate an additional base pair not in the stealth RNAi target sequence and the letter d indicates a base pair deletion from the stealth RNAi target sequence.

Stealth RNAi	**Sequence (5'-3')**
*Pmerg*DNV target	CCUAC<u>A</u>AGAAGAGGAG<u>G</u>AAAU<u>U</u>AUU
*Pmerg*DNV control	CCUA<u>G</u>AAGAdGAGGAG<u>U</u>AAAU<u>C</u>*<u>A</u>*AUU

1 Table 2: Experimental design for the knockdown of *Pmerg*DNV in *Acheta domesticus*

Treatment	**First Injection (0 hours)**	**Second Injection (24 hours)**
Unhandled controls	N/A	N/A
Placebo controls	Insect medium	Insect medium
Stealth RNA target only	Stealth RNAi for target gene	Insect medium
Target stealth RNA + *Pmerg*DNV	Stealth RNAi for target gene	*Pmerg*DNV
Control stealth RNA + *Pmerg*DNV	Stealth RNAi control	*Pmerg*DNV
*Pmerg*DNV	Insect medium	*Pmerg*DNV

2

APPENDIX J

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Intranuclear bacilliform virus and hepatopancreatic parvovirus (PmergDNV) in the mud crab Scylla serrata (Forskal) of Australia

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Intranuclear bacilliform virus and hepatopancreatic parvovirus (*Pmerg*DNV) in the mud crab *Scylla serrata* (Forskal) of Australia

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Abstract: The culture of mud crabs *Scylla serrata* (Forskal) is largely experimental in Australia but some operators are producing marketable quantities. In an effort to understand the bottlenecks in hatcheries, a number of disease studies were initiated. In one study, basophilic to particularly magenta, intranuclear inclusions consistent with intranuclear bacilliform virus were discovered in the hepatopancreas of ~9% of wild broodstock and ~13% of batches of their progeny larvae. The inclusions were highly focused in hepatopancreatic tubules with 13-42% of tubules infected and on average 12% of cells displaying inclusions within infected tubules. Concurrently, using quantitative real time Taqman PCR, ~74% of haemolymph samples of adults and 100% of inclusion body positive batches of larvae were positive for *Penaeus merguiensis Densovirus* (*Pmerg*DNV) (formerly hepatopancreatic parvovirus, HPV) with copy numbers ranging from 6×10^2 to 1.5×10^5. Sequencing of 2,475 base pairs of viral genome confirmed that the virus was 99% similar to *Pmerg*DNV. This is the first record of one of the members of the HPV group of viruses to be found outside penaeid prawns. This study demonstrates that there are at least two viruses circulating in mud crab populations in northern Australia.

Keywords: mud crab, *Scylla serrata*, intranuclear bacilliform virus, TaqMan PCR, hepatopancreatic parvovirus, *Pmerg*DNV

Running Title: Viruses in mud crab *S. serrata*

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INTRODUCTION

Crabs of the genus *Scylla* are often found associated with mangrove habitats throughout the tropical and subtropical Indo-West Pacific region and form a substantial component of inshore fisheries (Keenan 1999). Mud crabs, *Scylla serrata* (Forskal), are considered a quality food item and command high market prices due to such attractive qualities as delicate flavour and high meat yield. The market for mud crabs has been reported as being promising resulting from high market prices and high growth rates in crab consumption being experienced in many countries in Asia, America, Europe and Australia (Aldon & Dagoon 1997). Within Australia, the culture of mud crabs is not extensive with only few operators having produced marketable quantities (McCormack 1989).

Pathogen and diseases have been reported as being the major constraint preventing the establishment of a successful hatchery production (Yamaguchi 1991, Cholik 1999, Fielder & Heasman 1999). A thraustochytriid - like organism has recently been associated with egg mortality in *S. serrata* (Kvingedal et al. 2006). Boer et al. (1993) and Parenrengi et al. (1993) both reported that from a number of vibrio species that *Vibrio harveyi* was found to be the major contributor to disease. Other larval stages where mortalities occur, known as moulting death syndrome (MDS) (Fielder & Heasman 1999), have been reported as being around the time when zoea-V metamorphosed to megalopa. The underlying reason for the high mortalities associated with this particular larval phase is unclear however nutritional deficiency has been regarded as a factor. Zeng & Li (1999) reported that poor nutritional status during zoeal stages might play a crucial role in why larvae are unable to pass through this critical stage successfully.

Yet to be reported as major disease agents in larval *S. serrata*, are the viruses. However, viruses have been found in the adult crab stages of mud crabs. Anderson & Prior (1992) reported finding a non-occluded rod shaped nuclear virus in *S. serrata* from Darwin, Northern Territory, Australia. These infections were observed in juvenile, subadult and adult specimens but did not cause clinical disease at any stage. Recently in China, Weng et al. (2007) reported a reovirus that caused 80% mortalities in cohabitation studies of juvenile to subadult *S. serrata*.

During attempts to increase the success of the hatchery production of *S. serrata* by a better understanding of the disease agents, basophilic to magenta hepatopancreatic intranuclear inclusions were discovered first in locally caught broodstock and then progeny larvae from those broodstock. This paper reports the description of those inclusions and the subsequent indentification of a second virus which was indistinguishable from hepatopancreatic parvovirus from *Penaeus merguiensis* (*Pmerg*DNV).

MATERIALS AND METHODS

Origin of Scylla serrata

Wild-caught adult *Scylla serrata* were obtained from Ross River, Townsville northern Queensland in 2002 (n=20) and 2003 (n=25) and were subjected to histopathological investigation (see below). A further 10 crabs from the same site and another 9 from the Burdekin River, Ayr, northern Queensland only had haemolymph extracted for quantitation by real time polymerase chain reaction (see below).

Scylla serrata larvae were obtained from aquaculture facilities at James Cook University, Townsville, Queensland. Mud crab larvae that had been preserved in 70% ethanol were from the spawning of wild-caught adults obtained from Ross River. Fifteen batches of approximately 20 larvae were examined by histology and four batches of larvae (Day 0 and Day 12) including two batches with inclusion bodies and two batches without inclusion bodies were examined by real time polymerase chain reaction (PCR) (see below).

Histopathology

Whole larvae and the hepatopancrei of *S. serrata* adults were fixed in Davidson's fixative for 48 hours and subsequently transferred to 70% ethanol. Tissues were processed routinely for histology by passing through a dehydrating series of ethanol and embedded in paraffin wax. Sections were cut at 5 µm and stained with Mayer's haemotoxylin and eosin (H & E). Sections were viewed by light microscopy.

Nucleic acid extraction

As the broodstock crabs could not be sacrificed, DNA was extracted from the haemolymph of the broodstock and the total tissues of the larvae (Day 0 and Day 12) using the High Pure Template Preparation Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Haemolymph (500 µl-1 ml) was collected from adults using a 29-gauge needle and was immediately placed directly into ice-cold 0.06M sodium citrate at a ratio of 1:1 to prevent coagulation.

*Screening for Pmerg*DNV *by Taqman real-time PCR*

All *S. serrata* larvae and adults were screened for *Pmerg*DNV infection using real-time PCR, according to the protocol of La Fauce et al. (2007a). Briefly, reactions were performed in a total volume of 20 µl using Rotor-Gene 3000 (Corbett Robotics) 36-well rotor. The reaction mixture consisted of 10µl of TaqMan mastermix (Invitrogen), 0.5 mM of additional $MgCl_2$ (Invitrogen), 10 µM of each primer (Table 1), 50 nM of probe, 2.5 µl of DNA template and nuclease-free water to a final volume of 20 µl.

The cycling pattern consisted of an initial incubation at 95ºC for 10 minutes, followed by a two-stop cycle pattern consisting of forty cycles at 95ºC for 10 seconds and 60ºC for 45 seconds. Data acquisition and analysis was performed using Rotor-Gene 3000 (Corbett Robotics) and Microsoft Excel.

PCR amplification

Seven sets of PCR primers (Table 2) were used to sequence part of the genome of *Pmerg*DNV from *Pmerg*DNV real-time PCR positive samples. All primers were synthesised by Sigma-Genosys, Australia and had previously been used to sequence the genome of *Pmerg*DNV from Australian *Penaeus merguiensis* (*Pmerg*DNV) (GenBank Accession Number: DQ458781) (La Fauce et al. 2007b).

The PCR reaction mixture contained 1 x *Taq* buffer (750mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween20), 2.5 mM $MgCl_2$, 0.75 U *Taq* polymerase (MBI Fermentas), 200 µM each dNTP, 50 pmol of each primer and 20-50 ng of DNA template. The PCR reaction volume was adjusted with sterile distilled water to a final volume of 25 µl. Amplification was performed in an Eppendorf Mastercycler Gradient Thermocycler (Eppendorf, Germany) with a PCR profile consisting of an initial 94°C

for 7 minutes, 35 cycles at 94°C for 45 seconds denaturation, 55°C annealing for 45 seconds and polymerisation at 72°C for 1 minute and an additional 5 minutes at 72°C following the last cycle. PCR products were visualised by running all of the 25 µl PCR reaction mixtures on 1% agarose gel containing ethidium bromide at a concentration of 0.5 µg ml^{-1}.

Cloning and sequencing

DNA was purified from agarose gel bands using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and cloned into *Escherichia coli* JM 109 cells, using pGEM-T® Easy Vector System (Promega, USA), according to the manufacturer's instructions. Blue/white screening was used to screen for recombinant plasmids. Recombinant plasmids were purified from at least three white colonies using Wizard® Plus SV Minipreps DNA Purification System (Promega, USA), according to the manufacturer's instructions. Recombinant plasmids were digested with *Spe* I, followed by electrophoresis on 1% agarose gel to screen for DNA inserts. Recombinant plasmids containing DNA inserts were sent to Macrogen Inc (Seoul, Korea) for sequencing using M13 universal primers. Three forward and three reverse sequencing reactions were performed for each clone. Sequencher™ software (Gene Codes Corporation) was used to analyse and align overlapping sequences for each clone. Sequence results were compared to available databases using BLAST via the NCBI homepage.

RESULTS

Histopathology

On histopathological examination of adult *S. serrata,* basophilic to more commonly magenta, granular inclusion bodies were very focally distributed within hepatopancreatic tubules. Often one hepatopancreatic tubule showed nuclear pathology, while adjacent cells in adjacent tubules appeared to be normal (Figure 1a). Infected nuclear were significantly hypertrophied up to 4-10 times larger than normal with most often irregular or rarely, round in shape (Figure 1b). The nucleolus was hypertrophied, often marginated and tended to lie on top of the cell in section. The nucleus was completely occupied by inclusions. Viral inclusions were normally distributed in a dense cluster (Figure 1e).

In larvae, nuclear pathology of infected cells was similarly only seen in the hepatopancreas (Figure 1c, 1d). Early infected cells displayed slight hypertrophy of the nucleus, a pale nucleolus, a band of mildly basophilic chromatin with sporadic fine eosinophilic granules scattered throughout the nucleus. Nuclear pathology of advanced infected cells consisted of hypertrophied nuclei with inclusion becoming a darker purple-red colour (Figure 1f). The nucleolus was central and enlarged becoming more distinctive (Figure 1f). These inclusions are consistent with those described by Anderson & Prior (1992) and will be termed intranuclear bacilliform virus (IBV) inclusions from here onwards.

Prevalence of Infection

From Ross River, in 20 adult crabs sampled in 2002 and in 25 crabs sampled in 2003, only two crabs each year were observed to be infected with IBV (Table 3). The overall prevalence was 8.9% in wild populations of mud crab within Ross River. The intensity of tubules infected with IBV within the infected adult crab was 42 % in 2002 and 13 % in 2003 (Table 4). The 95% confidence limits of these two sampling years did not overlap, so it suggests there was real year to year variation in severity and is not possible to pool the data to give an overall estimate without further sampling. Approximately 12% of cells within infected tubules demonstrated inclusion bodies consistent with IBV (Table 5).

From 15 batches of larvae sampled, only two batches or 13% were infected as observed by light microscopy (Table 6). After examining the two infected batches, the percentage of larvae infected was 32.26% and 52.7% respectively with a mean prevalence of 40.7% (Table 7).

Real-time PCR

Fourteen adult *S. serrata* (~74%) and the two larval samples that were positive for inclusion bodies were positive for *Pmerg*DNV (see below) by real-time PCR. Larvae at Day 12 had substantially higher viral titres than those at Day 0 (1.95×10^4 and 6.03×10^2 respectively). *Pmerg*DNV numbers in positive animals were between 10^2 and 10^5 copies (Table 8).

Sequencing

The size of the *Pmerg*DNV genome from *Scylla serrata* that was sequenced was 2475 bp; just under half the genome. Nucleotide sequence comparisons revealed 99% homology with the genome of *Pmerg*DNV from Australian *Penaeus merguiensis* (DQ458781) (La Fauce et al. 2007b). The sequence data of *Pmerg*DNV from *S. serrata* is lodged in Genbank with the accession number EU073937.

DISCUSSION

When the magenta intranuclear inclusions were first visualised, there was an expectation that it would be the intranuclear bacilliform virus discovered by Anderson & Prior (1992) from Australia. The histopathology was consistent with the descriptions of Anderson and Prior and inclusions were subtly different to HPV. HPV inclusions are often almost round and strongly blue-purple rather than irregular and magenta as in this case. Furthermore, IBV has dispersed chromatin and the nucleolus is hypertrophied whilst in HPV, chromatin tends to marginate strongly and the nucleolus gets displaced marginally to give a typical "signet ring" appearance. No pathognomonic inclusion bodies,for HPV were seen in these crabs. This study extends the known range of the *Scylla serrata* IBV from a focus in Darwin (Anderson & Prior 1992) to Townsville, a distance of coastline of over 6,000 km, with the implication that the virus is probably widespread in between.

Initial attempts to use sequence from a conserved gene for the DNA binding protein found within the *Baculoviridae* and the *Whispoviridae* (white spot syndrome virus) did not produce PCR sequence consistent with these viruses (Liessmann 2006). Similarly, ***in situ*** hybridization using the same sequence was negative again suggesting a limited relationship between *Scylla serrata* IBV and other marine bacilliform viruses (Liessmann 2006).

Of interest, is that the *Pmerg*DNV PCR signal was found in the haemolymph of crabs demonstrating that the virus had crossed the gut/haemolymph barrier and that the signal was not just from ingested banana prawns (the common host of *Pmerg*DNV in Australia (La Fauce et al. 2007a)) in the gut of the crab. Whether the virus is replicating or just being sequestered is unanswered at the moment. The mud crab titres (10^2-10^5) are lower than in banana prawns (10^3-10^{13}) (La Fauce et al. 2007a) but

235 higher than in *Cherax quadricarinatus* (10^1-10^3) (La Fauce et al. 2007c). In non-
236 permissive hosts, we see a decay of qPCR titres over time with titres approaching
237 those in *C. quadricarinatus* which is not what appears to be happening here. Of
238 considerable interest was the fact the *Pmerg*DNV signal in the larvae was found at
239 day 0, the day of hatch before they had begun to feed, and therefore the virus must
240 have come from the parents.
241
242 The ecology of mud crabs and banana prawns are incredibly intertwined that sharing
243 of viruses should not be a surprise. Both species have their postlarval and juvenile life
244 stages in the mangrove-lined estuaries and the adults move offshore to breed and
245 broadcast their eggs for dispersal. Postlarval prawns would possibly feed on megalopa
246 of mudcrabs and later mudcrabs would feed on juvenile and adult prawns. Any virus
247 like *Pmerg*DNV that mutated to grow in both species would be selected for over a
248 monospecific-host variant as the chances of transmission would be greatly enhanced.
249 These findings complicate the epidemiology of *Pmerg*DNV, particularly in terms of
250 controlling the disease at the farm level for both host species. Not only will
251 production of virus-free stock be necessary, but exclusion of the alternative host will
252 also be required.
253
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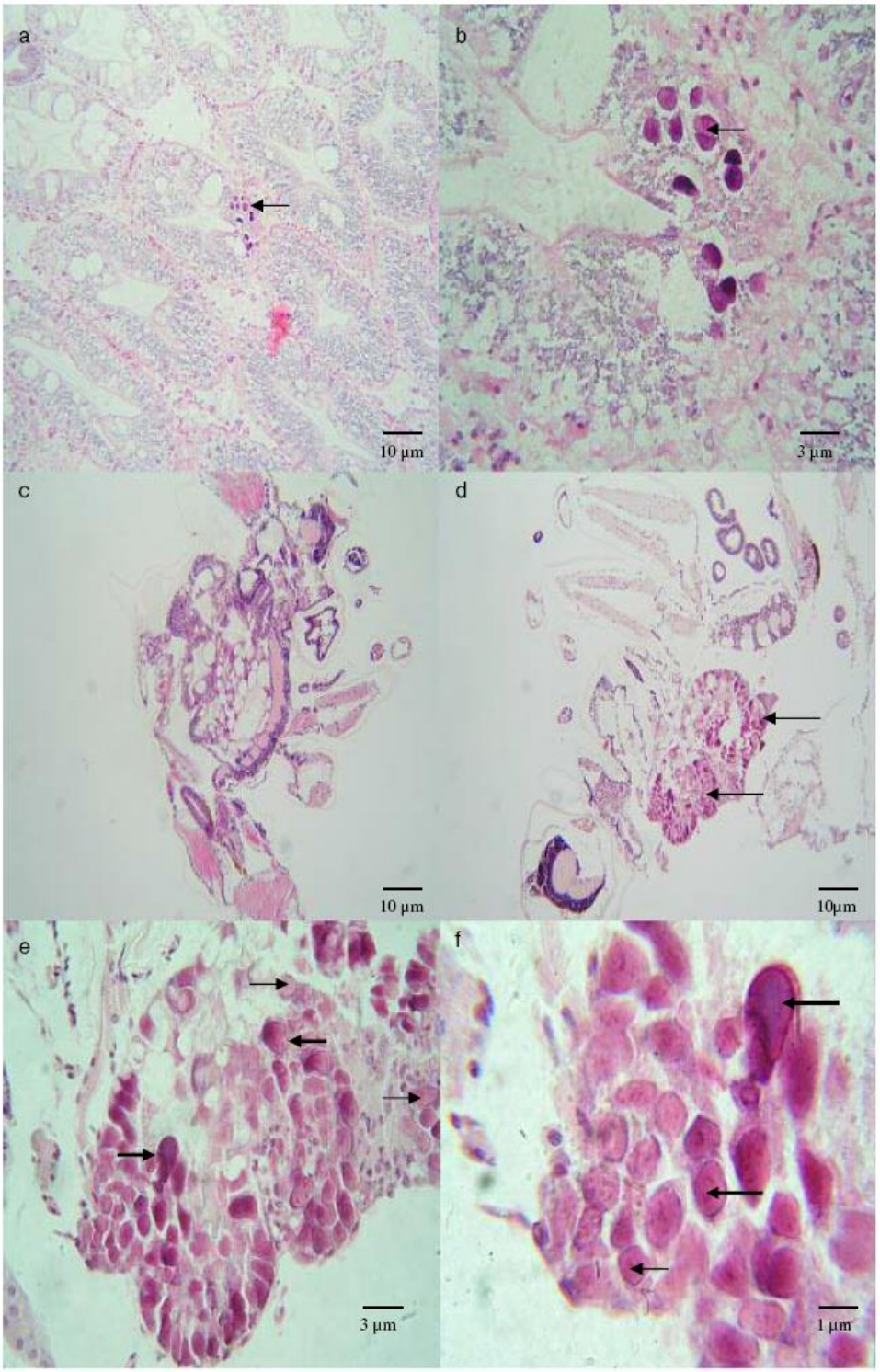
a
10 µm
b
3 µm
c
10 µm
d
10µm
e
3 µm
f
1 µm

Figure 1 Light micrograph of amorphous, basophilic intranuclear bacilliform viral inclusions in adult *S. serrata* hepatopancreatic tubule epithelial cells (a, b). Infected nuclei (arrows) are markedly hypertrophic and have thin marginal band of basophilic chromatin. Light micrograph of uninfected larvae of *S. serrata* (c). Light micrograph of basophilic intranuclear bacilliform viral inclusions in larvae of *S. serrata* (d,e,f). Infected nuclei are markedly hypertrophic. Advanced (large arrow) and early (small arrow) nuclear changes are present. All stained with haematoxylin and eosin.

355

356 Table 1: Oligonucleotide primers and probe for the *Pmerg*DNV Taqman Assay

Oligonucleotide	Sequence (5'-3')
HPV140F	CTA CTC CAA TGG AAA CTT CTG AGC
HPV140R	GTG GCG TTG GAA GGC ACT TC
HPV140probe	FAM TAC CGC CGC ACC GCA GCA GC TAMRA

357

358

359 Table 2: Overview of the primers used to sequence *Pmerg*DNV from *Scylla serrata*

Primer name	Primer sequence 5'-3'	Product length (bp)	Nucleotide position along 2475 bp
HPV823F	CATAGCCAAGAGCGAGGACAG	381	1-381
HPV500compR	CTTGTCGGGTTAGTTGAATCT		
HPVconfirmF	CACCGCAACAAACCTAT	495	159-653
HPVconfirmR	TGACTTTCTTTGCCATTACCA		
HPV140F	CTACTCCAATGGAAACTTCTGAGC	632	159-790
HPVconfirmR	TGACTTTCTTTGCCATTACCA		
HPV605F	AGCATTAGGGCAAGAGGAAAG	706	651-1356
HPV140R	GTGGCGTTGGAAGGCACTTC		
HPV823R	TGTCTGAAAATCCTGATGCGT	972	1103-2074
HPV623R	CGCCACCAGTAAAGTAATTGA		
HPV415F	TATTGGCAAGCATGCAGTATG	367	1708-2074
HPV623R	CGCCACCAGTAAAGTAATTGA		
HPVbegF	CGCTTTGACAGGATTAGTAA	768	1708-2475
HPV623R	CGCCACCAGTAAAGTAATTGA		

360

361

362 Table 3 Number of adult crabs sampled that displayed intranuclear bacilliform viral

363 inclusions.

Year sampled	No. of adult crabs sampled	No. of infected crabs	Prevalence of crabs infected (%)	95% confidence limits (%)
2002	20	2	10	0.1 - 32
2003	25	2	8	0.1 - 26
Total	45	4	8.9	0.25 - 21

364

365 Table 4 Prevalence of intranuclear bacilliform viral inclusions in tubules within
366 infected adult crabs.

Year sampled	No. of positive tubules	No. of tubules examined	Prevalence of tubules infected (%)	95% confidence limits (%)
2002	21	50	42	28 - 57
2003	5 0	386	13	9 - 16

367

368 Table 5 Intensity of intranuclear bacilliform viral inclusions within tubules in an
369 infected adult crab at the cellular level (n=20).

	Number of infected cells/infected tubule	Number of cells per tubule	% of infected cells per infected tubule
Range	2 – 22	34-167	3.7 – 37.9
Mean	7.55	62.5	12.1

370

371 Table 6 Prevalence of intranuclear bacilliform viral inclusions in batches of *S. serrata*
372 larvae in 2003

No. of batches sampled	No. of infected batches	Prevalence of batches infected (%)	95% Confidence limits (%)
15	2	13.3	2 - 40

373

374

375 Table 7 Prevalence of intranuclear bacilliform viral inclusions in cells within infected

376 batches of *S. serrata* larvae

Batch	No. of infected larvae	No. of larvae examined	Prevalence of larvae infected (%)	95% Confidence limits (%)
1	10	31	32.3	17 – 51
2	12	23	52.2	31 – 73
Total	22	54	40.7	28 – 55

377

Table 8 Calculated concentrations (number of copies) of *Pmerg*DNV in the haemolymph of *Scylla serrata* adults and tissues of larvae

	Number of copies
Scylla serrata adults	
	1.43×10^3
	1.26×10^4
	1.64×10^4
	1.80×10^4
	5.39×10^4
	1.59×10^4
	0
	4.15×10^4
	1.21×10^3
	0
	3.73×10^3
	1.10×10^4
	0
	4.01×10^4
	0
	6.81×10^3
	2.38×10^4
	1.51×10^5
	0
Scylla serrata larvae	
Batch 1: Day 0	6.03×10^2
Batch 2: Day 12	1.95×10^4
Batch 3: Day 0	0
Batch 4: Day 12	0